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**CRITICAL PERIODS ASSOCIATED WITH STRESSOR PROVOKED
ALTERATIONS OF THE ANTIBODY RESPONSE
TO SHEEP RED BLOOD CELLS**

Dissertation submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

Steve Zalcman
Department of Psychology
Carleton University

November, 1990

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acceptance of the thesis
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Abstract

A series of experiments assessed the effects of stressors on the splenic IgM-plaque forming cell (PFC) response and on serum antibody (IgG) titers to sheep red blood cells (SRBC) in male CD-1 mice. A critical period existed (72 hr) following antigen administration (SRBC: 10^6 cells, ip) during which footshock reduced the PFC response and antibody titers; however, when shock was applied at other intervals following immunization (0, 24, 48 or 95 hr), the PFC response was unaffected. The reduction of the PFC response was dependent on stressor severity, but not stressor controllability. The effect of an acute stressor was subject to adaptation, in that the immunosuppression was absent in mice repeatedly exposed to aversive stimuli, and typically favored immunopotentialiation.

An immunosuppression was provoked in mice that were exposed to footshock 2 weeks prior to immunization and reexposed to stressor-related cues 72 hr after inoculation. When the stressor was initially applied immediately after immunization, then reexposure to the stressor-related cues provoked an immunoenhancement. Moreover, footshock applied within 24 hr of inoculation counteracted the immunosuppression otherwise induced by shock at the 72 hr interval. Stressor application immediately after inoculation may have provoked an immunosuppression, as well as a compensatory response which was subject to conditioning-like effects. Reexposure to stressor-related cues 72 hr afterward induced the compensatory changes and hence an immunoenhancement.

In 9 month old mice the PFC response was approximately 50% that in 3 month old animals. The critical period for the stressor-provoked immunosuppression was not influenced by age; however, in the older mice the suppression could be provoked more readily than in the younger animals. In older mice, a stressor applied immediately or 24 hr

following immunization provoked a profound enhancement of the PFC response and antibody titers. The source for such an effect was not immediately apparent, but the possibility was entertained that stressor application in older mice may influence regulatory processes associated with an immune response being mounted, and the nature of these regulatory mechanisms may vary with time following antigenic challenge.

Finally, time-dependent alterations of central catecholamines were evident following SRBC inoculation. At the time of the peak immune response increased MHPG accumulation was evident in the hypothalamus, locus coeruleus and hippocampus, indicating an increase in norepinephrine activity in these regions. Moreover, alterations of mesocorticolimbic dopamine (DA) activity were apparent at or about the time of the peak antibody response. In contrast, striatal DA changes were not evident. These amine variations were reminiscent of those previously observed following inescapable footshock. Hence, the possibility was considered that antigenic challenge was interpreted as a stressor.

Taken together, the results of the present investigation suggest that under certain conditions stressful events may compromise immune functioning, and hence may increase vulnerability to illness associated with external pathogens. Conversely, excessive immunological activity promoted by stressful events may increase vulnerability to autoimmune disorders. Factors that increase vulnerability to the immunosuppression, such as aging, will similarly increase vulnerability to illness. Furthermore, failure of adaptive mechanisms, including the potentiation of immune activity following repeated stressor application, may render the organism more susceptible to disease states.

Acknowledgements

First and foremost, I would like to thank Hymie Anisman for all of the help, guidance and friendship he has provided over the course of these studies. Very special gratitude is also extended to Bob Zacharko who has been a great help and friend.

Warm thanks are extended to those around the lab who helped with experiments and/or in various ways made life more bearable and even enjoyable. In particular, Nola Shanks, who went through it all at the same time and was a pleasure to work with and to be around, as were Chris Prince, Jenna Griffiths, Glenda MacNeil, Rachel, Cindy, Nancy, Leslie, ela, Nando, Rog ...

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Appreciation is extended to Dr. Max Richter and Isabelle Trudel who provided helpful comments and information concerning immunological procedures.

Finally, I would like to thank my family, who were always there.

Glossary

Antigen: Any molecule which is recognized by the immune system and induces an immune reaction.¹ 2. An operational term for a genetically determined, typically glycoprotein cell surface marker on nucleated cells. When tissue is grafted onto another individual of the same species whose tissues do not carry the antigen, it may incite an immune response which leads to graft rejection.²

Antigen-Antibody Complex (Immune Complex): Antigen bound to antibody is termed an immune complex.¹

Autoantigen/Autoantibody: Refer to antigen/antibody systems where antibodies are formed to self molecules.¹

Autoimmunity: The reaction of the immune system against the body's own tissues.¹

Autoimmune Disease: Occurs when autoimmune reactions result in pathological tissue damage. In general they are either organ specific or organ non-specific.¹

Bone Marrow: The soft tissue that fills the cavity of bones. Red marrow is actively haemopoietic (i.e. blood forming) and is found in developing bone, ribs, vertebrae and parts of long bones. It contains the cells and corpuscles (with their precursors) of the circulating blood, and also megakaryocytes, reticulum cells, macrophages and plasma cells. It may be an important site of humoral antibody formation. In adult animals much of the red marrow is replaced by fatty tissue and becomes yellow marrow.²

Bursa of Fabricius: An organ which acts as a site of B cell development in birds. (Mammals do not possess this organ.) It develops as an outpocketing of the endoderm near the end of the gut and becomes

infiltrated with lymphocyte stem cells during embryonic development. It is a site of haemopoiesis as well as lymphopoiesis.¹

Cell-Mediated Immunity: Specific immunity which is dependent upon the presence of T lymphocytes. Cell-mediated immunity is responsible for reactions such as allograft rejection, delayed hypersensitivity, tubercullin test reactions and is important in defense against viral infections and against some bacteria.²

Complement: One of the serum enzyme systems. Its functions include mediating inflammation and causing membrane damage to pathogens.¹

Cross-Reacting Antibody: Antibody capable of combining with an antigen which did not specifically stimulate its production. This cross reaction may not be as strong as the reaction of the antibody with its own antigen.²

Humoral Immunity: Specific immunity mediated by antibodies which are present in the plasma, lymph, and tissue fluids of the body, and which may also become attached to cells (cytophilic antibody).²

Immunocompetence: The capacity and ability of the body to produce antibodies and/or to develop cell mediated immunity after having being exposed to harmful invaders.²

Immunoglobulin: Immunoglobulin is a synonym for antibody. Most antibodies are weakly charged at neutral pH and are found in the gamma globulin fraction of serum.¹

Inflammation: The response of tissue to injury, with the function of bringing serum molecules and cells of the immune system to the site of damage. The reaction consists of three components. 1) Increased blood supply to the region. 2) Increased capillary permeability in

the affected area. 3) Emigration of cells out of the blood vessels and into the tissues.¹

Interferons (IFN): A group of molecules which limit the spread of viral infection. There are three types, IFN-alpha and IFN-beta, produced by leukocytes and fibroblasts, and IFN-gamma, produced by activated T cells. Interferons from activated or virally-infected cells bind to receptors on nearby cells, inducing these cells to make anti-viral proteins. IFN-gamma also modulates immune responses and enhances natural killer cell activity.¹

Interleukins: A group of antigen non-specific factors involved in lymphocyte activation and differentiation.

Interleukin 1 (IL-1) is produced by macrophages and acts on T cells.

Interleukin 2 (IL-2) is produced by activated T cells. It is necessary for the long term proliferation of T cells and can also act on previously stimulated B cells.¹

Lymph nodes: Encapsulated organs which punctuate the lymphoid network, and contain aggregations of lymphocytes and antigen presenting cells.¹

Major Histocompatibility Complex (MHC): A cluster of genes important in immune recognition and signalling between cells of the immune system. The gene complex was originally identified as a locus encoding molecules present on cell surfaces, such that animals that differed at this locus would rapidly reject each others tissue grafts. All mammals have an MHC: H-2 is the mouse MHC and HLA is the human MHC.¹

Lymphocyte: A spherical cell, from 7-12 um in diameter, with a large, round nucleus (usually indented when observed under the electron microscope) and very scanty cytoplasm. The term lymphocyte is

essentially a morphological one that is used to refer to cells associated with all aspects of specific immunity, some (B lymphocytes) being associated with humoral immunity and others (T lymphocytes) with cell-mediated immunity.²

B-cells: are derived from bone marrow without passing through the thymus. B lymphocytes play a major role in humoral immunity. On stimulation by antigen they differentiate into antibody-forming plasma cells.²

T-cells: Cells that are derived from the thymus. Play a major role in the response against viruses, bacteria and fungi. Several subsets of these cells develop:¹

T-cytotoxic cell: A subset of T-lymphocytes which directly lyses virus-infected cells, tumor cells and transplanted tissue.¹

T-Helper cell: A subset of T-lymphocytes which stimulates antibody production. ¹

T-Suppressor cell: A subset of T-lymphocytes capable of regulating the magnitude of antibody production.¹

Lymphokines: A group of molecules, other than antibodies, produced by lymphocytes which are involved in signalling between cells of the immune system. The group includes some interleukins, B cell growth and differentiation factors, interferon gamma, and many less well characterized molecules, or 'activities'.¹

Macrophage: Large phagocytic cells found in most tissues and lining serous cavities in the lung. They are long-lived cells, and may remain in the tissues for years. Other macrophages recirculate through the secondary lymphoid organs, spleen and lymph nodes where they may function as antigen-presenting cells.¹

Mitogen: Molecule which induces differentiation and division (mitosis) of cells. A few are commonly used by immunologists to induce lymphocyte activation including:¹

Concanavalin A (Con-A): A T cell mitogen which stimulates both primed and unprimed cells.¹

Phytohaemagglutinin (PHA): A T cell mitogen which can also trigger cell functions and lymphokine production.¹

Pokeweed Mitogen (PWM): Stimulates both T and B cells, and can induce IgM synthesis by B cells.¹

Natural killer cells: Cells which are capable of killing a number of virally-infected and transformed target cells to which they have not been previously sensitized.¹

Phagocytosis: The process by which cells engulf particles and microorganisms. The particles first attach to the cell membrane of the phagocytic cell, either by non-specific receptors, or by receptors for opsonins such as IgG and C3b (i.e., a complement protein). Then the cell extends pseudopodia around the particle and internalizes it. Lysosomes then fuse with the phagosome. The lysosomal enzymes damage and digest the phagocytosed material and digestion products are finally released.¹

Plaque Forming Cell (PFC): Antibody secreting cell measured in an assay where each secreting cell produces a clear zone of lysis (plaque) in a layer of antigen sensitized red blood cells.¹

Plasma Cell: Terminally differentiated B-cells. They have an expanded cytoplasm with characterized parallel arrays of rough endoplasmic reticulum and are entirely devoted to the production of secreted antibody. Plasma cells are seen in the red pulp of the spleen, the

medulla of lymph nodes, the MALT (i.e. Mucosal Associated Lymphoid Tissue) and in small numbers at sites of inflammation.¹

Spleen: A secondary lymphoid organ, which lies in the peritoneum beneath the diaphragm and behind the stomach, contains two main types of tissue termed the red pulp and the white pulp.¹

Thymus: A primary lymphoid organ which overlies the heart. It is seeded by lymphoid stem cells from the bone marrow, which differentiate into T-lymphocytes. The thymus is bilobed and is organized into lobules separated by connective tissue septae (trabeculae). Each lobule is divided into a cortex and a medulla.¹

Virus: A group of minute infectious agents without any independent metabolism. They can only grow and reproduce within living cell hosts, which they do by invading the cell and taking over the material and machinery of the cell itself.¹

¹Male, D. Immunology: An Illustrated Outline. St. Louis: The C.V. Mosby Company, 1987.

²Herbert, W.J. & Wilkinson, P.C. A Dictionary of Immunology. Second Edition. Philadelphia: Blackwell Scientific Publications Ltd., 1977.

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For my parents, and Dorothy and Steve

I. Introduction

Considerable evidence has accumulated suggesting that in humans and infrahumans, exposure to stressors may influence immunological functioning, thereby increasing vulnerability to various disease states. In particular, it has been demonstrated that susceptibility to infectious, allergic, neoplastic and autoimmune disorders is enhanced by stressors (see Kelley & Dantzer, 1989). Moreover, some of the variables which influence stressor-provoked neurochemical changes, such as chronicity and controllability, as well as organismic variables (e.g., age), likewise affect immunocompetence (Irwin & Anisman, 1984). Accordingly, the view has been offered that immunologically related disorders may be secondary to the central neurochemical alterations engendered by stressors (see reviews in Irwin & Anisman, 1984; Sklar & Anisman, 1981).

Although pathways existing between the central nervous system (CNS) and the immune system have yet to be elucidated, it has been demonstrated that immune microenvironments in lymphoid organs are traversed by autonomic fibers (see Livnat, Felten, Felten, et al., 1988) and norepinephrine may contact cell surface receptors present on lymphocytes within spleen (Felten & Olschowka, 1987). Moreover, immune function has been shown to be influenced by lesions or stimulations of particular brain regions, suggesting a functional link between the two systems.

Just as the immune system is subject to modulation by neurochemical mechanisms, it appears that immunological processes may affect both neurochemical (Besedovsky, Del Rey, Dorkin, Da Prada, Burri, & Honegger, 1983; Carlson, Felten, Livnat, & Felten, 1987; Kabiersch, Del Rey, Honegger, & Besedovsky, 1988; Dunn, 1988a) and neuroendocrine activity (see Carr & Blalock, 1988). Interestingly, the profile of neurochemical

changes induced by antigenic stimulation, as well as the brain regions in which these may occur, are in some ways reminiscent of the transmitter alterations provoked by stressors. Accordingly, the possibility exists that immunological alterations may have behavioral repercussions.

The immune system is responsible for eliminating potentially harmful pathogens to which an organism may be exposed. Fortunately, this defense system possesses a great deal of autonomous activity and self-regulating capabilities, and is thus able to contend with an enormous range of pathogens. Typically, the successful elimination of foreign material depends on various cellular interactions, involving a complex array of regulator and effector mechanisms, ultimately leading to antibody production. Defects in these mechanisms could result in (i) a reduction in the ability to destroy pathogens, hence rendering an individual more vulnerable to disease (Heise, 1982), and/or (ii) an overactive immune response rendering an organism more susceptible to dysregulated effector activity thereby increasing the likelihood of autoimmune disease.

Although stressors appear to influence the development of immunologically based disease, only a few studies have systematically assessed the effects of stressor exposure on immune function (see Rabin, Cohen, Ganguli, Lysle, & Cunnick, 1989; Weiss, Sundar, Becker, & Cierpial, 1989). Not unexpectedly, stressor characteristics and psychological variables appear to be fundamental in determining whether immune function is suppressed, enhanced or unaffected by a stressor. However, there is reason to suppose that specific elements of the immune system or particular immune responses are differentially sensitive to stressors (Cunnick, Lysle, Kucinski & Rabin, 1990). Unfortunately, limited data are available that characterize the impact of a host of stressor variables,

known to affect neurochemical and neuroendocrine activity, on an orchestrated immune response. Accordingly the present series of experiments will assess the effects of stressor exposure on the antibody response to sheep red blood cells (SRBC). It will be determined whether stressor characteristics (chronicity, controllability, severity) as well as organismic factors (animal's stressor history, age) which influence the neurochemical and behavioral consequences of stressor exposure, likewise affect the antibody response to SRBC. Finally, it will be determined whether the profile of central neurochemical changes induced by antigenic stimulation, as well as the brain regions in which these occur, are reminiscent of the transmitter alterations provoked by stressors.

In the following sections, a brief overview will be presented concerning the processes operative in the production of an antibody response. Subsequent sections will review the evidence suggesting that stressors may provoke alterations of immune function and will examine some of the mechanisms that may mediate the stressor-induced immunological changes. Accordingly, a discussion of the neurochemical and hormonal consequences of stressor exposure will be presented with particular emphasis on possible communication between the CNS, endocrine and immune systems.

II. The Immune System and the Antibody Response

The immune system is designed to protect against foreign or 'non-self' material, typically in the form of life threatening infectious microorganisms such as bacteria, viruses and fungi. This is effected through the actions of a variety of cells which either patrol the body fluids (most notably the blood and lymph) or reside in various immune organs such as the spleen and lymph nodes (see Roitt, 1989).

When a foreign substance enters the body, a series of cellular and molecular interactions occurs, ultimately leading to the production of protein molecules called antibodies. These molecules are capable of recognizing and reacting with an almost limitless number of foreign substances, expending little of the body's resources in the process. Moreover, certain immunocytes possess "memory" capacity, such that once a given substance has been eliminated, these cells react robustly with the pathogen upon subsequent encounters with it.

The immune system has evolved recognition systems so that it can distinguish non-self from self material. Although a variety of safeguards against self-reactivity have developed, there are circumstances in which there is a breakdown in self-nonsel self discrimination. This may result in an 'autoimmune' condition, wherein the body's own structures are attacked as if they were foreign (Reinherz & Schlossman, 1980). If such a condition has pathological consequences, then it is referred to as an 'autoimmune disease' (Reinherz & Schlossman, 1980).

The antibody response is considered to be the hallmark of immune responses. There are several major characteristics of this response, namely recognition, specificity and memory (Roitt, 1985, Katz & Benacerraf, 1975). Essentially, immunocytes must identify invading material as foreign (recognition), mount a specific antibody attack (specificity) and be capable of destroying the pathogen should it later be reencountered (memory).

Elimination of foreign matter (operationally called 'antigens') is typically achieved by three cell types: the macrophage, which helps to identify, process and engulf antigens, the T-lymphocyte which serves to regulate the antibody response, and the B-lymphocyte which produces antibodies that help to mark cells for subsequent elimination (Claman &

Mosler, 1972). The system is regulated in a precise way by interactions that occur among these cells (Cantor & Gershon, 1977) (see later). Defects in the various mechanisms of cell interactions may (i) compromise in the organism's ability to eliminate harmful material, and (ii) provoke immune responses directed against the self.

The following section will describe the particular cells and interactions that are involved in the antibody response and how they are regulated.

a. Cell Populations

Immunocytes are white blood cells that arise from precursor cells in the bone marrow. Cells involved in the antibody response which are derived from this source circulate in the blood or migrate to various organs where they differentiate into cells belonging to either the 'phagocytic' or 'lymphoid' system. Cells belonging to the former are called 'macrophages', whereas those belonging to the latter are 'lymphocytes'.

(i) Macrophages

The macrophage is a long-lived phagocytic cell that differentiates from bone marrow stem cells into tissue specific macrophages (e.g. splenic, thymic, alveolar), providing a constant protection against invading pathogens (Unanue, 1979). Macrophages have numerous functions, the most prominent one being the elimination of foreign material (i.e. phagocytosis, or "cell eating"; the term refers to the process of destroying foreign material either by ingestion or by poisoning it with hydrogen peroxide). In addition to its role as a scavenger, the macrophage possesses secretory functions. In response to antigenic challenge, macrophages identify an antigen as foreign and manufacture and release

proteins called 'monokines' (e.g. interleukin-1 (IL-1)), which serve to activate other immunocytes that are involved in antigen elimination (see later) (Unanue & Benacerraf, 1984). It is thought antigen recognition is an important mechanism that prevents immunity to self by ensuring that lymphocytes respond only to non-self material.

(ii) Lymphocytes

Lymphocytes are small white blood cells which patrol the body's tissues for non-self material. They mature in the 'primary' immune organs, namely the thymus and, in birds, the bursa of Fabricius. (This organ is absent in humans, and it is thought that fetal liver and adult bone marrow serve the bursa's function in humans.) Some mature cells circulate through the tissues and are collected from them by the lymphatic vessels, which return them to the bloodstream through the subclavian vein. Alternatively, lymphocytes may become concentrated in the 'secondary' immune organs, namely the spleen or lymph nodes, which provide a suitable environment for the destruction of foreign molecules (see Tizard, 1984).

Lymphocytes are capable of recognizing and become activated by given antigens through receptors present on the surface of the antigen (cell surface receptors). These lymphocytes subsequently divide into cells of identical specificity with a long life-span (Playfair, 1971). There are two major classes of lymphocytes, namely T-cells and B-cells.

(ii.a) T-cells

T-cells, or "thymic-dependent lymphocytes", differentiate through several steps within the thymus gland. Several classes of T-cells develop, each involved with either a regulatory or effector role (Cantor & Gershon,

1977). Regulatory cells include helper cells and suppressor cells. When the organism is challenged by foreign matter, helper cells activate other lymphocytes (see later) through receptor binding and release of proteins referred to as 'lymphokines' (e.g. interleukin-2 (IL-2), interferon), while suppressor cells terminate antibody formation so that it does not continue unabated. The helper cells activate effector cells which kill antigens directly; these include cytotoxic T-cells and natural killer (NK) cells. (It should be noted that although NK cells may be a subset of T-cells, they are currently believed to be of undetermined lineage (Herberman & Ortaldo, 1981)). Cytotoxic T-cells help to destroy viruses which cannot be reached by antibodies during their intracellular growth phase. Markers on the viral cell surface activate specific cytotoxic T-cells which kill the infected cells (Roitt, 1985). Natural killer cells lack the specificity of T-cells, but may act as a first line of defense in the destruction of tumors and the rejection of transplants (Herberman & Ortaldo, 1981). There is evidence, moreover, that NK cells are involved in regulating the production of antibodies (Horwitz & Bakke, 1984).

(ii.b) B-cells

B-lymphocytes migrate from the bursa of Fabricius in fowl (or bone marrow in humans) to the spleen and lymph nodes. In response to antigenic challenge, these cells differentiate into "plasma cells", which are lymphocytes that produce antigen-specific antibodies upon initial exposure to the antigen. Plasma cells synthesize and secrete glycoproteins of various classes (G, A, M, E, D) which are called immunoglobulins (Ig) or antibodies (see Barrett, 1983). Antibodies provide the organism with the ability to recognize the diversity of foreign matter. There are millions of different antibodies present in each individual which are capable of

responding to as many foreign agents, and this accounts for the specificity of the immune system. There are cases, however, in which the antibody produced against one antigen will 'cross-react' with a second sufficiently similar antigen. It is important to note that while cross-reactivity should be high enough to ensure that given antibodies respond to more than one type of antigen, it must be low enough so that an antibody initially manufactured against a foreign substance does not attack the body's own structures, thereby initiating an autoimmune response (Burnet, 1970). Moreover, it appears that there are some antibodies (approximately 1 out of 10,000) that will bind strongly with any foreign structure (see Talmage, 1979). The largest immunoglobulin in the serum, IgM, occurs as five basic units and is the first immunoglobulin to appear following antigenic challenge. Moreover, of the five classes of antibodies, IgG is present in the greatest concentration in the blood, comprising about 75% of the total serum immunoglobulins. It is synthesized and secreted later in the antibody response and is the immunoglobulin typically associated with the response to a foreign agent.

Various antigens have been employed to examine the kinetics of a T-cell dependent response, including the sheep red blood cells (SRBC). Following SRBC inoculation various events occur culminating in the production and release of antigen-specific antibodies, primarily in spleen (see overview later). One method that is routinely employed to measure the peak splenic response is the plaque-forming cell (PFC) response. At the time of peak antibody production, splenocytes are harvested and the mononuclear cell band (which contains the antibody-forming cells) is isolated and incubated with SRBC as well as with complement (see later). The resulting 'antigen-antibody' complex is lysed by complement and one can observe a hemolysed or clear area, referred to as a 'plaque' in the

surrounding layer of SRBC. One simply counts the number of plaques evident in the field of SRBC's, thereby quantifying the number of PFC's for a given number of splenic mononuclear cells.

B-cell synthesize and secrete antibodies which can react to a multitude of antigens. Moreover, the system is energy-efficient in that it only expends energy when challenged by a micro-organism. These cells have the ability to respond specifically to a particular non-self infectious agent and to generate memory cells that have the ability to quickly and effectively eliminate the pathogen should it re-infect the organism.

In addition to these cell populations, a series of proteins, namely complement proteins, are intimately involved in the destruction of foreign particles (see Whicher, 1978). These proteins are synthesized by macrophages and normally circulate in the blood in an inactive form. Complement proteins become activated during an antibody response, such that when a sufficient number of these proteins bind with the antigen-antibody complexes, they result in its destruction.

b. The Genetics of Cell Interactions

Cells involved in the antibody response communicate with each other through cell to cell contact (Cantor & Gershon, 1977). It appears that cells contact each other through cell surface glycoprotein molecules which reside on the surface of immunocytes. These molecules (cell surface antigens) are genetically determined. (Unfortunately, both these cell surface markers and foreign substances are referred to as 'antigens'). Specifically, genes which code for cell surface antigens and immune response genes reside on the 'major histocompatibility complex' (MHC) of the human sixth chromosome (or the seventeenth chromosome in the mouse). The importance of the MHC was initially realized in

transplantation studies which demonstrated the existence of a genetic region that influences whether a transplanted tissue will be rejected or accepted. The human MHC consists of several loci (or regions) which are tightly linked together on the sixth chromosome and are typically referred to as the Human Leukocyte Antigen (HLA) region. The HLA region, in turn, houses four distinct loci, namely A, B, C and D, and these can be further divided into those which house Class I genes (A, B and C) or Class II genes (D or DR, which are also referred to as immune response (Ir) genes). The former class codes for antigens present on immune effector cells (cytotoxic T cells) while the products of the latter are found on regulatory cells (helper and suppressor cells). Functionally, it is through these cell surface determinants that cells contact each other during an antibody response (Katz & Benacerraf, 1975; Munro & Bright, 1976). Another MHC region expresses genes which code for complement proteins.

A given individual expresses one allele (or gene) for each locus of this polygenic effect; Class A is thought to have 20 alleles, Class B has 42 recognized alleles, Class C expresses 8, and the DR locus expresses 10 alleles (it is generally believed that each of these loci may express other presently unidentified alleles at each of these loci (see Gonwa, Peterlin & Stobo, 1983). Given the large number of alleles for this polygenic combination, there is potential for enormous interindividual variability. Interestingly, some phenotypes are expressed at rates that are much higher than would be expected from random independent assortment, suggesting that some of these genes are inherited together. This phenomenon, called "linkage disequilibrium", is an important characteristic of the MHC. Pathologically, some alleles appear to be more prevalent in patients with certain diseases (e.g. SLE) than in the normal population, possibly suggesting the influence of the genetic linkage.

c. Cell Interactions

Interactions among macrophages, T-cells and B-cells are typically required for an antibody response to occur (Claman & Mosler, 1972). While there are several models which outline the potential interactions among these three cell types (see Roitt, 1985), it appears that both direct cell to cell contact, and soluble helper factors (i.e. factors that are able to move through body fluids) are necessary for the response to occur (see Figure 1). Macrophages, patrolling the blood, are likely the first cells to contact invading foreign particles (Katz & Unanue, 1973) (i.e. after agents such as lysozymes and IgA type antibodies attempt to eliminate antigens in the skin, mucous membranes and the gut). The role of the macrophage at this stage is to (a) identify the antigen as foreign (and hence as a threat to the system) and eliminate most of these cells (as many as 90 per cent within the first few hours), (b) process the antigen by displaying parts of it (antigenic determinants) on its cell surface so that it can be identified by T-cells, (c) activate helper cells by presenting them with antigen through Class II receptors, and (d) induce helper cell proliferation (through the release and subsequent actions of interleukin-1 (IL-1)). Activated helper cells, which display the processed antigen on their membrane, subsequently produce interleukin-2 (IL-2) (also referred to as T cell growth factor (TCGF)), which appears to induce the proliferation of other helper cells and B-lymphocytes, which in turn proliferate and manufacture antigen-specific antibodies (IL-1 also appears to enhance B-cell differentiation). B-cell production of specific antibodies is additionally

Figure 1. Schematic representation of cell interactions in an antibody response. Immune attack on a pathogen involves both humoral (B cell) and cell-mediated (T cell) responses. Macrophages engulf the invader and display antigenic components to receptors on T cells. Additionally, they synthesize and release interleukin 1 (IL-1) which activates T helper cells. The helper cells multiply and produce lymphokines (including IL-2, interferons), which are chemical signals that regulate T- and B-cells. Interaction with macrophage and helper cells causes cytotoxic T cells to mature and roam the bloodstream, destroying infected cells. Meanwhile, antigenic determinants are presented to B cells which subsequently manufacture antigen-specific antibodies that bind antigens and neutralize them. Antigen-specific 'memory' cells are also generated which enable the immune system to combat the same invader in future encounters.

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stimulated by B-cell growth factor (BCGF) and B-cell differentiation factor (BCDF), which are produced by antigen activated helper cells (Howard, Nakamiski & Paul, 1984). Antibodies bind to antigen forming 'antigen-antibody complexes' which reside on the B-cell membrane. These complexes bind avidly to macrophages through an interaction of the antibody molecule with a specific surface receptor on the macrophage membrane. The phagocytosis of these complexes is greatly enhanced by the adherence of complement proteins to antibodies and macrophages (Raff, 1971). In addition, it is thought that complement proteins themselves may damage adhering micro-organisms by lysozomal enzyme release (injecting a poison into the pathogen) (Whicher, 1978). It is important to note that complement can disrupt large antibody-antigen complexes which are formed during an immune response and may lead to inflammatory reactions if they are not eliminated. Helper cells additionally produce macrophage activation factor (MAF) which appears to enhance the activities of macrophages, other helper cells and B-cells. The response is terminated by a variety of mechanisms, including the actions of suppressor cells and anti-idiotypic antibodies (see later) (Jerne, 1974). Finally, once the pathogen is effectively eliminated, B-cells which have produced antibody against the particular antigen proliferate thereby producing 'clones' of "memory cells" which will rapidly synthesize and secrete the antigen-specific antibody (IgG type) should the organism again encounter the antigen.

d. Regulation of The Antibody Response

A variety of mechanisms are involved in the regulation of the antibody response. These can be divided into those that are humoral

(associated with the synthesis and secretion of antibodies), cell-mediated (associated with specific cells), or genetic.

(i) Humoral Mechanisms

Jerne (1974) proposed the currently accepted 'network theory' of antibody regulation. According to this theory, once a sufficient number of antibodies have been formed against a particular antigen, other immunoglobulins, called 'anti-idiotypic antibodies', are formed against the original antibodies. (The term 'idiotype' refers to the specific region of an immunoglobulin molecule to which antibodies bind. Hence, such antibodies are called 'anti-idiotypic' antibodies.) The anti-idiotypes react with the original antibodies and the reaction is similar to an antigen-antibody reaction, in that the anti-idiotypes bind and neutralize their target. Furthermore, still other immunoglobulins can be formed against the anti-idiotypes. Jerne's theory thus states that a given immunoglobulin, whether it is produced against a foreign molecule or against another antibody, is subject to anti-idiotypic regulation, and this process generates a 'network' of antibodies that regulate other antibodies.

Antigen-antibody complexes can suppress B-cell activation (Roitt, 1985). In this regard, IgG complexes are often suppressive, whereas IgM complexes are often enhancing. In the former case, it appears that reduction in free antigen concentrations, as a result of complexes with antibody (IgG), serves to play a role in B-cell proliferation. Moreover, antigen catabolism appears to diminish the stimulatory effect of antigens.

(ii) Cell Mediated Mechanisms

Cell-mediated mechanisms involve the activities of regulator T-cells (helper or suppressor). It will be recalled that helper cells serve to induce

the proliferation of antibody forming cells, whereas suppressor cells, acting on helper cells or B-cells, are involved in terminating the response. It has been suggested that receptors present on suppressor cells may recognize either antigenic determinants or antibody idiotypes (see Webb, 1986). There appear to be two types of suppressor cells, namely antigen-specific and non-specific cells (Webb, 1986). The former binds to antigen and interacts with helper or B-cells by an antigen 'bridge', while the latter bind directly to receptors.

Helper cell activity is vital for the activation of other T-cells, B-cells and macrophages, while suppressor cells function to inactivate other regulatory or effector cells. The ratio of helper to suppressor cells thus appears to be important in determining whether given lymphocytes are activated (Roitt, 1985). A deficiency in either of these cell populations can result in a defective antibody response.

(iii) Genetic Mechanisms

The effective functioning of regulator T-cells requires the presence of Class II antigens (Gershon, 1979). These antigens determine (i) whether helper cells will be activated by foreign material, and (ii) the extent of subsequent cellular interactions (T-B interactions as well as T-T interactions). In effect, the cell surface antigens determine the relative activity of helper and suppressor cells (Benacerraf & Germain, 1978).

Cells lacking these antigens appear to be common sites of autoimmune disease (Feldmann, Londei & Essery, 1986). It is thought that the functional significance of this is a defect in immunoregulation reflecting a compromise of the system's ability to recognize foreign material, activate immunocytes and promote cell interactions (Reinherz & Schlossman, 1980).

e. Tolerance and Autoimmunity

The main task of the immune system is to protect the organism from foreign invasion. Under the normal course of events, however, there are conditions wherein the immune system ordinarily attacks the self. As indicated earlier, anti-idiotypic antibodies regulate (inhibit) the activity of other antibodies, and suppressor cells help to terminate an antibody response. However, rather than being seen as potentially destructive autoimmune reactions, these mechanisms are considered to be vital in the maintenance of 'autotolerance' by ensuring that antibody responses do not continue unabated and/or by limiting the activity of autoantibodies that may be present in the body (see Bowry, 1985). Despite the existence of such mechanisms, the immune system must learn to tolerate its own tissues during development and it is generally thought that the manner in which the immune system first encounters an antigen (e.g., a self-structure) determines whether tolerance to that antigen will develop. Burnet and Fenner (1949) claimed that the immune system learns in utero those components which are part of the self. Hence, self structures are 'tolerated' and not attacked by the immune system postnatally. Indeed, it had been previously reported by Traub (in Nossal, 1983) that mice infected in utero with a particular virus did not appear to mount an immune response directed against it later in life, whereas in the absence of this in utero treatment adult mice immunized with the same pathogen mounted a normal antibody response. Moreover, Owen (in Nossal, 1983) noted that twin cattle sharing a common placenta tolerated each other's erythrocytes and that this immunological tolerance persisted throughout their lives.

There exist, of course, several pathologies wherein the immune system attacks the self causing destruction of various tissues (i.e.,

autoimmune disorders). An autoimmune reaction is typically taken to represent the abrogation of tolerance to self-antigens. When this results in the destruction of the body's own tissues, the condition is referred to as an autoimmune disease (Reinherz & Schlossman, 1980). Autoimmune responses can be classified on the basis of immunopathological considerations (Gell, Coombs, & Lachmann, 1974). These include immediate and delayed hypersensitivity reactions, as well as 'antibody mediated' reactions. While the existence of autoimmune diseases has been firmly established, several different mechanisms have been implicated in the provocation of such illnesses. Among other things, complex interactions involving genetic factors, immunoregulatory defects and environmental phenomena (Smith & Steinberg, 1983) may trigger both the expression and severity of the disease (Nossal, 1983).

III. Stress and Immunity: Nervous, Endocrine and Immune Interactions

Immunologically-based disorders are characterized by defects in immunoregulation, and hence a disturbance in the balance of different immunocytes (Burnet, 1970). As will be recalled, the actions of the immune system are influenced by endocrine and neurochemical processes (Besedovsky & Sorkin, 1981). It has thus been suggested that events that affect the endocrine and nervous systems (such as exposure to stressors) may precipitate a defect in the immunological apparatus, which in turn may lead to pathology (Solomon & Moos, 1964; Irwin & Anisman, 1984; Rabin et al., 1989). For instance, many patients with infectious or autoimmune disorders have reported a preponderance of stressful life events prior to disease onset and immediately preceding exacerbations of disease activity (see Solomon & Amkraut, 1983). Conversely, there is

evidence suggesting that an activated immune system can influence nervous and endocrine activity (Besedovsky, Sorkin, Felix & Haas, 1977; see Blalock, Bost, & Smith, 1985). It is noteworthy that patients with certain immunological disorders, including certain autoimmune disturbances and acquired immune deficiency syndrome (AIDS) (see Kurstak, Lipowski & Morrow, 1987), have an increased prevalence of neurologic and psychiatric abnormalities, although these disturbances may be directly related to the immunological disturbance.

The following section will discuss the human and animal literature suggesting that (a) stressors can influence susceptibility to disease, and that (b) cellular components of the immune system are compromised after exposure to aversive stimuli. Additionally, evidence indicating that the nervous, endocrine and immune systems communicate bidirectionally will be presented.

a. Stress and Immunity: Human Studies

Studies which have assessed associations between psychological factors and disease in humans have been retrospective, prospective or prognostic in nature. Retrospective analyses have suggested that psychological variables are associated with the onset and course of various illnesses. For example, it was reported that in patients with tuberculosis a marked increase in stressful events occurred during the two years preceding the onset of symptoms (Holmes, Hawkins, Bowerman, Clark & Joffe, 1957). In contrast, disease severity decreased with the availability of social support systems (Holmes, Joffe, Ketcham, et al., 1961). Paralleling these findings, it was reported that the acquisition of upper respiratory infections (such as streptococcal infection) was shown to be highest in individuals from highly stressed families (Boyce, Cassel, Collier, Jensen,

Ramey & Smith, 1977). Likewise, in patients with acute necrotizing ulcerative gingivitis (trenchmouth) a marked elevation in negative life events as well as depression and anxiety were reported during the year preceding disease onset (Cohen-Cole et al., 1981).

It has been suggested that stressors not only influence infectious diseases, but can also affect the onset and course of certain autoimmune disorders. For example, Solomon and Amkraut (1981) described patients suffering from rheumatoid arthritis as introverted, overactive, rigid, excessively restricted in emotional expression and having an exaggerated need for independence. Many arthritics, moreover, have reported that stressors, or the inability to cope with stressors, preceded the onset of symptoms (Rimon, 1969; Baker & Brewerton, 1981; Solomon & Amkraut, 1981). Patients with other suspected autoimmune disorders, such as ulcerative colitis, multiple sclerosis and systemic lupus erythematosus (SLE) have similarly reported an increased prevalence of emotional tension prior to disease onset and symptom exacerbation (Engel, 1953; Mei-Tal, Meyerowitz & Engel 1970; Warren, Greenhill & Warren, 1982).

Although the preceding studies suggested that psychological variables influenced vulnerability to various illnesses, retrospective studies are plagued by various methodological problems, such as an individual's inaccurate (or biased) recall of past events (see Rabkin & Streuning, 1976; Cohen, 1981). Additionally, there is evidence to suggest that cognitive functioning may be impaired in some patients with immunologic disorders (see Kurstak, Lipowski & Morozov, 1987), hence rendering a personal account of a patient's stress history as being suspect. Prospective analyses, which do not suffer from these methodological constraints, have reinforced the contention that stressors influence vulnerability to disease. For example, Meyer & Haggerty (1962), who

followed 16 families for a period of 12 months to assess the occurrence of streptococcal respiratory illness (throat cultures were taken at regular intervals), observed that marked elevations in familial stress preceded streptococcal infection. In a study of female telephone operators, Hinkle (1974) reported that sadness tended to precede the onset of respiratory illness. Kasl, Evans & Wiedermann (1979) prospectively analyzed the development of infectious mononucleosis (IM) in West Point cadets, who were seronegative for antibodies against Epstein-Barr virus upon entering the academy (had no detectable antibodies against the virus). In contrast to students who were either seronegative or developed antibodies but did not contract IM, cadets who developed the disease were those who had felt the most pressure to succeed at the academy. As part of a fairly extensive series of studies, Kiecolt-Glaser et al. (1985) assessed medical students before, during and following final examinations for incidence of latent herpes virus activity (inferred from elevated blood antibody titers). The authors reported a higher incidence of viral activity during the examination period, especially in the loneliest students (assessed from self-reports). In a similar type of study, Dorian et al. (1986) assessed Medical and Psychiatric residents prior to, during, and following their Fellowship examinations. These investigators reported increases in respiratory infections in highly distressed individuals in the weeks after the examination. Although these studies indicated that psychological variables may influence disease susceptibility, it should be noted that some prospective studies have failed to find correlations between psychological variables and immune responses following challenge with influenza virus (e.g., Locke & Heisle, 1977; Green, Betts, Ochitill, Iker, & Douglas, 1978). However, it has been noted that factors other than psychological stress, such as gender, antigenic dose, number of prior

infections and small sample sizes may account for the divergent results observed (Jackson, Dowling, Anderson, Riff, Saporta, & Turck, 1960).

In addition to studies indicating that stressful events can influence the clinical course of disease, there is evidence that psychological factors can influence immunocompetence at the cellular level. For example, Bartrop et al. (1977) compared 26 adults following the death of their spouses with 26 controls matched for sex, age and race. It was reported that T-cell proliferation in response to mitogens was significantly impaired in bereaved subjects. Moreover, this effect was seen at 6 weeks, but not at 2 weeks following bereavement. Schleifer et al. (1983), who prospectively assessed 15 spouses of women dying with advanced breast carcinoma, likewise reported that both T- and B-cell mitogen responses were decreased 5 to 7 weeks following bereavement. In addition to effects on lymphocyte proliferative responses, Irwin et al. (1987) reported that bereaved women displayed reductions in NK cell and suppressor/cytotoxic cell activity, as well as marked alterations in helper to suppressor cell ratios. Furthermore, these effects were more pronounced in women displaying more severe depressive symptoms (as measured by the Hamilton Depression Scale). Interestingly, alterations in the ratio of helper to suppressor cells are evident in the majority of SLE patients and these are thought to be associated with an increased prevalence of autoantibody activity.

The notion that a link exists between psychological illnesses, such as depression, and immune functioning or neoplastic disease has a long history (see Sklar & Anisman, 1981). While the initial reports of such a relationship were essentially anecdotal, subsequent controlled studies revealed that hospitalized depressives showed significant reductions in lymphocyte counts and the ability of these cells to proliferate in response

to mitogenic stimulation (Kronfol, Silva, Greden, Dembinski, Gardner & Carroll 1983; Linn, Linn & Jensen, 1982; Schleifer, Keller, Meyeson, Raskin, Davis & Stein, 1984). Such findings were not evident in ambulatory depressives, who displayed less severe symptoms (Schleifer, Keller, Siris, Davis & Stein, 1985). Moreover, NK cell cytotoxicity (Irwin, Daniels, Bloom, Smith, & Weiner, 1987) and NK cell number (Evans, Pedersen, & Folds, 1988) were reduced in hospitalized depressives compared with matched controls. In contrast to these reports, however, Schleifer et al. (1989) found no significant changes of NK cell activity in various populations of depressives. In accordance with the aforementioned direct measures of immune functioning, it has been reported that increased levels of prostaglandin E (which are immunosuppressive) are evident in depressed patients (Calabrese, Skwerer, Barna et al., 1986). Additionally, it was demonstrated that depression is associated with a reduction in lymphocyte beta-adrenergic receptors (Mann, Brown, Halper, et al., 1985).

While it appears that cellular immunity is compromised in depressed patients, it is important to differentiate between the effects of the disease process itself and a drug therapy used to treat the illness. For example, tricyclic antidepressants, such as desmethylinipramine (DMI), reversibly inhibit lymphocyte proliferation (Baker, Santalo & Blumenstein, 1977, Audeus & Gordon, 1982) and natural killer cell activity (Miller, Asnis, van Praag, & Norin, 1986). Thus, the immunosuppression associated with depression may be a result of the drug used to treat the affective disorder, rather than a consequence of the depressive state. However, it was noted that the DMI concentrations necessary to provoke the NK disturbances are not typically present in the sera of depressives (Miller et al., 1986). Nevertheless, it remains unclear whether chronic exposure to

lower concentrations of DMI would have immunological consequences (Miller et al., 1986).

While the relationship between immune functioning and psychological state has largely focused on depression, several investigators have reported altered immune functioning in schizophrenic patients, including reductions in interferon production (Moises, Schindler & Kirchner, 1987), decreases in T-cell numbers (Nyland, Ness & Lund, 1980), and a marked reduction in suppressor/cytotoxic cell percentages (DeLisi, Goodman, Neckers & Wyatt, 1982). In addition, it was reported that autoimmunity may be increased in acute schizophrenic patients (Ganguli, Rabin, Kelly et al., 1987). In particular, it was reported that these patients had immunologic abnormalities, including reduced suppressor cell activity, increased helper cell numbers, as well as serum antibodies directed against brain antigens, possibly suggesting that the deficits in the immunologic apparatus were associated with the pathogenesis or etiology of schizophrenic symptoms.

b. Stress and Immunity: Animal Studies

Evidence from infrahuman experiments tend to confirm observations from human studies indicating that exposure to stressful events enhances vulnerability to various aspects of immunity. The nature of the effects are influenced by psychological variables and stressor characteristics, such as the chronicity and controllability of the aversive stimulation. Moreover, it appears that several components of the immune system, such as NK cell activity and T-cell proliferation are similarly affected by stressors; however, mounting evidence suggests that different mechanisms may mediate the stressor-provoked alterations of different cell types (Lysle, Cunnick, Fowler, & Rabin 1988). Additionally, a host of

events occur during the course of orchestrated immune responses which may be differentially influenced by stressors. Accordingly, in the present review, the effects of stressors on immunologically based disorders, various components of the immune system, as well as on orchestrated immune responses will be presented.

(i). Disease processes

It has been reported that susceptibility to viral challenge (e.g. poliomyelitis, vesicular stomatitis virus, coxsackie B-2, herpes simplex) was enhanced in animals exposed to an aversive stimulus (avoidance training, footshock or restraint) (Rasmussen, Spenser, & Marsh, 1957; Chang & Rasmussen, 1965; Friedman, Ader & Glasgow, 1965). Furthermore, it has been reported that exposure to a psychosocial stressor (overcrowding) resulted in an increased mortality in mice inoculated with the parasite, *Salmonella typhimurium* (Friedman & Glasgow, 1966; Edwards & Dean, 1977) and provoked a suppression of the antibody response to salmonella flagellin (Solomon, 1969). Additionally, the particular strain and sex of animals utilized influenced the effects of housing conditions on mortality following viral challenge (Friedman & Glasgow, 1973, see Rabin et al., 1989). Likewise, Sklar & Anisman (1980) demonstrated that social housing conditions or changes in such conditions differentially influenced tumor growth.

Stressors may influence the development of autoimmunity in animal models of these disorders. Amkraut, Solomon & Kraemer (1971) reported that arthritis developed more quickly and to a greater extent in rats exposed to overcrowding. These investigators also reported that the incidence of arthritis was increased in animals exposed to transportation and handling. Moreover, there is some evidence that stressors may

influence experimental autoimmune encephalomyelitis (EAE), which is an animal model of multiple sclerosis. In particular, it was reported that stressor exposure resulted in a lessening of symptom severity, owing to a stress-induced immunosuppression (Saltzman & Levine, 1987). In contrast to these findings, Rogers et al. (1979) demonstrated that only 3 per cent of rats exposed to a cat prior to being injected with an arthritis-inducing agent (collagen) developed arthritis. It has been suggested that the conflicting results may be associated with the particular stressor utilized, as well as the timing between stressor exposure and inoculation (Rogers, Trentham, McCune, Ginsberg, Renke, Reike & David, 1980). Indeed, neurochemical and immunologic activity may be dependent on the specific stressor employed (see Sklar & Anisman, 1981), and it was demonstrated that the timing between antigen administration and stressor exposure may determine whether the effects of a stressor are evident (see e.g., Riley, 1981).

(ii). Natural Killer Cell Activity

It will be recalled that NK cells appear to act as a first line of defense in the destruction of tumors and the rejection of transplants (Herberman & Ortaldo, 1981). Hence, reductions in the cytotoxic activities of these cells may render an individual more vulnerable to subsequent antigenic challenges. Several investigators have reported that NK cell activity is diminished following stressor application and that the nature of these alterations is related to stressor characteristics. For example, acute stressor exposure (e.g., cold water, restraint, surgical stress, transportation) provoked marked reductions of NK cell cytotoxicity (Aarstadt, Seljelid, & Gaudernack, 1982; Okimura et al., 1986; Irwin & Hauger, 1988; Toge, Hirai, Takiyama, & Hattori, 1981; Herberman &

Holden, 1978). It was additionally shown that a suppression of splenic NK cell activity was evident in rats exposed to footshock that provoked analgesia thought to be mediated by opioid peptides. In contrast, no effect was evident in animals exposed to a stressor regimen that induced an opioid-independent analgesia. Furthermore, the suppression was absent in animals pretreated with the opiate antagonist naloxone (Shavit et al., 1984). It has likewise been demonstrated that the footshock-induced NK cell suppression was blocked by pretreatment with naltrexone (Cunnick et al., 1988).

The effects of stressors on NK activity appear to vary as a function of organismic factors, as well as the characteristics of the stressor. For instance, it was reported that while a controllable (escapable) stressor did not appreciably influence NK cell activity, a pronounced immunosuppression was apparent in rats that received an identical amount of uncontrollable (inescapable) shock (Shavit et al., 1983). In effect, these data suggest that the psychological dimension of coping behaviorally with a stressor may be fundamental in determining the variations of NK activity. In addition to stressor controllability, it appears that NK activity may vary with stressor severity or chronicity. For instance, Irwin, Vale and Rivier (1990) reported that one session of footshock or restraint did not affect NK cell cytotoxicity, while 4 such sessions induced an immunosuppression. With continued stress over 10 days this effect disappeared. Likewise, Irwin, Livnat, Ader, Gallo and Anisman (1986) observed that while a single session of shock provoked a suppression of NK cell activity, shock applied over 14 consecutive days actually provoked an enhancement of NK functioning. Particularly interesting findings were also reported by Weiss, Sundar, Becker and Cierpial (1989) who found that bimodal variations of NK activity occurred

with increasing stressor severities. A mild stressor such as handling or a relatively severe stressor such as tailshock applied over 19 hr, provoked a suppression of NK activity. An intermediate stressor (3 shocks), in contrast, produced a significant elevation of NK cytotoxicity. Thus, it seems that a stressor may not only induce a suppression of NK functioning, but depending on the specific stressor regimen employed, this component of the immune system may be enhanced.

It should to be emphasized at this juncture that the NK cell suppression induced by a stressor may be relatively long lasting, and that such an effect may vary across strains of mice. In particular, Zalcman, Irwin and Anisman (1990) reported that in C57Bl/6J mice NK activity was reduced immediately, 24 hr and 48 hr following a single session of inescapable footshock. In C3H/HeJ mice, NK cell activity was not affected until 24 hr after footshock, and remained depressed at the 48 hr interval. In contrast to these strains, in Balb/cByJ mice, the suppression of NK activity was not evident until 24 hr after footshock, but returned to control levels by the 48 hr interval. In effect, these data not only suggest the importance of genetic factors in determining the response to stressors, but also suggest that individual differences need to be considered in evaluating the potential pathogenic action of environmental insults.

Although the impact of stressors on NK cell activity have been well documented, consensus has not been reached concerning the mechanisms subserving such effects. As already indicated, there is reason to suspect that stressor-induced alterations of opioid peptides contribute to the suppression (Cunnick et al., 1988; Shavit et al., 1984). However, it was demonstrated that beta (β)-endorphin, as well as peptide fragments (alpha-endorphin, gamma-endorphin, leu-enkephalin) may actually have a stimulatory effect on NK activity (see review in Fischer, 1988) which can

be antagonized by naloxone (Matthews, Froelich, Sibbitt & Bankhurst, 1983). It is important to underscore at this juncture that the evidence concerning opioid effects on NK cell activity are confusing and often contradictory. Nevertheless, it appears that among other things, the nature of the NK changes may be dependent on the particular opioid agonist employed, the specific dosage administered, and the number of administrations animals received. It will be recalled, as well, that the effects of stressors on NK cell cytotoxicity may be dependent on the particular stressor regimen used (Irwin et al., 1990; Weiss et al., 1989). Of particular importance to the present discussion was the finding that stressors could produce either an immunosuppression or an immunoenhancement, just as varying doses of opioid agonists could induce such effects (see Fischer, 1988). The effects of stressors on opioid activity are also believed to vary with the specific stressor regimen employed (see review in Anisman, Zalcman, Shanks, & Zacharko, 1990). It is not clear, however, whether the NK changes in the Weiss et al. (1989) and Irwin et al. (1990) studies corresponded with alterations in opioid peptide activity.

While not necessarily dismissing the contribution of opioid peptides, Irwin et al. (1987) recently reported that NK cell activity could be modulated by corticotropin-releasing factor (CRF), given that NK cell activity was suppressed following intraventricular infusion of CRF. Additionally, the blocking of central CRF activity (by anti CRF) antagonized the reduction of NK activity otherwise provoked by footshock (Irwin, Vale, & Rivier, 1990). In contrast, peripheral blocking of CRF and hence of pituitary-adrenal activation did not reverse the shock-induced immunosuppression.

In addition to CRF, the possibility exists that IL-1 may contribute to the effects of stressors on NK activity. It was demonstrated that acute footshock resulted in a suppression of NK cell functioning, as did intracerebroventricular infusion of small doses of IL-1 beta (Weiss, Sundar, Becker & Cierpial, 1989). The latter effect could be antagonized by alpha-melanocyte stimulating hormone (alpha-MSH). Inasmuch as IL-1 stimulates the gene for pro-opiomelanocortin, the precursor molecule for both ACTH and alpha-MSH, the possibility was introduced that IL-1 mediated the stressor-induced NK cell alterations. While admitting the speculative nature of this hypothesis, these investigators maintained that it deserves serious consideration.

Clearly, both the CRF and IL-1 hypotheses concerning the effects of stressors on NK activity are still tenable. In fact, it is conceivable that the central action of either of these substances may come to provoke peripheral amine changes which influence NK cell activity. For instance, Hellstrand et al. (1985) demonstrated that human NK cell activity is affected by β -adrenoceptor stimulation in vitro. In particular, addition of low doses of epinephrine to lymphocyte suspensions resulted in an enhancement of NK cell activity, and this effect was blocked by the β -adrenergic antagonist propranolol. In contrast, addition of high doses of epinephrine provoked a reduction of NK cell activity. Thus, central release of CRF or IL-1 could come to provoke the secretion of peripheral NE, which in turn modifies NK cell functioning.

(iii). T-cell proliferation

It is generally thought that in vitro T-cell proliferation following mitogenic stimulation is compromised by stressors. Furthermore, paralleling the effects on NK cell activity, characteristics of a stressor

determine the nature and magnitude of T-cell mitogenesis (see Rabin et al., 1989). However, it appears that stressor effects on T-cell activity may vary as a function of whether splenic or whole blood T-cell activity is considered (Lysle, Cunnick, Fowler, & Rabin, 1988). Acute exposure to various stressors (footshock, tailshock, restraint, rotation, isolation) have been shown to alter splenic T-cell proliferation in response to mitogenic stimulation (Keller, Weiss, Schleifer, Stein, & Miller, 1981; Gamzu, Vincent, Tare, Benjamin, Farrar, & Sullivan, 1984; Esterling & Rabin, 1987; Joasoo & Mackenzie, 1976). Although few studies have examined the duration of the effects, Lysle, Lyte, Fowler & Rabin (1987) reported that splenic T-cell proliferation in rats was reduced for approximately 24 hr following shock exposure, while whole blood T-cell division was reduced for 96 hr after stressor termination.

Weiss et al. (1989) recently reported that incubation of lymphocytes from nonstressed animals with serum from acutely stressed animals provoked large reductions of splenic T-cell proliferation in vitro. When dialyzed serum (containing molecules greater than 10,000 daltons, which includes steroids and catecholamines) was incubated with serum from lymphocytes of nonstressed animals, no effect on T-cell proliferation was evident. Hence, molecule(s) greater than 10 kilodaltons (such as catecholamines and steroids) may mediate the suppressive effects of stressors on T-cell mitogenesis in blood. It is of interest, in this regard, that Cunnick et al. (1990) demonstrated that administration of the β -adrenergic antagonist propranolol or nadolol prior to stressor application blocked the otherwise suppressive effects of footshock on splenic T-cell proliferation in vitro. Moreover, since nadolol does not cross the blood-brain barrier, it was suggested that modulation of T-cell mitogenesis in spleen is mediated by peripheral release of norepinephrine or

epinephrine. Interestingly, although shock provoked reductions of both splenic and blood T-cell proliferation, adrenalectomy reversed this effect only in blood T-cells, suggesting that adrenal steroids contribute to the immunosuppression engendered by shock in blood, but not in spleen.

Not surprisingly, stressor-provoked alterations of T-cell proliferation are related to the severity of the aversive stimulation. For example, Lysle et al. (1987) reported that the suppression of whole blood lymphocyte proliferation in response to Con A was related to the number of shocks that animals received irrespective of whether rats received 1, 3 or 5 sessions of footshock. The stressor-provoked reduction of lymphocyte proliferation in spleen was likewise related to the number of shocks to which rats were exposed, however, the immunosuppression was not evident following 5 sessions of shock. Keller et al. (1981) likewise demonstrated that whereas a limited degree of tailshock provoked a small nonsignificant reduction of T-cell proliferation in response to Con A or PHA, exposure to a more severe stressor provoked a greater immunosuppression in whole blood. Interestingly, this effect was evident even in animals that were adrenalectomized prior to the stressor treatment, suggesting that adrenal steroids were not responsible for the immunological alterations. Paralleling these findings, Weiss et al. (1989) reported that tailshock reduced splenic T-cell proliferation in response to PHA, as well as IL-2 and interferon production. These effects, like those seen in whole blood, were not appreciably altered by adrenalectomy.

It has been reported that the ability to cope behaviorally with a stressor and not the stressor per se may influence T-cell division in vitro. Specifically, Laudenslager et al. (1983) reported that the reduction of T-lymphocyte proliferation evident in rats exposed to uncontrollable

footshock was not seen in animals that were able to escape from shock. Mormede et al. (1988) demonstrated that after ten stressor sessions controllable and uncontrollable shock differentially influenced the response to a T-cell mitogen, phytohaemagglutinin (PHA), but not to Concanavalin A (Con A) or to Pokeweed mitogen. It is important to note, however, that stressor effects on immune functioning have been varied and often difficult to reproduce. For example, in contrast to Laudenslager et al. (1983), Gamzu et al. (1984) reported that controllable and uncontrollable shock influenced the B- but not the T-cell proliferative response. Moreover, Gamzu et al. (1984) made particular note of the fact that in their laboratory the effects of the stressor manipulation were not particularly reliable for reasons they were unable to determine. Indeed, Maier and Laudenslager (1988) have reported an inability to replicate their earlier findings (i.e., those of Laudenslager et al., 1983), and suggested in a companion paper (Laudenslager et al., 1988) that evaluation of antibody titers, rather than lymphocyte proliferation, was most sensitive to the effects of stressors.

The suppression of T-cell activity evident following acute exposure to a relatively potent stressor appears to be absent following repeated stressor application and several investigators have, in fact, observed an immunoenhancement following a chronic stressor regimen. For example, Monjan & Collector (1977) reported that while short-term exposure (1 week) to auditory stress markedly reduced the ability of T- and B-lymphocytes to proliferate *in vitro*, in mice exposed to the stressor over a long-term period (4 weeks) the proliferative responses were enhanced. Jessop, Gale, & Bayer (1987) reported that in rats exposed to isolation and/or water scheduling for 5 or 12 weeks, both splenic and blood lymphocyte proliferation in response to PHA were profoundly enhanced

relative to group housed animals. Unfortunately, lesser amounts of treatment were not employed, thus it is difficult to determine whether the immunoenhancement was related to the amount of stress to which animals had been exposed. In a subsequent paper, however, Jessop, Gale and Bayer (1988) reported that while 1 week of isolation was without effect, a significant enhancement of splenic and whole blood T-cell proliferation, as well as splenic B-cell proliferation, was induced by two weeks of isolation or water scheduling.

In contrast to the aforementioned studies, Batuman et al. (1990) recently reported that IL-2 production, as well as splenic T-cell proliferation in response to PHA or Con A was profoundly reduced in rats exposed to 7 or 14 days of footshock. Additionally, IL-2 production was reduced following repeated stressor application. However, while an immunosuppression was evident after 7 or 14 days of footshock exposure, an immunoadaptation may conceivably have been evident if animals were exposed to the stressor over a briefer or lengthier period. In particular, it seems that following a brief stressor an immunosuppression may be provoked, but as the stressor continues this is replaced by an immunoenhancement. If the stressor treatment continues still longer, a second, profound suppression of the immune response may be provoked (Weiss et al., 1989). Unfortunately, since the Batuman et al. (1990) study was not a parametric investigation, it is unclear whether adaptation of the immune response had already occurred during the course of the aversive regimen. In a similar fashion, Odio, Goliszek, Brodish, & Ricardo (1986) reported that T-cell proliferation in response to Con A was reduced 1 month following exposure to variable amounts of footshock that had been administered over a 6 month period, and that the magnitude of the suppression was related to the amount of shock animals received. It may

well be that during the course of the stressor regimen, an adaptation to the immunosuppressive effects of shock would have been evident but with the extensive course of the stressor regimen, a reduction of lymphocyte activity was engendered. It is nevertheless interesting that the stressor-induced immunosuppression was evident as long as 1 month following stressor termination.

It will be recalled that in evaluating the effects of stressors on immunity, it is important to consider the time of stressor application relative to measurement of the immune response. Indeed, several studies suggest that the time between termination of a chronic stressor regimen and assessment of T-cell proliferation is crucial in determining the direction of the effect. In this regard, Steplewski & Vogel (1986) assessed lymphocyte numbers and activity either immediately or 12 days following repeated exposure to immobilization. In the former group, the stressor induced a decrease in the number of T-lymphocytes (although percentage of T-cells did not change), but an increase in neutrophils and large granular lymphocytes. In contrast, following a 12 day period T-cell numbers and percentages increased above those of nonstressed animals.

(iv). Antibody Responses

Paralleling the effects of environmental insults on cellular immunity, it appears that stressors provoke alterations of an orchestrated immune response, particularly against T-cell dependent antigens. This is not surprising, since stressors appear to influence activities of cells and cytokines thought to be involved in antibody production. However, the antibody response is a dynamic process and particular events that are concerned with either the initiation, mounting or termination of the response may be differentially sensitive to the effects of a stressor. Hence,

the influence of a stressor on an antibody response might be expected to vary with the timing of its presentation relative to antigen administration.

Moynihan, Ader, Grotta, Schactman & Cohen (1990) have recently reported that in mice brief handling on 14 consecutive days provoked a reduction of the IgG response to the T-cell dependent antigen, keyhole limpet hemocyanin (KLH). In contrast, the IgM response was unaffected. It was suggested that cells involved in antigen recognition and processing may be less affected by stressor application than antibody forming cells. In addition to evaluating the effects of stressors on the primary antibody response, several experimenters have assessed the impact of aversive stimuli on the secondary immune response. For instance, it was shown that exposure to footshock 7 days prior to and 7 days after primary inoculation resulted in a reduction of the subsequent secondary response (Moynihan, et al., 1990). Furthermore, exposure to one session of shock 24 hr following primary immunization resulted in a decrease in the subsequent secondary or tertiary response to KLH. However, this effect was absent in mice exposed to 3 or 7 shock sessions commencing 24 hr after primary inoculation. Laudenslager et al. (1988) similarly reported that in rats exposure to tailshock provoked a reduction of antibody titers to KLH. However, this effect did not vary as a function of the number of shock sessions (1 or 3) animals received nor as a function of the portion of the light cycle during which the manipulations were performed. Of course, it is conceivable that the effect might have varied with other stressor parameters.

Paralleling the response to KLH, various stressors have been reported to provoke alterations of the antibody response to SRBC. For example, overcrowding has been shown to result in reduced primary and secondary antibody responses to SRBC (Solomon, 1969; Boranic, Pericic,

Radacic, Poljak-Blazzi, Sverkko & Miljenovic, 1982). Moreover, it was reported that housing influenced the antibody response to SRBC, but the effect varied with the duration of the housing condition. In contrast to group housed mice, PFC responses were lower in mice housed individually for 1-2 weeks; however this effect was not evident in mice individually housed for 3 or more weeks (see Rabin et al., 1989). Moreover, decreased antibody response to SRBC have been observed in chickens exposed to loud noises (Gross & Siegel, 1987) and in chronically isolated mice exposed to a dominant mouse (Beden & Brain, 1982).

It appears that the timing of stressor exposure relative to SRBC inoculation is important in determining the subsequent PFC response. For example, Okimura and Nigo (1986) demonstrated that in mice restraint on 2 consecutive days prior to immunization resulted in a marked suppression of the PFC response. In contrast, restraint on each of 2 days following antigen administration did not induce such an effect. Moreover, Esterling and Rabin (1987) reported an adrenal independent reduction of the PFC response in mice exposed to rotation stress on 3 consecutive days commencing 1 day following SRBC inoculation. Curiously, the PFC response was unaffected when the stressor regimen was begun immediately following immunization. It appeared as if stressor exposure soon after immunization had a protective effect, in that it prevented the immunosuppression ordinarily induced by a subsequently applied stressor. Like physical stressors, there is evidence suggesting that exposure to emotional stress may influence the PFC response and that the nature of the effect is also related to the timing of stressor application relative to antigen administration. In particular, Croiset et al. (1989) demonstrated that an enhancement of the splenic PFC response was induced in rats trained in a passive avoidance test. However, such an

effect was dependent upon the timing of SRBC inoculation relative to the time of testing, as well as the presentation of environmental cues associated with the stressor situation. Little data exists concerning the effects of repeated stressor application on the antibody response to SRBC. In a study conducted by Mormede et al. (1988) rats were exposed to daily sessions of controllable or uncontrollable footshock 5 days prior to- and 5 days following SRBC inoculation. Antibody titers were reduced in animals that had received the controllable stressor, but not in rats that had received repeated exposure to an uncontrollable stressor. It will be recalled, however, that with respect to the mitogen response, a greater suppression was produced by uncontrollable shock. These investigators concluded that while controllability may be an important factor in determining an immunological response, such an effect was dependent on the component of the immune response being examined. In addition, it should be considered that in this experiment the contribution of controllability may have interacted with the chronicity of the stressor (i.e., adaptation to a stressor effect was differentially influenced by controllable and uncontrollable stressors).

The mechanisms responsible for stressor-provoked alterations of antibody responses are not known. Nevertheless, there is some data indicating that modulation of β -adrenoceptor activity might influence the in vitro PFC response. In a series of studies conducted by Sanders & Munson (see Sanders & Munson, 1985) it was demonstrated that addition of β -adrenoceptor agonists, such as isoproterenol, during the early phases of the in vitro PFC response provoked an immunoenhancement. In contrast, these compounds had little or no effect when applied during the later phases of the response. Thus, it appears that activation of these receptors during the early phases of the response, or when the system is

concerned with initiation and mounting of an immune response may result in an enhancement of immune activity. During the later phases of the immune response, when suppressor mechanisms are activated, the contribution of B-NE receptors may be more limited. As such, these findings may be particularly pertinent when considering the previously mentioned findings concerning the importance of timing of stressor application relative to antigen treatment.

Several studies have demonstrated that peripheral depletion of NE following systemically administered 6-hydroxydopamine (6-OHDA) profoundly altered the antibody response to SRBC. The nature of the immunological response induced by the neurotoxin, however, varied across studies. For example, 6-OHDA administered peripherally immediately or 48 hours following SRBC inoculation suppressed the subsequent antibody response in adult mice (Kasahara, Tanaka, Ito & Hamashima, 1977; Hall, McClure, Hu, Tare, Seals & Goldstein, 1982). In contrast, 6-OHDA treatment prior to immunization was without effect (Miles, Quintans, Chelmicka-Schorr & Arnason, 1981). Moreover, an enhancement of the response was observed in adult mice that had received 6-OHDA treatment in addition to adrenalectomy soon after birth (Besedovsky, Del rey, Sorkin, Da Prada, & Keller, 1979). Walker & Codd (1985) have suggested several possible explanations for the discrepant findings. For example, the increased response observed in adult mice that had received 6-OHDA and adrenalectomy at birth may have been associated with splenic receptor supersensitivity or were perhaps related to a partial reinnervation of the spleen, a situation not uncommonly seen in neonatally treated rats. This proposition, however, does not account for the differential effects of 6-OHDA applied before or after immunization. Indeed, these findings are more comfortably interpreted in terms of the

Sanders and Munson (1985) findings showing that the effects of isoproterenol vary with time following immunization.

c. Conditioned Immunity

In addition to evidence indicating that stressor exposure influences immunocompetence, it has been reported that immune functioning can be suppressed through classical conditioning procedures (see Ader & Cohen, 1985). For example, using a taste aversion paradigm, where the immunosuppressive drug cyclophosphamide was paired with saccharine, subsequent saccharin consumption resulted in an immunosuppression (Ader & Cohen, 1974, 1975). Subsequent experiments carried out in other laboratories have reported similar attenuations of the antibody response, although two exposures to saccharine were typically necessary to elicit the immunosuppression (Rogers, Reich, Strom & Carpenter, 1976; Wayner, Flannery & Singer, 1978; Gorczynski, Macrae & Kennedy, 1984). Moreover, circadian rhythms appeared to influence whether immune functioning was suppressed, unaffected or enhanced by the conditioning procedures (Gorczynski et al., 1984).

Various other reactions have been shown to be subject to behavioral conditioning, including the Graft versus Host (GvH) reaction. In this reaction, donor cells that had been grafted onto a recipient react against the host's tissue. Bovjberg et al. (1982) injected parental lymphoid cells into the footpads of F₁ offspring. Whereas three injections of CY were required to markedly reduce the rejection in control mice (determined from the weights of the popliteal lymph nodes, which drain the area), the GvH reaction was suppressed in conditioned mice that were reexposed to saccharine (CS) and only one injection of CY. In effect, the conditioning procedure served as an effective adjunct to the CY in preventing the GvH

response. Conditioning procedures have also been used to decrease joint inflammation (Klosterhalfen & Klosterhalfen, 1983), delay mortality in New Zealand Black mice prone to a lupus-like disorder (Ader & Cohen, 1982), enhance NK cell cytotoxicity (Dyck et al., 1987, 1988; Ghanta et al., 1985) and induce mucosal mast cell release of the enzyme protease II (MacQueen et al., 1989).

In accordance with the aforementioned conditioning studies, it has been suggested that exposure to cues associated with a stressor may come to provoke immunological changes. For example, Lysle et al. (1988) reported that presentation of a neutral cue that had previously been paired with footshock induced a marked suppression of lymphocyte proliferation in response to Con A or PHA. Furthermore, this effect was extinguished following repeated presentation of the conditioned stimulus. Gorczynski and Macrae (1984) likewise reported that when mice were exposed to cues associated with rotation stress after SRBC inoculation, the subsequent antibody response was suppressed.

d. Nervous, Endocrine and Immune Systems Relationships

In addition to the human and animal literature suggesting that behavior can influence immune functioning, there is evidence suggesting that the nervous, endocrine and immune systems may affect one another. Several investigators demonstrated that lesions of certain brain regions influence immune functioning. For instance, Jankovic & Isakovic (1973) demonstrated that electrolytic lesions of the hypothalamus or reticular formation of rats markedly reduced the antibody response against bovine serum albumin. In another series of experiments, these authors reported that electrolytic lesions of the hypothalamus, reticular formation or superior colliculus significantly reduced the density of lymphocytes in the

thymus, spleen and lymph nodes (Isakovic & Jankovic, 1973). Other investigators have likewise observed that electrolytic lesions of the anterior hypothalamus in rats markedly reduced the number of cells in the spleen and thymus (Cross, Markesbery, Brooks & Roszman, 1980), suppressed NK cell cytotoxicity (Cross et al., 1985), attenuated the proliferative capabilities of lymphocytes (Roszman & Brooks, 1985) and decreased circulating antibody titers in response to antigenic challenge (Macris, Schiavi, Camerino & Stein, 1970). In contrast to these effects, lesions of the hippocampus and amygdala or hippocampus alone were shown to enhance the ability of thymus and spleen cells to proliferate in response to the T-cell mitogen, Con A, and these effects were attenuated by hypophysectomy (Brooks, Cross, Roszman & Markesbery, 1982). It is of interest that hippocampal lesions had immunoenhancing effects, since lesions of this region (or of the dorsal hippocampus) appear to have an inhibitory effect on stressor-provoked activation of the HPA-axis (see Sapolsky, Krey & McEwen, 1986). Taken together, these studies indicate that manipulations of various brain regions can influence immune functioning and that the pituitary-hypothalamic pathway may be an important mediator of CNS-immune system interactions.

Neuroanatomical studies in several species (mice, rats and rabbits) indicate that the primary and secondary immune organs (including the bone marrow, thymus, spleen and lymph nodes) receive sympathetic innervation (Bulloch & Moore, 1981; Felten, Overhage, Felten & Schmedtje, 1981, 1985; Giron, Crutcher & Davis, 1980; Williams & Felten, 1981; Williams, Peterson, Shea, Schmedtje, Bauer & Felten, 1981). It is thought that this innervation is associated with immunocompetence and not simply involved, for example, in splenic contraction, since the spleen has been shown to contract even when it has been freed from this innervation

(Bulloch, 1985). Moreover, lymphocytes and macrophages bear cell surface receptors for a vast array of neurotransmitters, hormones and steroids including norepinephrine, epinephrine, serotonin, acetylcholine, enkephalin, endorphin, histamine, substance P and corticotropin (ACTH) (Bourne, Lichtenstein, Melmon, Henney, Weinstein & Shearer, 1974; Hadden, Hadden & Middleton, 1970; Bulloch & Moore, 1980; Williams, Peterson, Shea, Schmedtje, Bauer & Felten, 1981; Wybran, Appelboom, Famacy, & Govaerts, 1979; see reviews by Hall & Goldstein, 1981, and Payan, McGillis & Goetzl, 1986). Furthermore, there is a higher density of adrenoceptors on activated immunocytes (T- and B-cells and macrophage) in comparison to resting cells, suggesting that these may serve an important regulatory function during the course of an immune response (Johnson & Gordon, 1980; reviewed by Livnat, Felten, Carlson, Bellinger & Felten, 1985; Sanders & Munson, 1985). It has been suggested that CNS and immune system interactions may be mediated through activation of surface receptors on lymphocytes which traverse microenvironments richly innervated by autonomic fibers (Williams & Felten, 1981). Hence, alterations in hypothalamic activity, which are known to influence peripheral neurotransmitters (Kvetnansky, 1981) may alter binding to lymphocyte surface receptors, thereby affecting immune responsivity.

Pharmacological studies have supported the contention that neurotransmitters can influence immunocompetence. For example, as indicated earlier, the administration of beta-adrenergic antagonists or agonists can influence lymphocyte proliferation in culture and modulate the *in vitro* PFC response against SRBC (see reviews in Livnat et al., 1985; Sanders & Munson, 1985). Moreover, catecholamines (NE or E) can markedly reduce the production of IL-1, possibly through actions on intracellular cAMP (Koff, Fann, Dunegan & Lachman, 1986), which has

been shown to reduce lymphocyte proliferation (Bourne, Lichtenstein, Melmon, et al., 1974). It is important to note that the transmitter concentrations required to achieve in vitro modulation of immune functioning are typically higher than physiological levels, suggesting that lymphocyte receptor activation, in vivo, is achieved through synergistic interactions of neurotransmitters (Johnson, Smith, Torres & Blalock, 1982; Koff et al, 1986).

It has been demonstrated that the in vitro antibody response to a variety of antigens (including SRBC) can be differentially modulated by endorphin, enkephalin and ACTH (Johnson et al., 1982). ACTH and alpha-endorphin produced the largest suppression of the antibody response, met- and leu-enkephalin produced moderate inhibition, whereas beta (β)- and gamma-endorphin were only slightly suppressive. While naloxone inhibited alpha-endorphin's suppressive effects, suggesting that these actions were mediated through an opiate-like receptor, it seems contradictory that β -endorphin and naloxone would exert similar actions. There are other data, however, suggesting that β -endorphin had stimulatory effects on lymphocyte proliferation (Gilman, Schwartz, Milner, Bloom & Feldman, 1982), NK activity (Matthews, Froelich, Sibbitt & Bankhurst, 1983) and IL-2 production (Gilmore & Weiner, 1988). Interestingly, while the endorphin effects on lymphocyte proliferation and NK activity were naloxone reversible (Hazum, Chang & Cuatrecasas, 1979, Matthews et al., 1983), this was not the case with respect to IL-2 production (Gilmore & Weiner, 1988). It has consequently been suggested that different immunocytes express different types of opioid receptors, thereby accounting for alpha-and β -endorphin's varied effects on immunocompetence (Gilmore & Weiner, 1988).

As discussed in the preceding section, there is considerable evidence suggesting an interdependence between nervous and endocrine activity (see review in Anisman, Kokkinidis & Sklar, 1978). It seems that to a great extent hypothalamic and pituitary factors may be fundamental in affecting immune functioning. In particular, tuberoinfundibular neurons of the hypothalamus contain peptide-releasing factors. These act on the anterior pituitary, through median eminence capillaries, to produce synthesis and secretion of ACTH, β -endorphin, TSH, PRL, LH and GH (Guillemin, 1978; Terry & Martin, 1978). Additionally, it seems that the secretion of these releasing factors is under neural control of DA, NE, 5-HT, histamine, ACh and GABA (see discussion in Irwin & Anisman, 1984). For example NE excites the release of PRL (Terry & Martin, 1978) and TSH (Reichlin, Martin & Jackson, 1978), while NE and DA inhibit corticotropin releasing factor (CRF) release (Ganong, 1976) and hence the release of ACTH and corticosteroids. Moreover, DA is thought to facilitate GH secretion (Durland, Martin & Brazeau, 1977). The actions of these hormones are clearly subject to neural modulation and their effects on immune function may, accordingly, be influenced by neural activity. Hence, stressor effects, which are thought to influence neurochemical (especially catecholamine) activity, can be seen to influence immune functioning (see Irwin & Anisman, 1984).

e. The Influence of the Immune System on Nervous and Endocrine Activity

Just as CNS manipulations may influence immune functioning, it appears that the immune system can influence nervous and endocrine activity. For example, Besedovsky et al. (1977) reported that activation of the immune system may alter hypothalamic norepinephrine activity. In

particular, it was observed that in rats which were injected with one of two non-toxic and non-infectious antigens (SRBC or a synthetic antigen against which antibodies are reliably formed) firing rates of neurons in the ventromedial nucleus of the hypothalamus were markedly increased. Moreover, the increased neuronal activity coincided with the time of the peak antibody response (96 hr following inoculation). In contrast, there was no change in electrical activity during the early phases of the immune response. Moreover, these central changes were not evident in animals that were non-responders (those that did not mount an antibody response). Thus, it appears that products from an activated immune system were responsible for the altered neuronal activity.

Subsequent to the demonstration that SRBC administration influenced electrical activity in hypothalamus, attempts were made to assess the central catecholamine variations that might be engendered by an antigen. It was observed that administration of an antigen resulted in a decrease in norepinephrine turnover and levels in hypothalamus at the time of the peak immune response, and this effect was related to the extent of the immune response that was mounted (Besedovsky, Del Rey, Sorkin, Da Prada, Burri & Honneger, 1983). Since this effect was not seen during the early phases of the response, the central alterations were not considered to be stress effects. Animals that were injected intraperitoneally with the supernatant from activated immune cells (i.e., cells taken from another animal that had been activated in vitro with Con A) showed similar alterations in hypothalamic NE, but this effect was found to occur within two hours of intraperitoneal injection. It thus appeared that products from an activated immune system mediated the NE variations, and it was further suggested that these amine changes may

have been responsible for the increase in medial hypothalamic electrical activity previously observed during the peak antibody response.

In accordance with the findings of Besedovsky et al. (1983) it was observed (Carlson, Felten, Livnat & Felten, 1987) that SRBC administration in mice resulted in a reduction of hypothalamic NE concentrations, but this effect appeared to be restricted to the paraventricular nucleus. While the hypothalamic alterations were most notable 4 days after immunization, hippocampal NE and serotonin alterations occurred considerably earlier (2 days of immunization). Thus, it appears that some aspect of the immune response, which occurs relatively soon after antigenic challenge (e.g., IL-1 activation) may come to elicit the hippocampal alterations. Interestingly, it has been reported that following systemic administration of IL-1, an increase in MHPG accumulation was evident in the hypothalamus, as well as in frontal cortex (Kabiersch, Del Rey, Honegger, & Besedovsky, 1988; Dunn, 1988). Similar findings were recently observed concerning the effects of IL-1, and the mitogens, lipopolysaccharide (LPS) and PWM, on hypothalamic MHPG accumulation (Mefford & Heyes, 1990). However, these effects were found to occur both 4 and 24 hr after antigen administration. In addition, these investigators also found that antigen treatment increased the accumulation of hypothalamic HVA and 5-HIAA. Taken together, it appears that several antigens and cytokines will reliably induce central transmitter alterations, and such effects are not restricted simply to NE, but are evident with respect to both DA and 5-HT activity.

In addition to the central amine alterations, it has been demonstrated that antigen administration can result in the production of neuroendocrine hormones. For example, ACTH and endorphins have been shown to be synthesized in response to tumor cells, bacterial

lipopolysaccharide, as well as to viruses, while production of thyrotropin is evoked by the mitogen staphylococcal enterotoxin A (Blalock et al., 1985). Studies in both mice and rats (Besedovsky, Sorkin, Keller & Muller, 1975; Shek & Sabiston, 1983) revealed that SRBC administration can raise plasma corticosterone levels, and that the degree of elevation paralleled the number of plaque forming cells in the spleen. Moreover, the injection of Newcastle Disease Virus into mice provoked a time-dependent increase in plasma corticosterone that was also present in hypophysectomized mice, suggesting a pituitary-independent production of corticosterone (Blalock et al., 1985). These investigators identified an ACTH-like substance in the spleen, indicating that the increase in corticosterone in the hypophysectomized mice was mediated through a 'lymphoid-adrenal axis'.

Particular lymphokines and monokines, notably IL-1 and alpha-interferon, have been implicated as messengers between the immune system and the CNS. These suspicions were derived, in part, from studies indicating that these peptides can influence neural activity. Dafny et al. (1985), for instance, noted that alpha-interferon microiontophoretically applied into somatosensory cortex or dorsal hippocampus increased single cell activity in a dose-dependent manner. When the drug was injected into the ventromedial hypothalamus or the parafasciculus thalamus, neuronal firing either decreased (at low doses of drug) or increased (at high doses of drug). In contrast, gamma-interferon was without effect when injected into these brain regions. Moreover, IL-1, which is structurally similar to the hypothalamic thermoregulatory peptide endogenous pyrogen (Oppenheim, Kovacs, Matsushima & Durum, 1986), and which can be produced by glial cells (Fontana, Weber & Dayer, 1984), has been shown to affect thermoregulation when injected into the anterior hypothalamus of rats (Stitt, 1981). This effect is of interest, since it is well known that

the immune system functions most effectively at elevated body temperatures, especially when confronted with a major antigenic challenge, such as might occur after exposure to a virus. A specific role for IL-1 in immune-brain interactions has been suggested by demonstrations that peripheral injection of IL-1 in mice evoked the release of ACTH from cultured pituitary cells (Woloski, Smith, Meyer, Fuller & Blalock 1985) and stimulated ACTH and corticosterone production (Besedovsky et al, 1975). Several papers have suggested that IL-1's central actions are mediated through the hypothalamus. It has been reported that the peripheral or central application of IL-1 induced the secretion of corticotropin-releasing factor (CRF), which in turn stimulated the pituitary to release ACTH (Sapolsky, Rivier, Yamamoto, Plotsky & Vale, 1987; Berkenbosch, van Oers, Del Rey, Tolders & Besedovsky, 1987). Furthermore, the enhancement of CRF release occurred without a concomitant increase in the production of other hypothalamic hormones (Sapolsky et al., 1987) and at subpyrogenic doses of IL-1 (Berkenbosch et al., 1987). These data suggest that both IL-1 and alpha-interferon can affect neuronal functioning and that the effects of IL-1 occur through the pituitary-adrenal axis. However, it was recently demonstrated that intracerebroventricular administration of IL-1 in rats resulted in a suppression of peripheral cellular immunity. In particular, soon after IL-1 infusion, splenic and blood T-lymphocyte proliferation, IL-2 production and NK cell cytotoxicity were suppressed (Sundar, Becker, Cierpial, et al., 1989). Furthermore, adrenalectomy did not completely block the immunosuppressive effects of IL-1 administration. The authors suggested that cytokine may be acting, at least partially, through sympathetic nerve innervation of the spleen, since central IL-1 infusion suppressed splenic lymphocyte activity at doses that did not affect blood lymphocytes.

In addition to the aforementioned neurochemical and hormonal effects of antigen administration, there is evidence suggesting that the application of immune complexes into the hypothalamus of rats can alter behavior. In particular, Hoffman et al. (1982) demonstrated that the injection of an antigen and its related antibody into the perifornical region of the hypothalamus of rats resulted in a decrease in water consumption. Schupf & Williams (1985), reported that such manipulations evoked eating in sated rats, as well as a potentiation of NE-elicited eating. It has been suggested that the antigen-antibody complexes may have exerted their central effects either by stimulating NE release or by acting on postsynaptic NE receptors (Schupf & Williams, 1985). Another possible explanation was that the complexes produced local inflammation, thereby interfering with normal neuronal functioning (Hoffman et al., 1982). However, it was recently reported that like antigen-antibody complexes, the behavior of animals is altered by low doses of IL-1. In particular, Spadaro, Berridge, & Dunn (1989) demonstrated that intracerebroventricular administration of IL-1 resulted in decrease in exploratory behavior. While it is conceivable that these behavioral changes were secondary to the central amine variations elicited by IL-1, it should be considered that the variations of exploration were, in fact, a consequence of alterations of body temperature induced by the cytokine.

Taken together, the aforementioned studies indicate that an activated immune system can influence neuronal activity in various brain regions. Furthermore, immune products, such as alpha-interferon or IL-1, may act as messengers between the immune and nervous systems. These peptides can modify neuronal activity when applied to discrete brain regions, possibly by actions mediated through the pituitary-adrenal axis. Additionally, an ongoing immune response appears to be capable of

inducing the production of neuroendocrine hormones, which may locally influence immune functioning.

Intriguing speculations about the nature of CNS-immune interactions have been expressed by Blalock and his associates (see Blalock, 1984; Blalock et al., 1985). In contrast to its ability to transduce sensory stimuli such as light or touch, the brain is only capable of recognizing the entry of foreign pathogens into the body through communication with the immune system. In effect, the immune system can be seen to perform the sensory role of identifying foreign matter such as viruses (Blalock, 1984; Blalock et al., 1985). This view may help to reconcile a fundamental paradox of behavioral immunology: while it is clear that many manipulations of the CNS do indeed affect immunocompetence, the majority of immunological responses can nevertheless be carried out in a test tube, in the absence of neural input. According to Blalock et al., the immune system is endowed with its own neuroendocrine capacities which provides it with a means to influence its own functioning, as well as to stimulate and also to be stimulated by the CNS.

f. Stressor-Provoked Alterations of Neurochemical Activity

As previously mentioned, there is substantial evidence indicating that manipulations affecting CNS activity, such as stressor application, may influence a variety of immune responses. Hence, it has been suggested that stressor-induced neurochemical changes may in turn impact on the immune system, thereby enhancing vulnerability to immunologically-based disorders. Moreover, mounting evidence exists that products from an activated immune system may influence neurochemical, neuroendocrine and behavioral activity, and in several ways these alterations are reminiscent of those provoked by stressors. Hence, in

assessing the association between stressors and immune functioning it is necessary to examine alterations of neurochemical activity provoked by stressors. Since detailed reviews concerning the effects of stressors on central neurotransmitter activity are available (Anisman, 1984; Zacharko & Anisman, 1989), only a brief overview will be provided here.

A variety of stressors, including footshock, tailshock, restraint, rotation, cold water, overcrowding, isolation, among others, have been shown to alter central neurochemical activity. The nature and magnitude of the changes vary with stressor characteristics, such as chronicity, controllability and severity, as well as with organismic variables such as the organism's previous stressor history, age and strain. Considerable evidence exists showing that stressors provoke alterations of NE levels and activity. When an organism is exposed to a stressor the utilization and synthesis of central NE are increased. However, if the stressor is relatively severe or if coping mechanisms are unavailable, then utilization may come to exceed synthesis, ultimately resulting in a reduction of NE levels (Anisman, 1984; Tsuda & Tanaka, 1985; Weiss, Glazer & Phorecky, 1976). Stressor-provoked alterations of NE activity appear to be most evident in hypothalamus; however, NE in locus coeruleus, hippocampus, amygdala and frontal cortex may also be altered by a stressor but of somewhat greater severity (Nakagawa, Tanaka, Kohno, Noda, & Nagasaki, 1981). Additionally, it seems that the amine alterations can be induced more readily in older animals than in mature adults (Ida, Tanaka, Kohno, et al., 1982; Ritter & Pelzer, 1978; Shanks, Zalcman, & Anisman, 1990).

It appears that the ability to cope behaviorally with a stressor is fundamental in determining whether NE alterations are evident. Whereas exposure to escapable footshock did not influence NE activity, inescapable shock markedly reduced NE levels (Anisman, Pizzino, & Sklar, 1980; Weiss

et al., 1976). In assessing the effects of a stressor on NE activity, it is important to consider the individual's previous stressor history. Specifically, although stressor-provoked NE changes are relatively short-lasting (Weiss et al., 1981), subsequent exposure to milder forms of the stressor or to stressor-related environmental cues will provoke the NE variations (Cassens, Roffman, Kuruc, Ursulak, & Schildkraut, 1980; Irwin, Ahluwalia, & Anisman, 1986). Thus, despite the relatively transient nature of the stressor-induced amine alterations, it is likely that the proactive consequences may have important implications with respect to the long-term impact of stressors on pathology.

The NE depletions following acute stressor exposure are not evident following repeated application of a stressor. It appears that following a chronic stressor regimen a compensatory increase of NE synthesis is engendered, thus preventing the depletion of NE (see Weiss et al., 1976). Interestingly, it seems that in chronically stressed animals, the enhanced synthesis persists following stressor termination, while the rate of utilization falls off. As a result the concentrations of NE come to exceed those of nonstressed animals (see Anisman & Zacharko, 1990). In addition to the alterations of amine turnover and levels, it has been reported that a chronic stressor regimen is associated with the down regulation of β -NE receptors and cAMP (see Stone, 1987). It is conceivable that these receptor changes play a fundamental role in influencing affective state, and conceivably might be important in the appreciation of the stressor-CNS-immune system topography (Anisman, Zalcman, Shanks, & Zacharko, 1990).

The effects of stressors on dopamine (DA) functioning appear to be more restricted than those of NE. For instance, stressors generally do not reduce DA in whole hypothalamus, but will provoke a reduction of the

amine in the arcuate nucleus (Kvetnansky et al., 1976). It seems that DA activity in mesocortical and mesolimbic structures are particularly responsive to stressors, while DA activity in nigrostriatal regions are hardly affected (Deutch, Tam & Roth, 1985; Herman, Guillonneau, Dantzer, Scatton, Semerdjian-Rouquier & LeMoal, 1982; Herve, Tassin Barthelemy, Blanc, Lavielle & Glowinski, 1979; Thierry, Tassin, Blanc & Glowinski, 1976). In particular, following stressor application, increases of DOPAC accumulation are observed in nucleus accumbens and frontal cortex but not in the substantia nigra. Moreover, stressors may proactively influence DA activity, in that stressor-related environmental cues provoked increases of frontal cortex DA activity (Herman et al., 1982; Deutch et al., 1985). Like NE neuronal activity, it appears that dopaminergic neurons adapt to repeated stressor exposure; however, it seems that this may result, at least in part, to moderation of the excessive DA utilization that is ordinarily engendered by acute stressors (Herman et al., 1984).

Limited data are available concerning the effects of stressors on brain serotonin activity. Nevertheless, stressors have been shown to alter activity of this amine in several brain regions, including the hippocampus, frontal cortex and lateral septum (Kennet & Joseph, 1981; Dunn, 1988b; Hellhammer, Hingtgen, Wade, Shea & Aprison, 1983). Moreover, stressors have been shown to increase serotonin activity in frontal cortex and hypothalamus in a strain-dependent manner (Shanks, Zalcman, & Anisman, 1988). Although it had been believed that a relatively severe stressor is necessary to provoke the changes (Thierry, Javoy, Glowinski, & Kety, 1968), it was recently proposed that 5-HT changes are actually induced very readily, but an adaptation effect may be engendered with continued stressor exposure (Dunn, 1988b). As a result, the 5-HT changes may have been obviated when the stressor regimen employed was too

protracted. Finally, there is reason to suspect that 5-HT alterations associated with aversive events may be influenced by the controllability of the stressor; however, to date differential effects of controllable and uncontrollable shock on 5-HT activity have been demonstrated only within the lateral septum (Sherman & Petty, 1980).

There is some evidence suggesting that stressors may influence central opiate activity. For example, following stressor exposure altered enkephalin and dynorphin levels were evident in the hypothalamus, ventral tegmentum (VTA) and frontal cortex (Fratta, Yang, Hong, & Costa, 1977; Rossier, Guillemin, & Floom, 1978; Morely, Elson, Levine, & Shafer, 1982). Additionally, stressors have been shown to alter the sensitivity of central opioid receptors (Nebeshima, Matsuno, & Kameyama, 1985). As in the case of NE and DA, it seems that opioid activity may be subject to conditioning-like effects in stress paradigms. In particular, Chance et al. (1978) reported that binding of [^3H]-N-Leu-enkephalin was decreased following exposure to stressor-related cues. Finally, there is evidence to suggest that endogenous opiates may serve to modulate stressor-induced DA changes. For example, Kalivas et al. (1983) demonstrated that the augmented DA activity observed in the nucleus accumbens and frontal cortex following stressor exposure was potentiated by infusion of enkephalin analogs into the VTA.

IV. Summary

Substantial evidence exists suggesting that stressors influence immunocyte proliferative capabilities and cytotoxic capacities and compromise orchestrated immune responses. It is generally thought that disturbances of the immune apparatus renders an individual more susceptible to disease. Moreover, the CNS and immune systems appear to

interact in a bidirectional manner. Manipulations which affect CNS activity, including environmental insults, may in turn modulate immune function. Conversely, immunological alterations may have CNS consequences, thereby influencing psychological state.

Despite the growing body of literature evaluating the effects of stressors on immune functioning, it is surprising that little information is available concerning the limiting conditions for stressors to influence an orchestrated immune response (i.e., PFC response). Likewise, even though it is often assumed that central nervous system events influence the impact of stressors on immune functioning, it remains to be fully documented whether psychological variables and stressor characteristics which influence neurochemical or behavioral deficits similarly influence immunological outcomes.

The present series of experiments was designed to (a) determine the impact of stressors on the PFC response and antibody titers to SRBC, (b) evaluate whether a critical period existed following immunization with SRBC during which stressors had the greatest impact, (c) assess whether factors such as stressor controllability and chronicity influenced the magnitude of the immunological changes, (d) determine whether the immune response was modifiable by cues that had been associated with a stressor, (e) assess whether the effects of a stressor are dependent on factors such as age of the organism, which is typically associated with reduced immunocompetence, and finally (f) to determine the profile of neurochemical changes that are provoked at various times following antigen administration.

EXPERIMENT 1a-1b

Exposure to Inescapable Footshock at Various Intervals Following Antigen Administration

Despite the pronounced effects of stressors on immune function, little work has been reported which systematically assessed whether a critical period exists following antigenic challenge during which the effects of stressors are maximal. Yet, several lines of evidence suggest that the time of stressor administration may be fundamental in influencing an immune response to antigenic challenge, as well as the growth of transplanted or virus-induced tumors (see reviews in Sklar & Anisman, 1981; Mon'án, 1981). Experiment 1a assessed the PFC response 96 hr after immunization with SRBC in mice exposed to footshock of 150 uA at various intervals following antigen administration. This particular stressor severity was based on previous studies, conducted in this laboratory, which demonstrated that this treatment induced alterations of brain catecholamines, and that escapable and inescapable treatments differentially influenced hypothalamic and hippocampal norepinephrine activity (see Zacharko & Anisman, 1989). Since time-dependent effects of a stressor on immune functioning might also vary with stressor severity (see Keller et al., 1981), Experiment 1b assessed the effectiveness of more intense footshock (300 uA) on the PFC response and antibody titers.

METHOD

Subjects. A total of 54 and 44 experimentally naive, male, CD-1 mice between 55 to 65 days of age (Charles River Canada Inc., St. Constance Quebec), served as subjects in Experiments 1a and 1b, respectively. Mice were acclimatized to laboratory conditions for 7 - 14 days before being used as experimental subjects. During experiments, mice were housed individually in standard polypropylene cages,

maintained on a 12 hour light/12 hour dark cycle and permitted ad lib access to food and water. The CD-1 strain of mice was used since we have accumulated an appreciable amount of data concerning the effects of stressors on behavioral and central neurochemical alterations in this strain. Accordingly, evaluating the effects of stressors on immune function in this strain afforded comparison between effects on central neurotransmitter alterations and immune response.

Immunization. Sheep red blood cells (SRBC) were prepared by washing citrated sheep blood (Woodlyn Laboratories Ltd., Guelph, Ontario) 3 times in sterile saline. Animals were immunized intraperitoneally with 10^6 cells in a volume of approximately 0.15 ml. Preliminary studies revealed that this dosage lead to a submaximal PFC response. Lower doses (e.g., 5×10^5 cells) yielded particularly variable PFC responses. Moreover, the 10^6 dosage provided a PFC response such that experimental manipulations could provoke either a decrease or an increase of the PFC response without the problem of ceiling or floor effects precluding such outcomes.

PFC Assay. Determination of the PFC response was made using a slight modification of the method of Cunningham and Szenberg (1968). Mice were decapitated, spleens removed and dispersed to a single cell suspension in RPMI medium (Grand Island Biological) supplemented with Hepes buffer (1.0 M solution), Gentamycin sulphate (40 mg/ml) and Streptomycin G (5000 units/ml: John's Scientific). The spleen cells were washed by centrifugation at 400 g for 10 minutes and resuspended in 2 ml of medium. The cells were then layered on a Ficoll-Hypaque gradient (density=1.1) and centrifuged at 700 g for 30 minutes. After centrifugation, the mononuclear cells at the interface were removed, resuspended in 5 ml RPMI and washed 3 times at 400 g. Twenty

microliters of the cell suspension (at 10^7 cells/ml) were combined with 20 ul of SRBC (approximately 2.5×10^8 c/ml) and 20 ul of guinea pig complement (absorbed with SRBC at a ratio of 2:1). The suspension was introduced by capillary action into microslides (Canlab, Pointe Claire, Quebec) with dimensions of 0.22 x 4 x 100 mm at a volume of 80 ul. The ends of the microslides were sealed with a 50% paraplast embedding medium and 50% vaseline mixture. The slides were incubated at 37°C for approximately 1 hour. Plaques were counted by microscopic examination of the microslides at a magnification of 10 X. Data were expressed as PFC/ 10^6 mononuclear cells.

Determination of antibody titer. Mice were decapitated, trunk blood collected and allowed to clot. Samples were subsequently centrifuged at 400 g for 10 min and the supernatant collected and spun at 600 g. Serum complement was then inactivated at 56°C for 30 min. Two fold serial dilutions of inactivated serum, saline, and a 1% SRBC solution was then made in glass microwells. The highest dilution at which aggregation of SRBC was most evident was considered to be the antibody titer and expressed in $\log_2$ units.

Apparatus. Four identical black Plexiglas chambers, measuring 30 x 14 x 15 cm, were employed to deliver inescapable shock. The chamber floors consisted of 0.32 cm stainless-steel rods spaced 1.0 cm apart (center to center) and were connected in series by neon bulbs. In addition, the end walls of the chambers were lined with stainless-steel plates which were connected in series with the grid floor. The lids of each box consisted of red translucent Plexiglas providing limited illumination. Footshock (150 or 300 uA, 60 Hz, AC) was delivered through a 3000-V source, thereby providing a relatively constant current.

Procedure. In Experiment 1a, mice were individually placed in the shock boxes and exposed to 360 shocks of 2 sec duration (150 uA; 9 sec intershock interval) at various intervals (0, 24, 48, 72 or 95 hr) following antigen administration (SRBC; 10^6 cells, ip). An additional group of mice received the antigen, was handled and placed in a new cage, rather than being exposed to shock. Mice were decapitated 96 hr after immunization, spleens were removed and the number of PFC's was subsequently determined. It will be noted that additional experiments conducted in this laboratory revealed that there were no differences in the PFC response between mice that were placed in the shock apparatus but not shocked 72 hr after antigen and mice that served as nonshocked home cage controls. Experiment 1b was identical to Experiment 1a, except that mice were exposed to a shock intensity of 300 uA, and trunk blood was additionally collected for subsequent determinations of serum antibody titers.

RESULTS AND DISCUSSION

Analyses of mononuclear cells in Experiment 1a, as well as in each of the ensuing experiments, revealed that the stressor treatments did not influence the total number of cells. Figure 2 depicts the PFC responses of animals exposed to one session of inescapable footshock applied at various intervals following antigen administration. A marked reduction (about 70%) in the number of PFC's was noted in mice exposed to the stressor 72 hours following immunization. Analysis of variance indicated a significant effect of shock on the number of PFC's, $F(5, 48)=7.59$, $p<.01$. Newman-Keuls multiple comparisons ($\alpha=.05$) revealed that mice shocked 72 hours following antigen administration differed significantly from each of the other groups. In contrast, shock applied at the 0, 24, 48 or 95 hr intervals was without effect on the PFC response.

Like the 150 uA treatment, exposure to 360 shocks of 300 uA (Experiment 1b) influenced the PFC response, $F(5, 38) = 3.73$, $p < .01$. As seen in Figure 3 and confirmed by Newman-Keuls multiple comparisons ($\alpha = .05$) a reduction in the PFC response was evident in mice exposed to the footshock treatment 72 hr after antigen administration. Shock applied at the other intervals did not significantly influence the response relative to nonshocked mice, although a moderate increase in the response was evident in mice that had been shocked 24 hr after immunization. Analysis of the antibody titers revealed a significant treatment effect, $F(5, 47) = 2.77$, $p < .05$ (see Table 1). Newman-Keuls multiple comparisons ($\alpha = .05$) revealed that in mice shocked 72 hr after immunization the antibody titers were significantly lower than in any of the remaining groups. In agreement with the change in spleen PFC, shock applied at other intervals was without effect on antibody titers.

EXPERIMENT 2

Effect of Varying the Number of Shock Presentations on the PFC Response

It has been demonstrated that the extent of central amine variations following uncontrollable footshock was related to the number of shock presentations to which animals were exposed. For example, while 30 shocks did not influence catecholamine turnover, exposure to 60 shocks provoked an increase of amine utilization, and 360 shocks provoked a reduction of both NE and DA (see Anisman and Zacharko, 1990). Likewise, it was reported that immunological changes following footshock were related to the number of shock presentations to which animals were exposed (Weiss et al., 1989). In particular, several components of the immune system were slightly suppressed after a mild stressor (e.g.,

Figure 2. Individual (open circles) and mean (arrowheads) PFC responses of mice that were not shocked (NS) or exposed to footshock (150 uA) at various intervals after immunization. Spleens were taken 96 hr after SRBC inoculation.

Figure 3. Individual (open circles) and mean (arrowheads) PFC responses of mice that were not shocked (NS) or exposed to footshock (300 uA) at various intervals after immunization.

Table 1
Antibody Titer to Sheep Red Blood Cells in Mice Shocked
at Various Intervals Following Immunization

Shock Treatment	Titer ($\text{Log}_2 \pm \text{S.E.M.}$)
NS	5.00 ± 0.43
0 hr	4.89 ± 0.48
24 hr	5.56 ± 0.41
48 hr	5.57 ± 0.97
72 hr	$3.00 \pm 0.69 *$
95 hr	5.62 ± 0.68

* $p < .05$ relative to each other group

handling), enhanced in response to a few brief shocks, and markedly suppressed after a relatively lengthy stressor session. Experiment 2 was conducted to assess whether varying the number of shock presentations would influence the PFC response.

METHOD

Subjects and Procedure. In Experiment 2, subject characteristics were the same as those employed in experiment 1a. All mice were immunized with SRBC (10^6 cells, ip) and assigned to one of 5 treatment groups. Mice were exposed to either 0, 10, 30, 60 or 360 shocks of 2 sec duration (150 uA) 72 hr after antigen administration. In each case the intershock interval was 9 sec, and thus the total amount of time spent in the shock apparatus also varied between groups. The nonshocked animals were handled but maintained in their home cages. Spleens of all animals were removed 96 hr after immunization and the number of PFC's subsequently determined.

RESULTS AND DISCUSSION

The extent of the stressor-induced suppression of the PFC response was found to be directly related to the number of shocks to which animals had been exposed 72 hr following antigen administration. Analysis of variance indicated a significant effect of Shock on the number of PFC's, $F(4,48) = 6.80$, $p < .01$. As seen in Figure 4, the extent of the suppression of the PFC response was directly related to the number of shocks that mice received. Newman-Keuls multiple comparisons ($\alpha = .05$) revealed that nonshocked mice and mice that received only 10 shocks exhibited a significantly higher PFC response than mice that received either 30, 60 or 360 shocks. The latter groups did not differ significantly from one another.

Figure 4. Individual (open circles) and mean (arrowheads) PFC responses of mice exposed to either 0 (NS), 10, 30, 60 or 360 shocks. Shock treatment was applied 72 hr following immunization and spleens taken for assay 24 hr later.

PFC/10⁶ MONONUCLEAR CELLS

Experiment 3

The Time Between Stressor Exposure and Sacrifice is not Critical in Provoking the Immunosuppression

The data of Experiments 1-2 suggested that there existed a critical period following antigen administration during which stressors were most effective in provoking an immunosuppression. In particular, an immunosuppression was evident in animals that had been shocked 72 hr after antigen administration and had their spleens assayed 24 hr afterward. No such suppression was apparent in mice that were shocked 48 hr after antigen administration and assayed 48 hr later. It is possible, however, that the critical parameter in suppression of the PFC response may have been the time between shock and sacrifice, rather than the time between antigen administration and stressor application. Accordingly, Experiment 3 was undertaken to determine whether the suppression of the PFC response induced by shock applied 72 hr after antigen administration would be evident both 24 and 48 hr after stressor application (i.e., whether the time between shock application and sacrifice was critical in producing the immunosuppression observed in Experiments 1-2).

METHOD

Subjects and Procedure. The subject characteristics and apparatus were the same as those of Experiment 1a. A total of 23 CD-1 mice were employed in Experiment 3. Mice received ip administration of the SRBC as in Experiment 1a, and were exposed to either 360 shocks (150 μ A, 2 sec duration) or no shock 72 hr afterward. Spleens of 5 shocked and 5 nonshocked animals were taken 24 hr later, while spleens of the remaining mice (6 shocked and 7 nonshocked) were taken 48 hr after the

stressor treatment. The PFC's were determined as described in Experiment 1a.

RESULTS AND DISCUSSION

Analysis of variance of the number of PFC's indicated a significant main effect of the Shock treatment, $F(1,19) = 5.78$, $p < .05$ (see Figure 5). The PFC response 120 hr after antigen administration was somewhat smaller than it was 96 hr following the SRBC treatment, although not significantly so, and in both cases the effects of the stressor treatment was apparent. Curiously, in this experiment the degree of overlap between shocked and nonshocked mice was greater than in the other experiments. The source for the between experiments difference is not immediately evident. Indeed, the reliability of the variance is not clear owing to the small number of subjects represented in this experiment relative to the other studies. The increased between groups overlap notwithstanding, it is clear from the results of Experiment 4 that the effects of the stressor applied 72 hr after antigen administration was evident 24 and 48 hr later. Thus, the critical parameter in determining the stressor provoked reduction of the PFC response appeared to be the time between immunization and application of the stressor (96 hr after SRBC injection).

EXPERIMENT 4

Serum Antibody Titers at Various Intervals

Following Stressor Exposure

The data from Experiments 1-3 suggested that a stressor applied 72 hr after antigen administration may have a pronounced effect on the PFC response, and this effect was evident 48 as well as 24 hr following stressor application. However, the number of antibody-forming cells in the spleen decrease dramatically following the day of the peak response (and

Figure 5. Individual (open circles) and mean (arrowheads) PFC responses in mice that were exposed to no shock (NS) or 360 shocks of 150 uA 72 hr after immunization and had spleens removed either 96 or 120 hr after immunization.

are not detectable 6-7 days following inoculation). Indeed, it is possible that the immunosuppression evident in spleen following stressor exposure was not reflective of the animal's overall response against the antigen. However, the concentration of serum antibodies (IgG) increases following the peak splenic response, being at highest concentrations approximately 10 days post-immunization. Experiment 4 was undertaken to provide a more general measure of the effect of a stressor on the animals' response to SRBC. Accordingly, serum antibody titers were determined at various intervals following stressor application.

METHOD

Subjects and Procedure. A total of 69 naive male CD-1 mice were employed in Experiment 4. The subject characteristics and apparatus specifications were the same as those described in Experiment 1a. Mice received intraperitoneal administration of SRBC as described earlier, and 72 hr later were exposed to either 360 shocks (300 uA, 2 sec duration) over a 1.1 hr period or were placed in the apparatus but not shocked. Trunk blood of these animals was taken at one of 4 intervals following immunization, namely 96, 120, 168 or 240 hr (i.e., 24, 48, 96 or 168 hr following stressor exposure) and antibody titers determined as described earlier.

RESULTS AND DISCUSSION

The mean serum antibody titers as a function of the treatment condition is shown in Table 2. Analysis of variance revealed that antibody titers varied as a function of the time at which the trunk blood was collected, $F(3, 59) = 3.44$, $p < .05$. Newman-Keuls multiple comparisons ($\alpha = .05$) confirmed that antibody titers increased over time, such that the 96 and 168 hr titers were significantly greater than those of 24 and 48 hr. The shock treatment was found to reduce antibody titers significantly,

(ANSI and ISO TEST CHART No 2)

Table 2

Antibody Titer to Sheep Red Blood Cells ($\text{Log}_2 \pm \text{S.E.M.}$) Determined 24, 48, 96 or 168 hr Following Stressor Exposure (i.e. 96, 120, 168 or 240 hr after antigen administration)

	24 hr	48 hr	96 hr	168 hr
No Shock	4.40 ± 0.40	4.17 ± 0.17	6.00 ± 0.53	5.71 ± 0.54
Shock	$2.33^* \pm 0.56$	$3.17^* \pm 0.60$	$3.67^* \pm 0.47$	$3.93^* \pm 0.48$

*shock treatment significantly reduced titers ($p < .01$) irrespective of the time at which blood was collected. Additionally, titers increased over days ($p < .05$).

irrespective of the time at which the blood was collected, $F(1, 59) = 21.99$, $p < .01$. Thus, it appears that the shock treatment had a fairly persistent effect, with antibody titers still reduced 168 hr after the shock treatment.

EXPERIMENT 5a-5b

Stressor Controllability and The PFC and Antibody Responses

While an appreciable amount of data exist concerning the effects of coping mechanisms on behavior (see Zacharko & Anisman, 1989), only limited data are available concerning the role of coping factors on immune functioning. As previously discussed, stressor controllability may be fundamental in determining the stressor-provoked alterations of lymphocyte proliferative responses and NK cell activity (Laudenslager et al., 1983; Shavit et al., 1983). However, Gamzu et al. (1984) reported that in their laboratory the effects of the stressor manipulations were not particularly reliable for reasons they were unable to determine. Indeed, Maier and Laudenslager (1988) have recently reported an inability to replicate their earlier findings (i.e., those of Laudenslager et al., 1983), and suggested in a companion paper (Laudenslager et al., 1988) that the antibody response, rather than lymphocyte proliferation, was most sensitive to the effects of stressors. Experiments 5a and 5b were conducted to determine whether stressor controllability influenced the suppression of the PFC response observed in mice shocked 72 hr following SRBC administration.

METHOD

Subjects and Apparatus. A total of 23 and 25 CD-1 mice served as subjects in Experiment 5a and 5b respectively. The subject characteristics were the same as those of Experiment 1a. Shock was delivered in black Plexiglas shuttleboxes, which measured 26.4 x 9.0 x 15.5 cm. The roof of

the boxes, the grid floor and its wiring, and the nature of the shock sources were identical to those of the shock boxes which delivered inescapable shock. Each shuttlebox was divided into two compartments by a stainless steel wall, partially made up of a solenoid-controlled, horizontally movable stainless steel gate. In the open-gate position, a 1.27 cm hurdle separated the two compartments, and a 5.2 x 6.1 cm space permitted access into the adjacent compartment. The hurdle was lined with stainless steel plates which were connected in series with the grid floor, thereby preventing the mice from avoiding shock by sitting on the hurdle. Situated 1.1 cm on either side of each hurdle were two infrared photocells 1.27 and 2.54 cm above the grid floor, respectively. The photocells were wired such that if the beams on both sides of the hurdle were crossed simultaneously, as would occur when the mouse was halfway between the gate, the cells would not trigger. When the mouse crossed the beam in the shock compartment and broke the beam on the safe side only, the cell was triggered. An additional set of photocells was located 2.54 cm from the end walls. If a mouse did not trigger the first set of cells (e.g. jumped over the cells), these latter cells were triggered. The photocells detected the sides of the apparatus in which the animal was located. Latencies to respond were printed independently for each of the shuttle boxes. All boxes were housed in sound attenuated chambers. The shuttleboxes, controlled by a microcomputer system (Carleton University Science Workshops), were programmed in pairs, such that one box was slaved to its partner. Among each pair, one box was fully functional, such that shock terminated when an animal crossed from the shock chamber to the adjacent compartment. In contrast, in the second box shock of the same intensity was delivered on exactly the same schedule, but the

photocells and gate were inoperative. Accordingly, shock in the slave box terminated only when the shock in the partner box terminated.

Procedure. In Experiment 5a, mice were immunized with SRBC, as described earlier, and were returned to their home cages. Seventy-two hours following antigen administration animals were individually placed in the shuttleboxes and exposed to one of three treatments. Mice of one group received 360 escapable shock (150 μ A) trials at 9 sec intervals between trials. On each trial shock was presented to the animals such that escape was not possible during the initial 0.5 sec. Thereafter the gate separating the compartments opened permitting access to the adjacent shock-free compartment. The escape delay procedure was instituted so that animals would receive approximately 2 sec of shock per trial. This procedure was previously found to influence differentially central norepinephrine concentrations as well as performance in several behaviors (see Zacharko & Anisman, 1989). For mice of the yoked group shock onset occurred at exactly the same time as it did for mice in the escapable group; however, shock offset was independent of the animal's responses. Rather, shock in this group terminated when the animal in the escapable group made an appropriate response. Thus animals of both groups received the same amount of shock at precisely the same time, but only in the former condition was shock offset dependent on the response emitted by the animal. Mice of the third group were not exposed to shock, but were placed in the shuttleboxes for an equivalent shock-free period. Experiment 5b was identical to Experiment 5a except that mice were exposed to 60 shocks.

RESULTS AND DISCUSSION

Figure 6 illustrates that uncontrollable and controllable shock (360 shocks) were found to be equally effective in suppressing plaque

formation. Analysis of variance indicated that the shock treatment significantly altered plaque formation, $F(2, 17) = 23.74$, $p < .01$. Newman-Keuls multiple comparisons ($\alpha = .05$) revealed that the escapable and inescapable shock treatments both reduced the PFC response relative to nonshocked animals (70 and 67%, respectively), but that the two shock groups did not differ from one another. When a smaller number of shock trials were used (Experiment 5b) a reduction of the PFC response of about 35% was induced in both the escapable and yoked groups. The analysis of variance, however, revealed that the shock treatment did not significantly affect plaque formation ($p > .05$).

EXPERIMENT 6

Immunosuppression Following Exposure to Stressor-Related Environmental Cues

Analyses of the central neurochemical consequences of stressors revealed that uncontrollable footshock resulted in NE utilization exceeding synthesis, and consequently transmitter concentrations declined (see reviews in Anisman, 1984; Anisman & Zacharko, 1990). The neurochemical changes appeared to be subject to conditioning like effects, in that the amine variations could again be established upon reexposure to a milder form of the stressor or to cues that had been associated with the stressor (Anisman & Sklar, 1979; Herman, et al., 1979; Irwin, Ahluwalia & Anisman, 1986; Cassens et al., 1981). It has been reported that exposure to cues associated with a stressor likewise induced a marked reduction of lymphocyte proliferation in response to Concanavalin A (Lysle et al., 1988). Experiment 6 was conducted to determine whether cues associated with a previously applied stressor would similarly influence the PFC response and antibody titers.

Figure 6. Individual (open circles) and mean (arrowheads) of mice that received either no shock (NS), 360 escapable shocks (escape) or an identical amount of inescapable shock (yoked) 72 hr after inoculation.

METHOD

Subjects and Apparatus. A total of 61 male, CD-1 mice served as subjects in experiment 6. The subject characteristics and the apparatus details were the same as those of Experiment 1.

Procedure. In experiment 6, mice were placed in the shock chambers and exposed to either 360 shocks (150 μ A, 2 sec) at 9 sec intervals or were handled but not shocked. Fourteen days afterward, mice were immunized intraperitoneally with SRBC (10^6 cells in a volume of approximately 0.15 ml) that had been washed 3 times in sterile saline. Mice were returned to their cages and 72 hr later (the time of greatest vulnerability to the stressor induced immunosuppression) were either exposed to the shock apparatus (without shock being delivered) for a 1.1 hr period, or handled but not placed in the apparatus. Spleens were removed and trunk blood collected 24 hr later for determination of PFC's and antibody titers.

RESULTS AND DISCUSSION

Analyses of mononuclear cells revealed that the stressor treatments did not influence the total number of cells. The PFC response of individual animals of each group is shown in Figure 7. Analysis of variance of the PFC response revealed a significant Initial Shock Treatment X Reexposure Treatment interaction, ($F(1,57) = 8.68, p < .01$). Newman-Keuls multiple comparisons ($\alpha = .05$) of the simple effects confirmed that exposure to cues that had been associated with the stressor provoked a suppression of the PFC response relative to mice that had not initially been shocked or in animals that had been initially shocked but were not reexposed to the apparatus. Paralleling the data for the PFC response, and as can be seen in Table 3, analysis of serum antibody titers revealed a significant Initial Shock Treatment x Reexposure Treatment interaction, $F(1,52) = 13.95$,

Figure 7. Individual (open circles) and mean (arrowheads) PFC responses. Mice were initially either handled (N) or exposed to 360 shocks (S). Animals were inoculated intraperitoneally with SRBC (10^6 cells) 14 days afterward and 72 hr later either handled (N) or reexposed to the shock apparatus without being shocked (A). Spleens were taken 96 hr following antigen administration.

PFC/10⁶ MONONUCLEAR CELLS

Table 3

Antibody Titer ($\text{Log}_2 \pm \text{S.E.M.}$) To Sheep Red Blood Cells In Mice Initially Exposed to No Treatment or 360 Shocks and Reexposed to No Treatment or Apparatus

	No Treatment	Apparatus
No Treatment	4.86 ± 0.26	4.64 ± 0.21
Shock	4.79 ± 0.33	$2.29 \pm 0.28^*$

* $p < .05$ relative to each other group

$p < .01$). Newman-Keuls comparison ($\alpha = .05$) of the simple effects confirmed that mice initially shocked and then reexposed to the shock apparatus had lower serum antibody titers than mice that were initially shocked but not reexposed to the apparatus or animals that had initially not been shocked and then placed in the apparatus. The latter two groups did not differ from one another or from mice that had not been exposed to the shock or to the test apparatus subsequently. Evidently, just as a stressor applied 72 hr after inoculation may result in a suppression of the PFC response and serum antibody titers, cues that had been previously paired with a stressor may likewise induce the immunosuppression.

Experiment 7a-7b

Proactive Effects of a Stressor Applied in Close Temporal Congruity with Immunization

Although a stressor applied 72 hr after immunization appears to compromise immune functioning, there is reason to believe that there exists a brief period following immunization during which a stressor may either enhance immune functioning, or at least, limit the effects of a subsequently applied stressor. Esterling and Rabin (1987) found that when a stressor (rotation) was applied 24, 48 and 72 hr after immunization, the PFC response was reduced. However, when the stressor was applied 0, 24, 48, and 72 hr after immunization, then the PFC response was comparable to that of nonstressed animals. It was almost as if a stressor applied at time 0 had a protective effect which limited the reduction of the PFC response ordinarily provoked by subsequently applied stressors. Experiment 7a was conducted to confirm that stressor exposure soon after inoculation would prevent the effects provoked by a single session of uncontrollable footshock applied 72 hr later.

Furthermore, if such an effect is due to proactive effects of the initial treatment, possibly involving a compensatory-like immunological response, then it might be expected that cues associated with a stressor applied immediately after immunization would subsequently produce an immunoenhancement.

METHOD

Subjects, Apparatus and Procedure. A total of 90, male, CD-1 mice served as subjects in Experiment 7a. The subject and apparatus details were the same as those described in Experiment 1. Mice received SRBC as in the preceding studies, and immediately thereafter were exposed to either footshock (360 shocks, 2 sec duration, 150 uA at intervals of 9 sec) or handling without being shocked. These groups were further subdivided, such that 72 hr following immunization they were either reexposed to the shock treatment (360 shocks, 150 uA, 2 sec), exposed to the shock apparatus, or handled but neither shocked nor exposed to the shock apparatus. Spleens and trunk blood were taken 96 hr after inoculation.

RESULTS AND DISCUSSION

Analyses of variance of the PFC response and of the serum antibody titers revealed a significant interaction between the Initial Shock Treatment and the Reexposure Condition $F(2,84 \text{ and } 2, 71)=4.40 \text{ and } 3.51$, $p<.05$, respectively. Several blood samples were lost and hence the number of samples in the antibody analysis differed from the number represented in the analysis of the PFC response. As seen in Figure 8 and Table 4, and confirmed by Newman-Keuls multiple comparisons ($\alpha=.05$), in previously nonshocked mice exposure to footshock 72 hr after immunization resulted in a reduction of the serum antibody titers and of the PFC response relative to mice that had not been

Figure 8. Individual (open circles) and mean (arrowheads) PFC responses. Mice were immunized with SRBC (10^6 cells) and immediately thereafter either handled (N) or reexposed to 360 footshocks (S). Seventy-two hours later, animals were either handled (N), reexposed to the shock apparatus without being shocked (A) or reexposed to 360 shocks (S). Spleens were taken 96 hr following antigen administration.

PFC/10⁶ MONONUCLEAR CELLS

Table 4

Antibody Titer to Sheep Red Blood Cells ($\text{Log}_2 \pm \text{S.E.M.}$) in Mice Initially Exposed to No Treatment or 360 Shocks and Reexposed to Either No Treatment, Apparatus or 360 Shocks

<u>Initial Treatment</u>	<u>Reexposure Treatment</u>		
	No Treatment	Apparatus	Shock
No Treatment	4.67 ± 0.28	4.31 ± 0.37	$2.85 \pm 0.40^*$
Shock	4.40 ± 0.43	$5.67 \pm 0.27^*$	4.33 ± 0.33

* $p < .05$ relative to nontreated animals

exposed to a stressor. In the absence of any further treatment, exposure to a stressor immediately after antigen administration did not influence either antibody titers or the PFC response relative to nonstressed animals. However, mice that received shock immediately after inoculation and were again shocked 72 hr later exhibited scores that did not differ from those of mice that received only the initial stressor treatment, and significantly exceeded those of mice that were shocked only at the 72 hr interval. Of particular interest was the finding that in animals initially exposed to footshock and then later reexposed to the cues associated with shock (i.e., the shock apparatus) the antibody titers and the PFC response significantly exceeded those of mice that had either not been shocked, received only the initial shock treatment or had been shocked on both occasions. Taken together, these data indicate that a stressor applied immediately after immunization will effectively counteract the immunosuppression ordinarily provoked by a stressor applied 72 hr after antigen administration. Moreover, simply presenting the cues that had been associated with the stressor effectively enhanced the immunological response.

While shock-related cues provoked an immunoenhancement, it is not clear whether the initial shock treatment was essential for this effect. After all, exposure to the cues that had been associated with the antigen may have been sufficient to produce the immunological change. To evaluate this possibility, in Experiment 7b mice were injected with the antigen and then placed in the apparatus without shock being delivered or were simply handled. These groups were subdivided and 72 hr later were either reexposed to the same apparatus or were handled ($n=7-8/\text{group}$). Analyses of the serum antibody titers and of the PFC response revealed that none of the groups differed from one another (see Table 5). Thus the

Table 5

Mean PFC Response (/10⁶ Mononuclear Cells $\pm$ S.E.M.) and Antibody Titer ($\log_2 \pm$ S.E.M.) to Sheep Red Blood Cells in Mice Exposed to No Treatment or Apparatus Immediately Following Immunization and Reexposed to Either No Treatment or Apparatus 72 Hours Afterward

Treatment	PFC Response	Antibody Titer
No Treatment + No Treatment	3007 $\pm$ 411	4.25 $\pm$ 0.38
No Treatment + Apparatus	3276 $\pm$ 435	4.43 $\pm$ 0.47
Apparatus + No Treatment	2694 $\pm$ 229	4.29 $\pm$ 0.28
Apparatus + Apparatus	2450 $\pm$ 373	4.00 $\pm$ 0.50

immunoenhancement was not due simply to the pairing of the antigen with particular environmental cues. Rather application of the stressor in the presence of these cues was fundamental to the immunological change. It might be argued that in the present experiment pairing of the apparatus cues with the antigen may simply not have been a sufficiently powerful stimulus to result in conditioning. However, this position is not supported by the present findings, since it would necessarily have been expected that reexposure to shock (if it were acting only as a stimulus) would have resulted in a greater conditioned enhancement than simply reexposure to the apparatus cues.

EXPERIMENT 8a-8c

Effects of Repeated Stressor Exposure on the PFC and Antibody Responses

It has been demonstrated that immunological deficits ordinarily evident following an acute stressor are absent in animals repeatedly exposed to the stressor. In fact, it has been reported that immune functioning may be enhanced after chronic stressor exposure. For example, Monjan and Collector (1977) reported that while short-term exposure to auditory stress markedly reduced the ability of T- and B-lymphocytes to proliferate *in vitro*, proliferative responses were enhanced in rats that received long-term stressor exposure. Likewise, it has been reported (Livnat, Irwin, Ader, Gallo & Anisman, 1986) that although NK cell activity was suppressed by acute stressors, a chronic stressor regimen enhanced NK activity. The data from Experiment 7a indicated that exposure to footshock immediately following immunization had the effect of counteracting the immunosuppression otherwise provoked by shock applied 72 hr after antigen administration. Experiment 8a assessed whether the reduction of the PFC response ordinarily induced by shock

administered 72 hr after immunization would be absent in animals repeatedly exposed to footshock commencing immediately after SRBC inoculation. Experiment 8b was conducted to determine whether repeated exposure to footshock prior to inoculation would likewise result in an adaptation of the immunosuppressive effect ordinarily associated with an acute stressor. Inasmuch as a stressor applied soon after inoculation may limit the immunosuppression ordinarily elicited by a stressor applied 72 hr after immunization, in Experiment 8b the stressor regimen terminated 24 hr prior to antigen administration. Finally, since stressor application either 24 hr before or immediately following inoculation appears to counteract the immunosuppressive effects of subsequent exposure to the stressor (i.e., 72 hr after inoculation), in Experiment 8c the final stressor session was applied 48 hr before antigen administration.

METHOD

Subjects and Apparatus. Experiments 8a-8c employed 158 male, CD-1 mice. The subject and apparatus specifications were the same as those described in Experiment 1.

Procedure. In Experiment 8a, mice received SRBC (10^6 cells). Independent groups of mice then received either, (i) shock (150 μ A, 2 sec duration, 9 sec ITI) 72 hr after immunization ($n=9$), (ii) shock treatment (150 μ A, 2 sec duration, 9 sec ITI) on 5 occasions: 0, 24, 48, 72 and 95 hr after immunization ($n=8$), or (iii) left undisturbed in their home cages ($n=8$). Animals were sacrificed 96 hr after inoculation, whereupon spleens were taken for subsequent determinations of the PFC responses and trunk blood collected for evaluations of serum antibody titers. In experiment 8b, mice were either (i) exposed to footshock on each of 15 successive days (150 μ A, 2 sec duration, 9 sec ITI), (ii) left undisturbed in their home cage for 14 successive days and exposed to one session of footshock on day 15,

or (iii) left undisturbed in their home cages for 15 successive days. Twenty-four hr following the last stressor session, mice were inoculated with SRBC (10^6 cells) and 72 hr thereafter mice in each group were subdivided and either received one shock session (150 μ A, 2 sec duration, 9 sec ITI) or were exposed to the shock apparatus without shock being delivered. In Experiment 8c, mice were either (i) exposed to footshock (as described in Experiment 8b) on 13 successive days, (ii) left undisturbed in their home cage for 12 successive days and exposed to one session of shock (as described in Experiment 8b) on day 13, or (iii) left undisturbed in their home cage. Forty-eight hours following the final shock session mice were inoculated with SRBC and 72 hours later were subdivided and either were exposed to shock (as in Experiment 8b), placed in the shock apparatus without shock being delivered, or left undisturbed in their home cages. In both Experiments 8b and 8c spleens were taken 96 hr after inoculation for PFC determinations and trunk blood was collected for determinations of antibody titers.

RESULTS AND DISCUSSION

Figure 9 and Table 6 show the mean PFC response and antibody titer for each of the groups in Experiment 8a. Several samples were lost during the course of the experiment, and hence the degrees of freedom for the two variables differed in Experiment 8a, as well as in Experiments 8b-8c. Analysis of variance revealed that in Experiment 8a the stressor treatment influenced both the PFC response $F(2,22)=8.07$, $p<.05$ and antibody titers, $(2, 18) = 3.67$, $p<.05$. Consistent with earlier experiments, Newman-Keuls multiple comparisons ($\alpha = .05$) confirmed that a single session of shock applied 72 hr after immunization reduced both measures of the immune response relative to nonstressed mice or mice that received repeated footshock exposure. The latter two groups did not differ

from one another. While these data suggest that the effects of an acute stressor may be eliminated by repeated exposure to the stressor, it will be recalled that footshock applied on the day of inoculation also served to antagonize the effects of shock applied 72 hr after inoculation (Experiment 7a). Thus, the apparent adaptation may be a reflection of the stressor applied on the day of immunization as opposed to the effects of repeated stressor exposure.

Experiment 8b was conducted to determine whether repeated exposure to footshock prior to inoculation would likewise result in an adaptation to the immunosuppressive effect ordinarily associated with an acute stressor. Inasmuch as a stressor applied soon after inoculation may limit the immunosuppression ordinarily elicited by a stressor applied 72 hr after inoculation, in Experiment 8b the stressor regimen terminated 24 hr prior to SRBC inoculation. The mean PFC response as a function of the shock treatment is shown in Figure 10. Analysis of variance confirmed that the PFC response varied as a function of the Initial Shock Treatment x Reexposure Treatment that mice received, $F(2, 65)=6.31$, $p<.01$. Newman-Keuls multiple comparisons of the means comprising this interaction revealed that in mice that initially had not been shocked (i.e., prior to antigen administration) exposure to footshock 72 hr after inoculation resulted in a marked reduction of the PFC response relative to nonshocked mice. In contrast, in mice that were exposed to the stressor 24 hr prior to inoculation, later reexposure to the stressor 72 hr after antigen did not significantly influence the PFC response. Indeed, the PFC response in the reexposed mice exceeded those of animals that had received a single session of footshock 72 hr after inoculation. It will be recalled that Experiment 7a indicated that in mice exposed to footshock soon after inoculation, subsequent reexposure to stressor related cues resulted in an

enhancement of the PFC response. In the present experiment, when the initial stressor session was applied 24 hr prior to antigen treatment, later exposure to the stressor related cues provoked a 30% increase of the PFC response relative to mice that were exposed to the stressor, but this effect was not statistically significant. Finally, in mice that received repeated exposure to the stressor over 15 days, subsequent reexposure to the stressor did not result in the immunosuppression seen after acute footshock. In fact, upon reexposure to the stressor, the PFC response significantly exceeded that of animals that had not been reexposed to the stressor. In effect, the repeated shock treatment not only prevented the reduction of the PFC response ordinarily associated with acute footshock, but actually conferred an immunoenhancement in mice that were reexposed to the stressor.

Although the effects of footshock on antibody titers were found in the preceding studies to be congruent with the variations of the PFC response, this parallel was not maintained in Experiment 8b. While acute footshock applied 72 hr following inoculation provoked a small reduction of antibody titers, this effect was not statistically significant (see Table 7). The analysis of variance did reveal, however, that the initial shock manipulations influenced antibody titers, $F(2,68)=4.78$, $p<.05$. Multiple comparisons confirmed that a single session of footshock applied 24 hr prior to inoculation or repeated exposure to the stressor increased antibody titers relative to animals that had not been exposed to the stressor prior to SRBC inoculation.

The results of Experiment 8b confirmed that a chronic stressor regimen applied prior to antigen administration (and terminating 24 hr before SRBC inoculation) prevented the immunosuppression ordinarily elicited by footshock administered 72 hr after inoculation. In fact, this

treatment provoked a significant immunoenhancement. Yet, in animals that received the shock treatment 24 hr prior to immunization the immunosuppressive effect was also prevented. Thus, it is possible that the timing of the initial stressor treatment relative to inoculation may have contributed to this adaptation. Accordingly, Experiment 8c was conducted to establish whether the effects of a chronic stressor regimen that terminated 2 days prior to inoculation would influence the immunosuppression engendered by a stressor applied to 72 hr after SRBC inoculation. Analysis of variance confirmed that the PFC response varied as a function of the Initial Shock treatment x Reexposure treatment interaction, $F(4,53)=3.63$, $p<.05$. As seen in Figure 11, and confirmed by Newman-Keuls multiple comparisons ($\alpha=.05$), in the absence of the chronic shock treatment, exposure to footshock 72 hr after SRBC inoculation resulted in a marked reduction of the PFC response relative to apparatus exposed and nontreated animals. Among mice that received either acute footshock 48 hr prior to inoculation or mice that had received the chronic stressor prior to immunization, subsequent apparatus exposure did not enhance the immune response. Thus it appears that if the initial stressor session and SRBC inoculation are separated by 48 hr, later exposure to stressor related cues will not provoke an immunoenhancement (as observed when the initial stressor exposure and inoculation are administered in closer temporal congruity (i.e., as in Experiment 7a)). It should be noted, however, that in the acutely stressed mice, later reexposure to the stressor at the 72 hr interval did not elicit the immunosuppression, while in chronically stressed animals reexposure to the stressor itself resulted in a significant immunoenhancement relative to chronically stressed mice that had not been reexposed to the stressor.

The effects of the stressor on antibody titers were in most respects comparable to the effects on the PFC response (see Table 8). The analysis of variance revealed that antibody titers varied as a function of the Initial Shock treatment x Reexposure treatment, $F(4,57)=5.43$, $p<.05$. Newman-Keuls multiple comparisons ($\alpha=.05$) of the simple effects revealed that in mice that had not been stressed prior to inoculation a marked reduction of antibody titers was induced by footshock applied 72 hr after SRBC inoculation. As in the case of the PFC response, the shock treatment applied to mice that had been exposed to the stressor 48 hr prior to immunization did not differ from that of mice that had not been reexposed to the stressor. However, reexposure to the apparatus in which mice had initially been shocked prior to inoculation resulted in a significant reduction of the PFC response. Finally, in chronically shocked mice reexposure to either the stressor or to cues associated with the stressor did not result in a reduction of antibody titers. In fact, upon reexposure to the stressor antibody titers exceeded those of mice that had initially received acute shock or no shock and then exposed to footshock, or animals that had received the chronic shock treatment and then either not stressed or exposed to the cues associated with the stressor. Thus, it seems that an acute stressor applied even as long as 48 hr prior to SRBC inoculation can prevent the immunosuppression provoked by a subsequently applied stressor, while a chronic stressor treatment may favor the development of an immunoenhancement.

Figure 9. Individual (open circles) and mean (arrowheads) PFC responses. Mice were immunized with SRBC and either received no shock (NS), one session of shock 72 hr after inoculation (72 hr) or shock on 5 days commencing immediately after inoculation (0-95 hr).

Table 6

Antibody Titer ($\text{Log}_2 \pm \text{S.E.M.}$) to Sheep Red Blood Cells in Mice Immunized with SRBC and Exposed to Either No shock (NS), One session of Shock 72 hr after inoculation (72 hr) or Shock on 5 days Commencing Immediately After Inoculation (0-95 hr).

Treatment	Antibody Titer
NS	4.67 ± 0.80
72 hr	$3.12 \pm 0.44^*$
0-95 hr	4.86 ± 0.26

* $p < .05$ relative to each other group

Figure 10. Mean ($\pm$ S.E.M.) PFC responses. Mice were individually caged and either exposed to chronic footshock (C) (360 shocks on each of 15 days), one session of shock (S) (on day 15) or were left undisturbed in their home-cage for 15 days (N). Mice were immunized with SRBC on day 16 and 72 hr later exposed to either 360 shocks (S), or to the shock apparatus without being shocked (A).

Table 7

Antibody Titer to Sheep Red Blood Cells ($\text{Log}_2 \pm \text{S.E.M.}$) in Mice Initially Exposed to No Treatment, Acute Shock or Chronic Shock and Reexposed to Either Apparatus or Shock

<u>Initial Treatment</u>	<u>Reexposure Treatment</u>	
	Apparatus	Shock
No Treatment	3.54 ± 0.41	2.50 ± 0.51
Acute Shock	4.84 ± 0.46	4.23 ± 0.57
Chronic Shock	4.23 ± 0.54	4.50 ± 0.61

Figure 11. Mice were individually caged and either exposed to repeated footshock (C) (360 shocks on each of 13 days), one session of shock (S) (on day 13) or were left undisturbed in their home-cage for 13 days (N). Mice were immunized with SRBC on day 14 and 72 hr later exposed to either 360 shocks (S), to the shock apparatus without being shocked (A) or received no treatment (N). The immunosuppression evident in animals receiving one shock treatment 72 hr after inoculation was absent in animals repeatedly exposed to the stressor.

Table 8

Antibody Titer to Sheep Red Blood Cells ($\text{Log}_2 \pm \text{S.E.M.}$) in Mice Initially Exposed to No Treatment, Acute Shock or Chronic Shock and Reexposed to Exposed to No Treatment, Acute Shock or Chronic Shock

	<u>Reexposure Treatment</u>		
	No Treatment	Apparatus	Shock
<u>Initial Treatment</u>			
No Treatment	5.28 $\pm$ 0.84	5.14 $\pm$ 0.26	2.71 $\pm$ 0.56
Acute Shock	4.86 $\pm$ 0.55	2.71 $\pm$ 0.78*	3.55 $\pm$ 0.53
Chronic Shock	5.14 $\pm$ 0.51	4.62 $\pm$ 0.42	6.71 $\pm$ 0.52**

* $p < .05$ relative to mice initially left undisturbed in their home cage

** $p < .05$ relative to mice that had initially received acute shock or no shock and then exposed to footshock, or animals that had received the chronic shock treatment and then either not stressed or exposed to the cues associated with the stressor.

EXPERIMENT 9

Age-Related Alterations of the PFC Response and Antibody Titers

Following Stressor Exposure

It is well established that aging is associated with alterations of immune functioning. For example, the antibody response to SRBC in mice appears to peak between 3 and 6 months of age and begins to decline thereafter (see Makinodan & Kay, 1980). Additionally, it has been reported (Thoman & Weigle, 1981) that the ability of T-cells to proliferate *in vitro* in response to a mitogen and to produce chemical messengers (e.g., IL-2) is impaired in aging organisms. Although vulnerability to disease processes appears to increase with age, few data are available regarding the effects of stressors on immunocompetence as a function of the age of the organism. The data from Experiment 1a-1b indicated that in 2-3 month old CD-1 mice footshock applied 72 hr following SRBC inoculation resulted in a marked suppression of immune functioning, while at other intervals after immunization (i.e., 0, 24, 48 or 95 hr) immune functioning was unaffected. Experiment 9 was undertaken to determine whether an acute stressor would (a) have more pronounced effects on the PFC response and on antibody titers in 9 month than in 3 month old mice, and (b) influence the critical period during which stressors may come to affect immune functioning.

METHOD

Subjects, Apparatus and Procedure. A total of 86 and 82 male, CD-1 mice, 3 and 9 months of age, respectively, served as subjects. The 3 month old mice (Charles River Canada, Inc., St. Constance, Quebec) were obtained at 6 weeks of age and maintained in our laboratory until time of testing (3 or 9 months of age). Additionally, 4 month old retired breeders were procured from Charles River Canada, Inc. and maintained in our

laboratory until they reached 9 months of age, whereupon they were employed as experimental subjects. Mice were housed in groups of 5 in standard polypropylene cages, maintained on a 12 hour light/12 hour dark cycle and permitted ad lib access to food and water.

Mice were immunized with SRBC as in previous experiments. Preliminary studies indicated that in 9 month old mice immunized with 10^6 cells the PFC response was approximately 50% lower than the responses of 3 month old mice. As in previous experiments, this SRBC dose permitted detection of both suppression and enhancement of immune functioning resulting from the experimental manipulations. At various intervals following inoculation (0, 24, 48, 72 or 95 hr) independent groups of mice were exposed to 360 shocks of 2 sec duration (150 μ A, 9 sec intershock interval) or were left undisturbed in their cages. Mice were decapitated and spleens removed 96 hr following antigen administration for PFC determination. Additionally, trunk blood was collected for subsequent determination of serum antibody titers.

RESULTS AND DISCUSSION

The mean PFC response for each group is shown in Figure 12. Analysis of variance of the PFC scores revealed a significant Age x Shock treatment interaction, $F(5,156) = 2.45$, $p < .05$. Newman-Keuls multiple comparisons ($\alpha = .05$) of the simple effects indicated that the PFC responses of nonshocked 9 month old mice were significantly lower than those of nonshocked 3 month old mice. In both age groups the PFC response was reduced in mice that were shocked 72 hr following immunization. As predicted, in the 9 month old mice, shock applied 72 hr after inoculation produced a greater reduction of the PFC response than in similarly treated 3 month old animals. As previously observed (see Experiment 1), in the 3 month old mice, shock applied at intervals other than 72 hr was without

effect on the PFC response relative to nonshocked animals, although there was some trend towards an increase of the PFC response in mice shocked immediately after inoculation ($p < .10$). Surprisingly, in the 9 month old mice exposure to shock immediately or 24 hr following immunization resulted in a marked increase of the PFC response. While unexpected, this immunofacilitation was not a spurious one and was noted in each of 4 independent replications. These data indicate that the time frame for vulnerability to the immunosuppressive effects of shock is unchanged in the older mice, but that exposure to shock during the early phases of the response may potentiate the PFC and antibody responses of older mice.

Analysis of variance of antibody titers revealed significant main effects of Age, $F(1,167)=19.64$, $p < .05$, and Shock treatment, $F(5,167)=6.09$, $p < .05$ (see Table 9). Although a significant Age x Shock treatment interaction was not obtained, Newman-Keuls multiple comparisons ($\alpha = .05$) were performed since a priori predictions had been made on the basis of Experiment 1. Antibody titers were significantly decreased in nonshocked 9 month old mice compared with nonshocked 3 month old animals. Consistent with the PFC results, antibody titers were significantly reduced in 3 month old mice shocked 72 hr after immunization compared with 3 month animals in the remaining groups. However, compared with other 9 month old mice, stressor exposure did not influence antibody titers in 9 month old mice. This, however, may have been due to a floor effect, since antibody titers of nonshocked older mice were particularly low. Paralleling the PFC results, a marked enhancement of antibody titers was evident in older mice shocked within 24 hr of inoculation, compared with 9 month old animals in the remaining groups.

Figure 12. Mean ($\pm$ S.E.M.) PFC responses of mice (3 or 9 months of age) that were not shocked (NS) or exposed to footshock (150 μ A) at various intervals (hr) after SRBC inoculation (10^6 cells). Spleens were taken 96 hr after immunization.

PFC/10⁶ MONONUCLEAR CELLS

Table 9

Antibody Titer ($\text{Log}_2 \pm \text{S.E.M.}$) to Sheep Red Blood Cells in 3 and 9 Month Old Mice
Shocked at Various Intervals Following Immunization

	NS	0	24	48	72	95
3 Months	4.40 $\pm$.33	4.71 $\pm$.56	4.80 $\pm$.41	4.27 $\pm$.39	2.93 $\pm$.37	4.00 $\pm$.48
9 Months	1.92 $\pm$.22 ^a	4.59 $\pm$.47 ^b	4.00 $\pm$.52 ^b	2.60 $\pm$.21	1.86 $\pm$.24	2.79 $\pm$.34

^a Antibody titers of non-shocked 9 month old mice were lower ($p < .05$) than those of non-shocked 3 month old animals.

^b A marked increase of antibody titers ($p < .05$) was evident in 9 month old mice exposed to the stressor immediately or 24 hr after inoculation relative to all other 9 month old animals.

EXPERIMENT 10

Age-Related Vulnerability to Stressor Provoked Immunosuppression in Older Mice

The data of Experiment 9 indicated that although the critical period for the shock induced immunosuppression was comparable in the 3 and 9 month old mice, the PFC response of 9 month old mice that had been shocked 72 hr after inoculation was lower than that of comparably treated 3 month old animals. Inasmuch as PFC values in nontreated 9 month old mice were lower than those of nontreated 3 month old animals, it was not certain whether the age of the organism actually increased vulnerability to the stressor induced immunosuppression. Accordingly, Experiment 10 was conducted to determine whether, in fact, the immunosuppression in older mice could more readily be attained (i.e., after fewer shock presentations) than in the younger animals.

METHOD

Subjects and Apparatus. A total of 42, male CD-1 mice 3 months of age and 39 male CD-1 mice 9 months old were employed as subjects in Experiment 10. The subject characteristics and apparatus specifications were the same as those of Experiment 9.

Procedure. Mice were immunized with SRBC (10^6 cells, ip) and housed individually. Seventy-two hours after immunization mice of each age group were exposed to either 0, 10, 60 or 360 inescapable footshocks of 2 sec duration (150 μ A, 9 sec intershock interval) or were left undisturbed in their home cage. Mice were decapitated and spleens removed 96 hr after antigen administration for subsequent PFC determinations. Trunk blood was collected for determination of serum antibody titers.

RESULTS AND DISCUSSION

Analyses of mononuclear cells in Experiment 10 revealed that the stressor treatments did not influence the total number of cells. As seen in Figure 13 and Table 10, the immunosuppression evident in mice shocked 72 hr following immunization was related to the number of shocks that mice received, as well as with the age of the organism. Analyses of variance of PFC responses and antibody titers revealed a significant Age x Shock treatment interaction, $F(3, 125)=2.82$, $p<.05$, and $F(3,126)=2.71$, $p<.05$. Newman-Keuls multiple comparisons ($\alpha=.05$) of the simple effects confirmed that the PFC response of nonshocked 9 month old mice was significantly lower than that of 3 month old mice. In the 3 month old mice the PFC response was significantly reduced by 60 and by 360 shocks, while exposure to 10 shock trials was without effect. In contrast, in the older mice a significant suppression of the PFC response was evident irrespective of the number of shocks (10, 60 or 360) received. Indeed, in the 9 month old animals the immunosuppression was as marked after 10 shocks as it was after 360 shocks.

EXPERIMENT 11

Age-Related Immunoenhancement Following Mild Stressor Exposure

It was demonstrated in Experiment 2 that in 2-3 month old mice the extent of the immunosuppression varied directly with the number of shock presentations to which animals had been exposed. As indicated earlier, Weiss et al., (1988) demonstrated that in rats several components of the immune system were slightly suppressed after a mild stressor (i.e., handling), enhanced in response to a few brief shocks and markedly suppressed after a relatively lengthy stressor session. Experiment 11 was

Figure 13. Mean ($\pm$ S.E.M.) PFC responses of mice (3 or 9 months of age) exposed to either 0 (NS), 10, 60 or 360 shocks. Shock treatment was applied 72 hr following immunization and spleens were taken for assay 24 hr later.

PFC/10⁶ MONONUCLEAR CELLS

Table 10

Antibody Titer ($\text{Log}_2 \pm \text{S.E.M.}$) to Sheep Red Blood Cells in 3 and 9 Month Old Mice Exposed to No Treatment (NT) or to 10, 60 or 360 Shocks 72 hr Following Antigen Administration

	NT	10	60	360
3 Months	3.72 ± 0.36	3.70 ± 0.32	2.58 ± 0.31^b	1.78 ± 0.38^b
9 Months	2.00 ± 0.34^a	1.10 ± 0.28^c	0.73 ± 0.25^b	1.00 ± 0.26^b

^a Antibody titers of non-shocked 9 month old mice were lower ($p < .05$) than those of 3 month old mice.

^b In the 3 month old animals antibody titers were reduced ($p < .05$) by 60 and by 360 shocks, while exposure to 10 shock trials was without effect. In contrast, in the older mice a significant suppression of antibody titers was evident irrespective of the number of shocks (10, 60 or 360) received.

conducted to assess whether the immunological consequences of relatively mild stressor application (handling or handling plus exposure to a novel environment) might vary with the age of the organism.

METHOD

Subjects and Procedure. A total of 25 male, 3 month old and 24 male, 9 month old mice were employed as subjects in Experiment 11. The subject characteristics were the same as those of Experiment 9. Mice (3 and 9 months of age) were immunized with SBC (10^6 cells, ip) and 72 hr later were either (i) placed in the shock boxes for 1.1 hr without shock being delivered, (ii) lifted by the tail but not placed in the shock apparatus or, (iii) left undisturbed in their home cage.

RESULTS AND DISCUSSION

The mean PFC response and antibody titer ($\pm$ S.E.M.) for each group is shown in Figure 14 and Table 11. Analysis of variance revealed that the PFC response and antibody titers were higher in the 3 than in the 9 month old mice, $F's(1,76 \text{ and } 1,86)=20.83 \text{ and } 21.75$, $p's<.01$, and also varied as a function of the treatment mice received, $F's(2,76 \text{ and } 2,86)=3.53 \text{ and } 4.70$, $p's<.05$. Although the Age $\times$ Treatment interactions were not statistically significant, Newman-Keuls multiple comparisons of the simple effects were conducted, since a priori predictions had been made on the basis of the Weiss et al. (1989) findings and those of Experiments 9 and 10. These comparisons confirmed that the 3 month old mice neither handling nor apparatus exposure influenced the PFC response and antibody titers. Likewise, in the 9 month old mice handling itself was without effect, but apparatus exposure provoked an appreciable increase of the PFC response and antibody titers. Thus, the present results suggest that a mild stressor such as exposure to a novel environment may enhance immune responsivity, but such an effect is dependent on the age of the animal. It

is difficult to equate the severity of the stressors used in the present study with those employed by Weiss et al. (1989). It is significant, however, that a relatively mild stressor applied 72 hr after inoculation may result in an increase of the PFC response, while the more severe stressor leads to an immunosuppression.

EXPERIMENT 12

Time-Dependent Alterations of Central Catecholamines Following Administration of Sheep Red Blood Cells

Although the immune system has long been considered as independent of other endogenous systems, evidence has accumulated suggesting that in addition to being subject to external modulation it is capable of influencing neurochemical (Besedovsky et al., 1983; Carlson et al., 1987; Kabiersch et al., 1988, Dunn, 1988) and neuroendocrine activity (see Carr & Blalock, 1988). For example, Besedovsky et al. (1983) reported that in rats SRBC administration influenced hypothalamic NE concentrations at the time of the peak antibody response, and was additionally related to the extent of the immune response that was mounted. Likewise Carlson et al. (1987) reported that SRBC administration in mice provoked a reduction of hypothalamic NE concentrations which appeared to be restricted to the paraventricular nucleus, and that this effect was most notable on the peak day of antibody production. In contrast, hippocampal NE and serotonin (5-HT) alterations were noted during the early phases of the response (2 days after immunization).

Experiment 12 was conducted to confirm some of the earlier findings and to determine (a) whether antigen treatment would influence DA activity, and (b) to assess the neurochemical changes in mesocorticolimbic structures, as well as in hypothalamus. It will be

Figure 14. Mean ($\pm$ S.E.M.) PFC responses. Mice (3 or 9 months of age) were immunized with SRBC and 72 hr later either left undisturbed in their home cage (NS), placed in the test apparatus for 1.1 hr without shock being delivered (APP), or lifted by the tail but not placed in the test apparatus (H). Spleens were taken 96 hr after immunization.

PFC/ 10^6 MONONUCLEAR CELLS

Table 11

Antibody Titer ($\text{Log}_2 \pm \text{S.E.M.}$) to Sheep Red Blood Cells in 3 and 9 Month Old Mice Either Left Undisturbed in Their Home Cage (N), Lifted by the Tail and Returned to their Home Cage (H), or Exposed to the Shock Box for 1.1 hr Without Shock being Delivered (A) 72 hr After Immunization

	N	H	A
3 Months	4.31 ± 0.37	4.20 ± 0.49	4.87 ± 0.35
9 Months	2.33 ± 0.27^a	2.67 ± 0.37	3.94 ± 0.43^b

^a Antibody titers of non-shocked 9 month old mice were lower ($p < .05$) than those of non-shocked 3 month old mice.

^b Antibody titers were elevated ($p < .05$) in 9 month old mice exposed to a mild stressor (i.e., a novel environment) 72 hr after inoculation relative to nonstressed 9 month old animals.

recalled that stressful stimuli provoke pronounced alterations in the turnover and concentrations of NE and DA; however, it seems that these variations are more readily attained in some regions than in others (see Anisman & Zacharko, 1990). It was of interest to determine whether the profile of neurochemical changes induced by an antigen would be reminiscent of those induced by stressors.

METHOD

Subjects and Apparatus. A total of 138 mice served as subjects in Experiment 12. The subject characteristics were the same as those of Experiment 1.

Procedure. Mice were injected intraperitoneally with SRBC (as in Experiment 1) or saline. At various intervals (1, 2, 3, 4, 5, or 6 days) thereafter, mice were decapitated, brains removed and sectioned on dry ice for subsequent HPLC determination of NE and MHPG, as well as DA and its metabolites DOPAC and HVA. Additionally, trunk blood was collected for determinations of plasma corticosterone levels, and spleens were taken for assessments of the PFC responses of mice that had been inoculated 2, 3, 4, 5, or 6 days previously.

Brain Microdissection. Brain regions were sectioned on a dissecting block fashioned from 0.5 mm thick aluminum templates and separated by brass plates (0.25 mm). The slots created by the brass plates served as guides for single-edged razor blades. The brain sections were teased from the blades onto glass slides and frozen on dry ice. A petri dish filled with powdered dry ice served as a cold stage for sectioning and was situated under a stereomicroscope, which was illuminated by a fiber optic cold light source. Under low magnification, the frontal cortex, nucleus accumbens, caudate nucleus, dorsal hippocampus, and locus coeruleus were punched with hollow microdissection needles, which ranged in

diameter from 22 to 16 gauge, while the hypothalamus was taken in its entirety prior to microdissection. The punched sections were placed in round-welled titer trays and were frozen at -70°C . High performance liquid chromatography (HPLC) with coulometric detection following the procedure of Seegal et al. (1986) was employed to determine amine and metabolite concentrations in the various brain regions.

HPLC Procedure. Frozen tissue were sonified in 0.3M monochloroacetic acid containing 0.1mM disodium EDTA. The protein content of each sample was measured using the method of Lowry et al. (1951). The homogenate was then centrifuged and the supernatant filtered through a 0.2 μm regenerated cellulose filter. An aliquot of this filtrate was injected onto a C_{18} reversed-phase column (Chromatography Sciences Company Inc.) in a high performance liquid chromatography (HPLC) system (Waters) equipped with a coulometric detector (ESA). The output from the detector was plotted and measured using a Hewlett-Packard HP-3390A plotting integrator. The mobile phase used with this aliquot (0.1M phosphate buffer with 20% acetonitrile) allowed for the separation of 3-methoxy, 4-hydroxyphenylethylene glycol (MHPG) and 3, 4-dihydroxyphenylacetic acid (DOPAC), the metabolites of NE and DA, respectively. Sodium octyl sulphate (.2 mM) was added as an ion-pairing agent and EDTA (.05 mM) was added as an anti-oxidant. A separate analysis of the samples using 50% acetonitrile added to the phosphate buffer was performed in order to obtain homovanillic acid (HVA).

RESULTS AND DISCUSSION

Separate statistical analyses were conducted for each of the amines and metabolites assessed. A number of samples were lost during the course of tissue sampling or in the HPLC analyses, and hence the degrees of freedom for the analyses varied across brain regions. Since it had been

previously demonstrated that central catecholamine variations following SRBC inoculation occurred around the time of the peak immune response (Besedovsky et al., 1984; Carlson et al., 1987), except for hippocampal NE which was previously shown to vary 2 days after inoculation (Carlson et al., 1987), in the present investigation planned contrasts were conducted to assess statistical significance.

The number of splenic antibody-forming cells at various intervals following SRBC inoculation is seen in Figure 15. The peak immune response occurred 4 days post-inoculation.

Norepinephrine

The mean concentrations ($\pm$ S.E.M.) of NE and MHPG in several brain regions at various times after saline or SRBC inoculation are shown in Figure 16. Planned contrasts revealed that at the time of the peak antibody response MHPG accumulation was markedly increased in hypothalamus, locus coeruleus and hippocampus, relative to saline treated animals, $F(1,126; 1,83 \text{ and } 1,107) = 9.56, 6.69 \text{ and } 11.30, p < .01, p < .02 \text{ and } p < .01$, respectively. In contrast, no antigen-associated changes in MHPG accumulation were evident in the frontal cortex following inoculation. Moreover, NE levels were not significantly altered within each of these brain regions by SRBC inoculation. The levels of NE within the hypothalamus were decreased by approximately 20% in SRBC treated mice, but this increase did not reach statistical significance ($p = .057$).

As indicated earlier, Besedovsky et al. (1983) and Carlson et al. (1987) both reported that SRBC treatment resulted in a reduction in hypothalamic NE concentrations. Thus, the decline of NE in the present investigation, although not significant, was consistent with these earlier reports. The fact that the magnitude of the NE decline was smaller than in these previous studies may be attributable to any number of factors,

including the species or strain differences between studies and the relatively low dose of antigen used in the present investigation.

Dopamine

The mean ($\pm$ S.E.M.) concentrations of DA, DOPAC and HVA within the nucleus accumbens, frontal cortex and caudate nucleus for each of the groups is shown in Figure 17. Compared with day-matched saline injected animals, SRBC treatment provoked profound reductions of DA levels within the nucleus accumbens around the time of the peak antibody response, i.e. on Day 3, $F(1,98)=6.49$, $p<.02$, and Day 4 post-immunization, $F(1,98)=6.94$, $p<.02$. DOPAC accumulation also decreased on day 3, $F(1,100)=7.67$, $p<.01$, but to a considerable extent this derived from relatively high levels of DOPAC in saline treated animals 3 days after saline injection (i.e., 22.22 ng/mg protein vs 16.99 ng/mg protein for all saline treated animals). The antigen treatment did not significantly affect HVA accumulation.

Within the frontal cortex, a marked increase in DOPAC accumulation was evident on Day 4 in SRBC treated mice, $F(1,22)=12.33$, $p<.01$. An increase was likewise evident 2 days post-immunization, although this effect was marginally significant, $p=.027$. HVA concentrations did not vary as a function of the antigen treatment. Likewise, DA levels within the frontal cortex were unaffected by the SRBC treatment.

In contrast to the dopaminergic changes observed within the nucleus accumbens and prefrontal cortex, no alterations of DA, DOPAC or HVA, were evident in the caudate nucleus after SRBC inoculation. This pattern of changes in DA activity following antigen administration, i.e., being evident in mesocorticolimbic structures but not in nigrostriatal regions, is consistent with the pattern of DA changes seen in this and other

laboratories following exposure to footshock (see Anisman & Zacharko, 1990).

Figure 15. Splenic IgM plaque-forming cell (PFC) response at various intervals (2, 3, 4, 5 or 6 days) following intraperitoneal administration of SRBC (10^6 cells).

PFC/ 10^6 MONONUCLEAR CELLS

Figure 16. Mean ($\pm$ S.E.M.) norepinephrine (NE) and 3-methoxy, 4-hydroxyphenylethylene glycol (MHPG) concentrations (ng/mg protein) at various intervals (1, 2, 3, 4, 5 or 6 days) following SRBC inoculation.

DAYS AFTER SRBC INOCULATION

DAYS AFTER SRBC INOCULATION

Figure 17. Mean ($\pm$ S.E.M.) dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) concentrations (ng/mg protein) at various intervals (1, 2, 3, 4, 5 or 6 days) following SRBC inoculation.

SRBC

Control

General Discussion

In accordance with earlier reports (Esterling & Rabin, 1987; Okimura, & Nigo, 1986), the results of the present investigation indicated that application of a stressor may compromise the immune response to a T-cell dependent antigen. The magnitude of the stressor-induced immunosuppression appeared to be directly related to the number of shock presentations mice received within a stressor session. Moreover, it appeared that a stressor could proactively influence the immune response, in that cues that had previously been paired with a stressor could reduce immune activity. Of particular interest with respect to each of these findings was the fact that the immunosuppression was evident only when the treatment was applied 72 hr after SRBC inoculation. A stressor applied at other intervals (0, 24, 48, 95 hr after immunization) did not elicit the immunosuppression.

The mechanisms associated with the time-dependent effects of a stressor cannot be determined on the basis of the present investigation. A substantial number of physiological changes occur over time following antigen administration which could impact on the immune response. For instance, it appears that dynamic variations of catecholamine activity and receptor sensitivity occur over the course of an immune response being mounted. In particular, it was reported that 4 days after antigen administration β -adrenoceptor expression on lymphocytes in responding lymphoid organs increases, possibly rendering these cells more sensitive to stressor-evoked sympathetic or adrenomedullary catecholamines (see review in Livnat et al., 1985). Additionally, it has been known for some time that β -NE agonists and antagonists differentially influenced the *in vitro* PFC response, depending on the time of their administration following immunization (see Sanders & Munson, 1985). Application of β -

NE agonists during the early phases of the *in vitro* IgM PFC response resulted in an immunoenhancement, while such an effect was less pronounced during the later phases of the immune response (Sanders & Munson, 1984). Additionally, Strom and Carpenter (1980) reported that during the initial phases of the immune response, a reduction in T-cell β -NE binding sites occurred, whereas, the binding of β -NE receptors increased during the later phases of the immune response. Consistent with the aforementioned reports, it was demonstrated that during the early phase of an immune response C57Bl/6 mice displayed an increase of B-cell reactivity to NE; however, during the later phases of the immune response this effect was no longer evident, but B-cell reactivity was increased in response to acetylcholine (ACh) (Ado & Goldshtein, 1988). Commensurate with the Sanders and Munson, (1985) findings, it was reported that in the C57Bl/6 mice β -NE receptor density was reduced 2-5 days following SRBC inoculation (Fuchs, Albright & Albright, 1988). It should be noted that these findings are inconsistent with those of Strom and Carpenter (1980), who observed that the initial decline was followed by an increase in β -NE receptor density. The latter inconsistencies notwithstanding, these data suggest that while NE and ACh receptor activity may influence immune functioning, the contribution of these transmitters or receptors vary over the course of an immune response being mounted. Moreover, the variations in the response to NE or its agonists may represent a down regulation of β -NE receptors, possibly reflecting a regulatory action to limit the magnitude of the immune response elicited by NE receptor stimulation (see Sanders & Munson, 1985).

In addition to a role for NE, it has been suggested, as indicated earlier, that adrenal corticoids as well as endorphins, both of which are

affected by stressors, may influence the immune response. Given the wide array of factors that may contribute either directly or indirectly to immune functioning, coupled with the fact that several of these processes vary over time following antigen administration, several viable candidates exist that might account for the time-dependent effects of a stressor on immune functioning. It should be underscored, however, that different aspects of the immune response (e.g., cell proliferation in response to a mitogen in spleen vs blood, NK cell activity, PFC response, etc.) may be differentially influenced by central or peripheral neurotransmitters and/or steroids. Accordingly, it would be premature at this juncture to even attempt to offer a single mechanism as being more or less important than others in accounting for stressor-provoked immunological alterations. Nevertheless, the possibility ought to be considered that a stressor applied soon after SRBC inoculation would favor an enhancement of immune functioning owing to the release of peripheral catecholamine induced by the stressor. Concurrently, however, other factors such as the corticoid release would act in an opposite direction. Application of a stressor at a later phase would not result in a NE release favoring an immunoenhancement, owing to the down regulation of the NE receptor system. Accordingly, any suppressive effects engendered by stressor activation of a second system (whether it is one involving ACh, corticoids, endorphins or an as yet undetermined mechanism) would be manifested.

In considering mechanisms which might subserve stressor-induced reductions of the antibody response, it should be noted that although a variety of stressors may come to provoke an immunosuppression, it is not necessarily the case that they do so via common processes. It is known, for instance, that stressors will provoke variations of NE, DA, 5-HT and endorphins, and in addition it seems that these variations may be subject

to an adaptation with continued exposure to stressors. However, it is not necessarily the case that the adaptation occurs equally readily in response to different stressors, and it is certainly not the case that the adaptation will develop at similar rates in all brain regions (Deutch, Tam, & Roth, 1985). As will be discussed in greater detail in ensuing sections, it seems that different aspects of an immune response may be subject to modulation by different mechanisms. Accordingly, it should not be surprising to find that the effects of stressors on CNS or peripheral neurotransmitters do not necessarily parallel immunological changes, even if the latter effects are assumed to be related to particular transmitter alterations. After all, even if a particular transmitter were related to immune functioning, the contribution of this factor in response to an experimental manipulation may be obscured by the induction of other physiological processes which may affect immune activity.

In contrast to reports showing that controllability is fundamental in the provocation of stressor-induced suppression of NK cell activity (Shavit et al., 1983) [the data concerning the effects of controllability on lymphocyte proliferation (Laudenslager et al., 1983) have since been withdrawn (Maier & Laudenslager, 1988)], this factor did not appear to contribute to the suppression of the PFC response in the present investigation. These stressor parameters were previously shown to differentially influence central NE activity, as well as several behaviors (see Anisman, 1984; Anisman, & Zacharko, 1990), and thus the lack of a differential effect on immune activity cannot be attributed to the selection of inappropriate stressor parameters. Rather, the PFC response appears to be particularly sensitive to the stressor, irrespective of its controllability.

The exquisite sensitivity of the PFC response and antibody titers to a stressor is underscored by the demonstration that 72 hr after SRBC

inoculation an immunosuppression is elicited by exposure to a relatively mild stressor (light) or to stressor-related odors. The fact that stimuli such as light exposure, stressor-related odors, and cues associated with the stressor, elicit the immunosuppression not only point to the sensitivity of the immune response, but also indicate that psychological, as opposed to physical stressors, are sufficient to engender the immunosuppression. It is not unreasonable to assume that exposure to odors associated with a stressed animal, like cues that had previously been associated with a stressor, might serve to alert an organism to a potential danger, and hence acted to elicit a preparatory-like response. Inasmuch as the immunosuppression is engendered under such conditions the possibility exists that it may, in fact, be an adaptive response to a stressor, rather than a maladaptive change engendered as a secondary response to other adaptive changes (e.g., corticoid secretion engendered by a stressor). Indeed, as discussed earlier, Munck et al. (1984) argued that the corticoid secretion elicited by a stressor, and the resultant immunosuppression engendered by the steroid secretion, may serve to limit immunological activation which might otherwise favor the development of an autoimmune response. In fact, under some conditions stressors may come to provoke an immunoenhancement (e.g., Weiss et al., 1989; see also Experiment 9 and 11) which could have adverse physical consequences.

Although there appears to be a discrete period following inoculation during which a stressor is most effective in suppressing the PFC response, it seems that a second phase exists during which stressor application prevents or minimizes the effects of a subsequently applied stressor. It will be recalled that when animals are exposed to a stressor several weeks prior to immunization, then reexposure to the stressor or to the stressor related cues 72 hr after inoculation resulted in a reduction of antibody

titers and the PFC response. Interestingly, however, if the stressor was applied soon after SRBC treatment, then the immunosuppression ordinarily associated with a stressor applied 72 hr after inoculation was abrogated, while reexposure to stressor-related cues actually provoked an immunoenhancement.

The finding that cues associated with a stressor that had been applied weeks prior to immunization would subsequently elicit an immunosuppression is consistent with reports that immune functioning may be subject to conditioning processes (Ader, & Cohen, 1975; Ader, Cohen, & Bovjberg, 1982; Gorczynski, Macrae, & Kennedy, 1982). The procedure of the present investigation, however, should be distinguished from these earlier reports since footshock, rather than cyclophosphamide, was paired with the environmental cues (see also Gorczynski, & Kennedy, 1984; Lysle et al., 1988). Moreover, it is not clear whether the conditioning effect involved some aspect of the immune system directly or was secondary to central or peripheral transmitter or hormonal changes elicited by footshock. As indicated earlier, stressors have been shown to alter central neurotransmitter turnover or concentrations, and these variations may be subject to conditioning-like effects (Cassens et al., 1980; Irwin et al., 1986). Thus upon reexposure to the stressor-related cues the neurotransmitter alterations may again be provoked (e.g., alterations of hypothalamic NE activity), and could in turn lead to alterations of immune functioning.

It is not possible at this juncture to identify the mechanisms subserving the immunoenhancement induced by cues associated with a stressor which had been applied soon after antigen administration. Several hypotheses can be offered, although it should be understood that these are highly speculative. In their analyses of behavioral tolerance to

morphine, Siegel (1976, 1983) and Stewart and Eikelboom (1987) suggested that the administration of the drug elicits both an analgesic reaction, as well as a compensatory type of response which acts in an opposite direction. The compensatory response is thought to come under stimulus control, so that in the absence of the primary stimulus (the morphine), cues associated with the drug may come to produce a hypoanalgesic response. It is likewise conceivable that stressor application soon after inoculation provokes two independent processes; one favoring a suppression of immune functioning and the second being a compensatory response acting against the suppression. Thus, the effects of this manipulation will itself not come to influence antibody titers or the PFC response. The latter effect, however, may be subject to conditioning processes so that upon reexposure to stressor-related cues only the compensatory changes (immunoenhancement) are provoked. Of course, while the present data are suggestive of conditioned compensatory immunofacilitation and immune suppression (Experiments 7a and 6, respectively), such conclusions must be considered as being provisional, pending the inclusion of additional control procedures (e.g., the effects of a CS following random CS-shock pairings) or manipulations which are known to affect the response to a previously established CS (e.g., extinction) (see discussion in Ader & Cohen, 1985).

An alternative to this proposition concerns the possibility that soon after antigenic challenge several immunological changes occur, which ordinarily serve to potentiate an immune response (e.g., release of IL-1 from macrophages). In animals that are exposed to a stressor soon after inoculation and then reexposed to stressor related cues 72 hr afterward, these same mechanisms may be activated thereby promoting an immunoenhancement. If, however, the initial stressor is applied in the

absence of these immunological processes being activated (e.g., two weeks prior to antigenic challenge), then reexposure to stressor related cues will not promote these same immunological changes and hence an immunofacilitation will not be evident. Of course, in providing this alternative to a conditioned compensatory hypothesis, any number of immunological mechanisms might contribute to the potentiation of an immune response. The specific mechanisms involved in this respect remain to be fully characterized.

In contrast to the immunosuppression associated with an acute stressor, an adaptation occurs following a chronic stressor regimen. Such an effect was not only evident with respect to antibody titers and the PFC response (Experiments 8a-8c), but also in terms of the proliferative response to a mitogen (Lysle et al., 1988; Jessop et al., 1987; Monjan & Collector, 1977) and natural killer cell activity (Irwin et al., 1990; Livnat et al., 1986). It should be emphasized that the chronic stressor did not simply result in immune functioning returning to levels equivalent to that of nonstressed animals, but this treatment actually potentiated immune activity well beyond that observed in the absence of a stressor. In effect, it seems that the chronic stressor resulted in dynamic variations which served to enhance immune activity.

In attempting to determine the mechanisms that might subserve the adaptation to a chronic stressor it is essential to recognize that the neurochemical, hormonal and immunological effects of the environmental insults may interact with those changes elicited by an antigen itself. As mentioned earlier, when animals were exposed to a stressor on 5 separate occasions commencing immediately following antigen administration, the reduced PFC response ordinarily engendered by a stressor applied 72 hr after immunization was prevented. However, given that a stressor

applied within 24 hr of inoculation likewise counteracted the effects of a subsequent environmental challenge, it is likely that the apparent adaptation may be a reflection of a conditioned compensatory response established by stressing animals on the day of inoculation, rather than an adaptation associated with repeated exposure to the stressor. It might be argued that compensatory mechanisms might similarly account for the adaptation which is evident when the chronic stressor was applied prior to inoculation. That is, a chronic regimen that terminated within 24-48 hr of inoculation might engender a compensatory immunological response upon subsequent reexposure to the stressor (i.e., 72 hr after inoculation). However, in the present investigation it was observed that a stressor applied 72 hr after inoculation resulted in a significant immunoenhancement among mice that had been exposed to a chronic regimen that terminated 48 hr prior to inoculation. No such enhancement was apparent when animals were initially exposed to an acute stressor 48 hr prior to inoculation and then later reexposed to the stressor. In effect, the chronic regimen enhanced immune activity well beyond that provoked by an acute stressor applied in relatively close temporal congruity with the antigen. On the basis of these data it is not possible to dismiss the contribution of a conditioned compensatory response entirely, but it does seem that the chronic regimen is responsible for provoking an enhancement beyond that elicited by an acute stressor administered within 48 hr of antigen treatment.

It should be underscored that the development of the adaptation may well be dependent upon the background conditions upon which the stressor is applied. It was recently reported (Lysle & Cunnick, 1990) that the adaptation of a mitogen response associated with a chronic stressor was not evident when the test stressor was administered in an

environment different from that in which the adaptation had developed. Thus, these investigators suggested that the adaptation reflected a situation-specific compensatory response that opposed the effect ordinarily provoked by the stressor. In a like fashion, the adaptation in the present investigation may reflect such a compensatory process; however, this effect was not necessarily dependent upon the stressor being paired with the antigen treatment. As such, this adaptation should be distinguished from the immunoenhancement that is observed by cues that had been paired with an acute stressor applied at the time of antigen treatment.

It will be recalled that like the adaptation to the immunosuppressive effects of a stressor, the reductions of NE, DA and 5-HT associated with an acute stressor may be absent following a chronic regimen (see Anisman & Zacharko, 1990). Moreover, it was observed that the chronic stressor regimen produced an increase of amine levels beyond that of nonstressed animals, possibly suggesting that the chronic treatment was associated with a diminution of the excessive amine utilization associated with an acute stressor and/or an increase in amine synthesis. Given the parallels between the neurochemical and immunological consequences of acute and chronic stressors, it is tempting to speculate that these physiological changes might be related to one another. Of course, at this juncture there is no evidence suggesting a causal relationship between the two, nor is it possible to differentiate the relative contributions (if any) of these three amines. At the very least, additional data are necessary to determine whether instances exist wherein adaptation develops with respect to the central amine changes, but not in terms of the immunological alterations (or vice versa).

The impact of acute and chronic stressors on immune functioning, as well as on central neurotransmitter activity, appears to be dependent on the backdrop upon which the aversive stimulation is superimposed. In particular, it has been well established that the NE changes introduced by an uncontrollable stressor may be prevented if animals are permitted to engage in (reflexive) fighting or if animals are presented with a prod which can be attacked (Stolk, Conner, Levine, & Barchas, 1974). Presumably, fighting acted as a coping mechanism, thereby minimizing the impact of the stressor (Anisman, 1978). Along the same lines it has been shown that the enhancement in tumor growth associated with the stress of a change in housing conditions could be offset if animals engaged in fighting (Sklar & Anisman, 1981). Finally, it has been reported that the marked enhancement of NK cell activity associated with a chronic stressor was eliminated in those animals that were continuously attacked by a dominant cage mate (Livnat et al., 1986). In fact, it was observed that in submissive animals that were exposed to a repeated stressor a very marked suppression of NK cell activity was noted relative to nonstressed animals. This outcome contrasts with the immunoenhancement associated with the chronic stressor in the absence of fighting. A much more common environmental condition which may interact with the impact of a stressor, namely that of individual vs. grouped housing conditions, often is not considered at all. In particular, it is known that housing conditions (or change in housing conditions) may influence the course of transplanted tumor growth (Sklar & Anisman, 1981). Likewise, it has been reported that in mice individual housing for two weeks results in a diminution of immune functioning, but such an effect disappears with three or more weeks of isolation (see Rabin et al., 1989). In effect, initial isolation exerts an effect on immune functioning which is subject to adaptation over a

three week period. Since in most studies that assessed the immunological consequences of chronic stressors animals were housed in isolation, it is conceivable that the effects of the stressor were confounded with the housing conditions themselves. Of course, in these studies animals that received either no stress or acute stress were similarly housed, but there is no a priori reason to assume that the chronic stressor would interact with housing condition in the same way as would an acute stressor. Moreover, whatever effect a chronic stressor has on immune activity may also vary with the duration which animals are housed individually (i.e. 2 weeks vs. three weeks or longer). In the present investigation animals were housed in isolation from the time of antigen administration to the time of sacrifice (in acute studies) or for 14 days prior to antigen exposure until sacrifice and 4 days after inoculation (in chronic studies). Accordingly, the results of the present investigation must be considered in the context of the aforementioned caveats concerning the potential contribution of housing conditions. To be sure, the potential for Housing x Stressor interactions does not detract from the significance of the present results, but it is essential to consider the effects of stressors in the context in which environmental or psychological challenges were applied.

While adrenal corticosteroids are certainly known to influence immune functioning, there is reason to suspect that corticosterone alterations associated with a stressor were not responsible for the results observed in the present investigation. In particular, using stressor parameters identical to those employed in the present investigation we observed that no such adaptation was not evident with respect to the acute stressor-provoked enhancement of plasma corticosterone (Irwin, Ahluwalia, Zacharko, & Anisman, 1986; Shanks, Griffiths, Zalcman, & Anisman, 1990). Furthermore, footshock-induced corticosterone variations

did not occur in the graded fashion that was evident in the case of the PFC response (Prince, Ahluwalia, & Anisman, 1986). The fact that the variations of the PFC response were not paralleled by similar corticosterone changes, suggests that this particular steroid itself was not responsible for the stressor induced immunosuppression. This should not be taken to imply that other adrenal factors do not contribute to stressor-provoked variations of some components of the immune system. Indeed, it has been reported (Esterling & Rabin, 1987) that alterations of the PFC response, as well as Ly-1 and Ly-2 lymphocytes, may be dependent on the time of stressor application relative to SRBC inoculation. These investigators noted that although adrenalectomy did not affect the stressor-provoked alterations of the PFC response, the variations of Ly-2 lymphocyte activity varied as a function of the interaction of time of stressor application and adrenalectomy. Furthermore, it has been reported that the antibody response to SRBC can be modified by 6-OHDA, but such an effect was pronounced only in animals that had undergone adrenalectomy (Besedovsky et al., 1979). Further to the same issue, it has been reported that while acute administration of the synthetic corticoid, dexamethasone, resulted in a suppression of splenic lymphocytes, no such effect was evident following repeated treatment with this compound (Paciotti, Skwerer & Tamarkin, 1987). Thus, these investigators suggested that chronic stressor exposure may result in the desensitization of lymphocytes to corticosterone. In effect, it seems that while corticosterone may not be solely responsible for the immunosuppressive effects of an acute stressor in the present investigation, it is still premature to reject a secondary or modulatory role for adrenal corticoids in affecting immune activity.

In considering the potential contribution of corticoids and catecholamines in determining the immunosuppressive effects of stressors it is important to recognize, as indicated earlier, that various compartments of the immune response may be affected by different mechanisms. Additionally, there is reason to suspect that the mechanisms associated with a given index of immune functioning may vary across lymphoid organs or blood. For instance, it was reported that the footshock provoked reduction of T-cell proliferation in response to mitogenic stimulation (Con A) in blood was antagonized by adrenalectomy, whereas the stressor induced reduction of the mitogen response in spleen was unaffected by this treatment (Cunnick et al., 1990). Conversely, the β -adrenergic receptor antagonists, propranolol and nadolol (the latter does not readily enter brain tissue), effectively antagonized the shock-provoked reduction of the mitogen response in spleen, but not in blood. Thus, it was suggested that the splenic mitogen response associated with a stressor was adrenal independent, possibly involving catecholamine activity, while the mitogen response in blood was adrenal dependent. It is interesting to note at this juncture as well, that the adaptation of immune activity with a chronic stressor is likewise dependent upon the immune compartment examined. In particular, while an adaptation is evident with respect to the splenic mitogen response, no such adaptation is seen in blood (Lysle et al., 1987). It is significant that the central and peripheral NE changes associated with a stressor are similarly subject to an adaptation with a chronic stressor regimen, while the variations of plasma corticosterone are typically not diminished with repeated stressor application (see Anisman et al., 1990). Taken together, these data suggest that corticoids may play a role in accounting for the variations of the mitogen response seen in blood, while catecholamines are more intricately

involved in determining the response in spleen. Whether similar differentiations exist with respect to the splenic PFC response and plasma antibody titers remains to be determined. However, the possibility that the antibody response measured in blood, which ought to be a reflection of the splenic PFC response, is to some degree influenced by corticosterone cannot be dismissed.

As repeatedly suggested in earlier sections, it might be expected that any number of organismic or experiential variables might influence the nature and magnitude of stressor effects on immune functioning. Of course, it is well documented that some aspects of immune functioning decline with age, and thus it is not unreasonable to suppose that aged organism might likewise be more vulnerable to a variety of disease states. In the present investigation we began to assess the possibility that the impact of stressors might, in fact, influence immune activity more profoundly in older animals than in young mature animals. While mice 9-months of age are not typically considered to be aged (see Kay, 1980), it is clear that in these animals immune functioning was appreciably reduced relative to that of 3 month old CD-1 mice. Other investigators (Doria, Adorini & Frasca, 1987) have likewise reported that in 6 month old mice several aspects of the immune response (IL-2 production, expression of IL-2 receptors on mitogen activated spleen cells, helper T-cell activity) are reduced relative to that seen in 3 month old mice.

Consistent with Experiment 1, it was demonstrated that acute footshock provoked a suppression of both plasma antibody titers and the splenic PFC response. Moreover, there existed a critical period following antigen administration (72 hr) during which a stressor was effective in eliciting such an outcome. The period for the induction of the immunosuppression did not differ between the 3 and 9 month old mice;

however, the suppression could be provoked more readily in the older animals. In particular, in the 9 month old animals as few as 10 shocks applied 72 hr after inoculation was sufficient to elicit the immunosuppression, whereas in the 3 month old animals such a treatment was without effect. Thus, it appears that not only does the immune response decline with age, but vulnerability to stressor induced immunosuppression is exacerbated as well. Curiously, however, in contrast to the effect of a relatively strong stressor the mild stress of being exposed to a novel environment resulted in a marked immunoenhancement in the 9 month old mice, whereas this treatment was without effect in the younger animals. Although it has typically been observed that acutely applied stressors provoke an immunosuppression, it has been reported that mild stressors may actually enhance immune functioning (Weiss, Sundar & Becker, 1988). Thus, the finding of an immunoenhancement following exposure to a novel environment in the present investigation is not particularly surprising. It is not clear, however, why such an effect would occur in 9- but not 3-month old animals.

Although the effects of a stressor appeared to have a greater suppressive effect in the 9- than in the 3-month old mice, this conclusion must be somewhat tempered. Specifically, in the absence of a stressor the antibody response in the older animals was diminished relative to that seen in the younger animals. Accordingly, it could be argued that the immune response might thus be more susceptible to disruption by application of an environmental insult. Thus, it is necessary to determine whether a greater stressor-provoked immunosuppression would be evident in older mice treated with a higher dose of antigen that would ordinarily result in an antibody response equivalent to that seen in

younger mice. This notwithstanding, it is certainly the case that other things being equal immune functioning in older animals is more readily disrupted than in younger animals, and it might thus be expected that the older animals would be more vulnerable to viral challenge.

In contrast to young animals, which were hardly affected by a stressor applied soon after antigen exposure, when a relatively potent stressor was applied immediately or 24 hr after SRBC inoculation, a very dramatic increase of the PFC and antibody titers was apparent in 9 month old mice. The source for the enhanced immune response in the older animals was not immediately evident, and intuitively it is not likely that the enhanced PFC response was a consequence of augmented effector activity (e.g., enhanced cytotoxic T-cell or B-cell activity). It has been suggested that in older animals regulatory mechanisms are more tightly controlled (e.g., helper and suppressor cells and antiidiotype antibodies), thereby limiting the potential antibody response (see Kay, 1980). In particular, it seems that in the aged animal T-suppressor cells may be more vulnerable to disruption, thereby modifying the response to an antigen. In fact, it has been demonstrated that treatments that reduced levels of suppressor cells to those of young animals restored immune functioning (Segre & Segre, 1987). The possibility exists that these regulatory mechanisms, and particularly suppressor cells, are more readily affected by stressors in the older mice than in the younger animals. Thus, exposure to a relatively mild stressor or application of a stronger stressor during the early phase of the immune response being mounted may preferentially affect this regulatory mechanism, thereby resulting in the apparent immunoenhancement.

An alternative account for the immunoenhancement evident in older mice exposed to footshock soon after SRBC inoculation concerns the

alterations of regulatory cells involved in the early phases of the immune response being mounted. It is generally assumed that the functional integrity of B-cells is not compromised with age (Doria, Adorini, & Frasca, 1987). However, fewer T-cells appear to be activated following stimulation and there is a concomitant decline in IL-2 production (Miller & Harrison, 1987; Thoman & Weigle, 1983). This would result in the generation of fewer antibody-forming cells following antigenic challenge. Inasmuch as the enhancement of the PFC response in older animals occurred when the stressor was applied soon after inoculation, it is conceivable that this reflects an effect related to T-helper cell functioning (e.g., activation of helper cells or IL-2 production). Since the functional integrity of B-cells is intact in older animals, these cells would respond normally to signals from T-helper cells, and hence antibody production comparable to that of nonstressed younger animals would result. Of course, this proposition does not exclude the previous notion that in the older animals the stressor preferentially affects T-suppressor cell functioning. In fact, the stressor-provoked enhancement of antibody production may reflect the conjoint action of increased T-helper and reduced T-suppressor cell activity.

It may be relevant in the present context that in mature adult animals, there is a downregulation of lymphocyte β -adrenoreceptor activity following stressor exposure. This presumably adaptive response may render the cell less vulnerable to the effects of a stressor (e.g., increased sympathetic activity). In contrast to the changes seen in young animals, it appears that in aged rats the down-regulation does not occur as readily, and as a result sympathetic activity will be potentiated and more persistent (De Blasi et al., 1986). Inasmuch as the application of β -adrenergic agonists during the early phases of the in vitro PFC response to

SRBC resulted in an enhancement of the subsequent PFC response (see Sanders & Munson, 1985), it is conceivable that the immunoenhancement observed in older animals in the present investigation was likewise related to retardation of the down-regulation of β -NE receptors.

It is worth noting at this juncture, that in aged rats there appears to be a large decline in splenic NE content, number of NE terminals, as well as in overall uptake of the amine. Interestingly, however, this is compensated for, at least in part, by an appreciable increase in the efficiency of NE uptake (per terminal), possibly reflecting an increase in the density of high affinity uptake sites for NE (Bellinger, Lorton, Felten, & Felten, 1990). Inasmuch as NE appears to play its most prominent role during the early phase of an immune response being mounted (Sanders & Munson, 1985), it might be expected that age-related differences in the effects of a stressor on immune functioning would be most apparent at this time. In effect, the suggestion being offered here is that the mechanism supporting the stressor induced immunoenhancement in the aged mice may be related to age and stressor dependent variations of NE, while the immunological alterations evident in mice stressed 72 hr after inoculation are attributable to an NE-independent mechanism. Of course, as indicated earlier, the mice of the present investigation were only 9 months of age, and it remains to be determined whether changes in NE and in β -NE downregulation are, in fact, retarded in these animals relative to three month old mice. It might be tempting to speculate, as well, that in the older mice the augmented immunosuppression induced by a stressor applied 72 hr after SRBC inoculation might have been due to particularly marked increases of corticoid activity that is often associated with a stressor (see Sapolsky & Donnelly, 1984). However, we were unable to detect differential effects of inescapable footshock on plasma

corticosterone accumulation in 3 and 9 month old CD-1 mice (Shanks et al., 1990).

The findings discussed to this point suggest that environmental stressors and psychological factors associated with such stressors may influence immune functioning. In fact, the basic tenets that were provisionally offered was that stressors, by virtue of their central transmitter effects, may be responsible for the observed alterations of immune functioning. Additionally, the view was entertained that the effects of stressors may interact with potential central amine alterations that might have been engendered by antigenic challenge. In accordance with several earlier reports (Besedovsky et al., 1983; Carlson et al., 1987), it was indeed observed, that variations of central catecholamines were evident following SRBC inoculation. Furthermore, these changes coincided with the time of the peak immune response and were not evident at relatively brief intervals following inoculation. Thus, it is unlikely that the effects observed were attributable to the stress of handling or injection per se. Rather, it is likely that the changes were related to the immunological effects provoked in response to the antigen.

The alterations of NE activity observed in the present investigation are distinguishable from some of the previously reported findings. Whereas the data of Carlson et al. (1987) focussed on the NE alterations in specific hypothalamic nuclei and in the hippocampus, in the present investigation additional brain regions were examined and the utilization of NE, as gauged by MHPG accumulation, was also determined. It was observed that MHPG accumulation in the hypothalamus, hippocampus and locus coeruleus was increased 4 days after inoculation. Furthermore, whereas Carlson et al. noted that hippocampal NE was reduced during the

rising portion of the immune response (2 days after SRBC treatment), no such effect was evident in the present investigation.

As indicated earlier, Besedovsky et al. (1983) and Carlson et al. (1987) both reported that SRBC treatment resulted in a reduction in hypothalamic NE concentrations. Thus, the decline of NE in the present investigation, although not significant, was consistent with these earlier reports. The fact that the magnitude of the NE decline was smaller than in these previous studies may be attributable to any number of factors, including the species or strain differences between studies and the relatively low dose of antigen used in the present investigation. The results of the present study also differed from those of Besedovsky et al. (1983) who reported that SRBC treatment resulted in a decrease, rather than an increase of NE turnover. The fact that the increased turnover was noted in three of the four regions examined in the present investigation suggests that this outcome was, in fact, a reliable one. We cannot readily determine why the decline of NE reported by Besedovsky et al. (1983) was not accompanied by increased turnover. However, there were numerous procedural problems associated with the Besedovsky et al. report. Among other things, the method of estimating turnover involved treatment with the tyrosine hydroxylase inhibitor, α -MPT which does not necessarily provide the most accurate index of turnover, particularly since the form of α -MPT used did not include a salt (i.e. α -MPT methyl ester) and may have had toxic effects. Moreover, the reduction of NE was only measured 2 hr after α -MPT, rather than at several intervals which would have provided a better index of turnover. Third, not all SRBC treated animals exhibited the "reduced turnover" but only those which displayed a relatively low PFC response. Finally, since NE levels were reduced in the SRBC treated animals, the further decline of NE following tyrosine

hydroxylase inhibition may not provide an accurate index of turnover (see discussion in Stone, 1975).

In addition to the NE variations, SRBC treatment in the present investigation was found to markedly influence DA activity in some brain regions. While DA activity and levels within the caudate were unaltered in SRBC treated animals, an appreciable increase of DOPAC accumulation was evident within the frontal cortex. In the nucleus accumbens, DA levels were appreciably reduced, although an accompanying increase of DOPAC was not evident. We have reported previously that although the DA reductions associated with a stressor are typically accompanied by increased accumulation of the metabolite, DOPAC, this is not always the case in the nucleus accumbens (see Anisman & Zacharko, 1990). Indeed, we observed that instead of increased DOPAC, the stressor-induced DA reductions may be accompanied by increased accumulation of HVA. As such, it was suggested that the stressor acted to alter the route of DA degradation. In the present investigation, however, no such increase of HVA was apparent. However, it must be recognized that in the case of stressors, such as footshock, the increased utilization occurs over a brief period of time (i.e., during and very shortly after stressor termination) and hence the accumulation of DA metabolites can be detected fairly readily. In the case of antigen administration the altered transmitter activity may be less pronounced, but more sustained. Thus, at any given time the accumulation of metabolites would be less marked than in the case of stressor exposure. In fact, as yet unpublished data that have recently been collected in rats using *in vivo* microdialysis indicated that antigen administration resulted in DA release from nucleus accumbens commencing 2 days after inoculation and persisting over approximately 4 days. As in the present report, increases of DOPAC and HVA accumulation

were not detectable (Merali, Zalcman & Anisman, 1990 unpublished report).

It is of particular interest that analyses of stressor effects have typically indicated that DA activity within the mesocortex is readily affected by stressors, alterations of DA in the nucleus accumbens occur somewhat less readily, while in the substantia nigra and caudate DA activity is not affected at all (see Anisman & Zacharko, 1990). The effects of the SRBC treatment in the present investigation provoked a similar region specific profile. On the one hand, it is possible that caudate DA activity is generally less modifiable than DA activity within mesocorticolimbic neurons, irrespective of the stimuli to which animals are exposed (i.e., stressor or nonstressor-related stimuli). Alternatively, it is possible that the antigen treatment is being interpreted as a stressor and as a result leads to the region-specific DA alterations. A similar suggestion was offered by Dunn et al. (1989) since infection with influenza virus, like application of a stressor, was found to increase secretion of adrenal corticosterone and also to increase the accumulation of MHPG and the cerebral content of tryptophan. In his seminal work, Blalock (1984) argued that the immune system may be acting as a sensory organ, such that activated lymphocytes release an ACTH-like substance which could, in turn, influence other aspects of immune and peripheral nervous system functioning. The results of the present investigation, like those of other investigators (Besedovsky et al., 1984; Carlson et al., 1987; Dunn et al., 1988; Kabiersch et al., 1988; Mefford & Heyes, 1990), suggest that immunological alterations may come to influence central nervous system transmitters. In this respect, the amine alterations are not restricted to the hypothalamus but may affect mesocorticolimbic structures which have

been implicated in subserving emotional and affective states (Anisman et al., 1990).

Summary

Taken together, the data of the present investigation indicated that the immune response is exquisitely sensitive to the effects of a stressor. A critical period existed following SRBC inoculation during which physical and psychological stressors profoundly influenced the PFC response and antibody titers to SRBC. The nature and magnitude of these effects were related to stressor characteristics, including severity and chronicity, as well as to organismic variables such as the animal's previous stressor history and age. The possibility exists that under stressor conditions where immunocompetence is compromised vulnerability to illnesses related to external pathogens may be increased. It follows that variables which favor the immunosuppression, such as aging, or factors which prevent or limit the adaptation associated with a chronic stressor, will similarly favor vulnerability to illness (see Anisman et al., 1990; Kelley & Dantzer, 1989; Kiecolt-Glaser & Glaser, 1986). It should also be considered that excessive immune activity, such as that observed when older animals are exposed to a stressor shortly after antigenic challenge, may render the organism more vulnerable to autoimmune disorders. The contribution of various regulatory mechanisms may vary over time following antigen exposure. Accordingly, in older animals where these regulatory mechanisms are more readily disturbed, stressor exposure may lead to either immunoenhancement or suppression, depending upon the time of its application relative to antigenic challenge. Such an hypothesis is consistent with the paradoxical findings that stressful events may exacerbate both autoimmune disorders, as well as vulnerability to disease states associated with pathogenic challenge.

In assessing the relationship between stressor effects on immune functioning and the development of disease states, it should be recognized that not only do CNS events influence immune activity, but also immunological alterations may come to affect central neurotransmitter functioning. Moreover, it is reasonable to assume that the effects of a stressor on an immune response may interact with neurochemical and neuroendocrine changes provoked following antigen administration. Thus, psychological disturbances that are typically associated with alterations of central neurotransmitters, such as depression, may not only be provoked or exacerbated by psychological stressors, but also by external antigens or interactions between stressor and antigenic effects.

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Appendix A

Assay Procedures

I. Plaque-Forming Cell Assay

Determination of the PFC response was made using a slight modification of the method of Cunningham and Szenberg (1968). Mice were decapitated, spleens removed and dispersed to a single cell suspension in RPMI medium (Grand Island Biological) supplemented with Hepes buffer (1.0 M solution), Gentamycin sulphate (40 mg/ml) and Streptomycin G (5000 units/ml: John's Scientific). The spleen cells were washed by centrifugation at 400 g for 10 minutes and resuspended in 2 ml of medium. The cells were then layered on a Ficoll-Hypaque gradient (density=1.1) and centrifuged at 700 g for 30 minutes. After centrifugation, the mononuclear cells at the interface were removed, resuspended in 5 ml RPMI and washed 3 times at 400 g. Twenty microliters of the cell suspension (at 10^7 cells/ml) were combined with 20 ul of SRBC (approximately 2.5×10^8 c/ml) and 20 ul of guinea pig complement (absorbed with SRBC at a ratio of 2:1). The suspension was introduced by capillary action into microslides (Canlab, Pointe Claire, Quebec) with dimensions of 0.22 x 4 x 100 mm at a volume of 80 ul. The ends of the microslides were sealed with a 50% paraplast embedding medium and 50% vaseline mixture. The slides were incubated at 37°C for approximately 1 hour. Plaques were counted by microscopic examination of the microslides at a magnification of 10 X. Data were expressed as PFC/ 10^6 mononuclear cells.

II. Determination of Serum Antibody Titers

Mice were decapitated, trunk blood collected and allowed to clot. Samples were subsequently centrifuged at 400 g for 10 min and the supernatant collected and spun at 600 g. Serum complement was then inactivated at 56°C for 30 min. Two fold serial dilutions of inactivated serum, saline, and a 1% SRBC solution was then made in glass microwells. The highest dilution at which aggregation of SRBC was most evident was considered to be the antibody titer and expressed in $\log_2$ units.

III. HPLC Procedure

Frozen tissue were sonified in 0.3M monochloroacetic acid containing 0.1mM disodium EDTA. The protein content of each sample was measured using the method of Lowry et al. (1951). The homogenate was then centrifuged and the supernatant filtered through a 0.2 μ m regenerated cellulose filter. An aliquot of this filtrate was injected onto a C₁₈ reversed-phase column (Chromatography Sciences Company Inc.) in a high performance liquid chromatography (HPLC) system (Waters) equipped with a coulometric detector (ESA). The output from the detector was plotted and measured using a Hewlett-Packard HP-3390A plotting integrator. The mobile phase used with this aliquot (0.1M phosphate buffer with 20% acetonitrile) allowed for the separation of 3-methoxy, 4-hydroxyphenylethylene glycol (MHPG) and 3, 4-dihydroxyphenylacetic acid (DOPAC), the metabolites of NE and DA, respectively. Sodium octyl sulphate (.2 mM) was added as an ion-pairing agent and EDTA (.05 mM) was added as an anti-oxidant. A separate analysis of the samples using 50% acetonitrile added to the phosphate buffer was performed in order to obtain homovanillic acid (HVA).

Appendix B

Immunosuppression Induced by Exposure to a Mild Stressor
or Stressor-Related Odors

It has been demonstrated in several laboratories that acute exposure to stressors may result in a suppression of immune activity (see Dantzer & Kelley, 1989). Such effects have been noted with respect to natural killer cell activity, T-cell proliferation following mitogen stimulation, as well as the plaque forming cell (PFC) response and antibody titers in response to antigens (Shavit, Lewis, Gale, & Liebskind, 1983; Lysle, Cunnick, Fowler, & Rabin, 1987). The magnitude of the immunosuppression, as measured by the PFC response and antibody titers, appears to be directly related to the severity of the stressor employed (see Experiment 2). Interestingly, it has been demonstrated that particularly mild stressors (e.g., repeated handling and apparatus exposure) may suppress NK cell activity and IL-2 production, but an immunoenhancement is apparent with a somewhat more severe stressor (Weiss, Sundar, Becker, & Cierpial, 1989), followed by a suppression once again if animals are exposed to a severe stressor.

While the physical impact of aversive stimuli may contribute to the immunosuppression, it is clear that psychological factors might likewise be fundamental in provoking immunological changes. In particular, it was demonstrated that cues explicitly paired with a stressor may come to provoke the suppression of a mitogen response much like that provoked by the stressor itself (Lysle et al., 1988). Likewise, it was reported that exposure to apparatus cues that had been associated with a stressor may influence the PFC response and antibody titers in response to SRBC (see Experiment 6-7). The nature of the latter effect (i.e., immunosuppression

or immunoenhancement) was also found to vary as a function of the time at which the initial stressor treatment was applied relative to SRBC inoculation. During the course of a series of experiments to evaluate the immunological effects of a stimulus (light) explicitly paired with a stressor, we observed that even fairly innocuous stimuli, which would have been thought not to influence immune functioning, actually had profound effects. The present series of experiments demonstrate that the immune response is exquisitely sensitive to relatively mild stimulation (light exposure). Moreover, an immunosuppression can be elicited in a conspecific which is exposed to odor cues associated with a stressed animal.

Experiment 1a-1b

The initial purpose of the investigation was to assess the immunological effects of a CS (weak light) that had previously been paired with footshock (i.e., to determine whether the immunosuppression provoked by exposure to stressor-related cues in Experiment 6 was the result of a conditioned response). Prior to conducting this experiment, however, it was necessary to ascertain that the stressor parameters to be employed would, in fact, lead to a suppression of the PFC response and antibody titers, and that the CS to be used would have no such effect (i.e., that the light stimulus was indeed a neutral cue). Inasmuch as mild stressors such as handling or apparatus exposure could conceivably influence the immune response, these factors were considered, as well.

METHOD

Subjects and apparatus. The subject characteristics and apparatus specifications were the same as those described in Experiment 1a.

Procedure. Mice received intraperitoneal SRBC (10^6 cells) administration and were exposed to one of four experimental treatments 72 hr

afterward. Mice of one group were placed in the shock boxes and exposed to 65 shocks (5 sec duration, 150 μ A) at intervals of 30 sec. Mice of a second group were likewise placed in thoroughly cleaned chambers, but shock was not delivered. Animals in a third group were handled (lifted by the tail for approximately 2 sec), and immediately returned to their home cages (i.e., these animals were neither exposed to the apparatus nor shocked), while animals in the fourth group were left undisturbed in the cages. All mice were then returned to their cages and 24 hr afterward sacrificed, spleens and trunk blood collected, for subsequent PFC and antibody determinations.

Experiment 1b assessed the effects of the light stimulus on immune functioning. Mice received the SRBC treatment as in Experiment 1 and 72 hr later placed in the apparatus. For half the mice 2 light bulbs (emitting 5 foot candles of light) were present in the chamber, while for half the animals 4 light bulbs were present. In the latter condition one light bulb was located on the outside of each end wall of the chamber and could diffuse into the chamber through a translucent disc. Two additional bulbs were located at either end of the chamber beneath the grid floor, approximately 1.0 cm from the end walls. For those groups for which two light bulbs were present one light was present on the end wall and one beneath the grid floor at the same end of the chamber. For half the mice in each of these groups the lights were illuminated on 165 occasions for 5 sec at intervals of 30 sec between stimulus presentations, while for the remaining mice these bulbs were not illuminated. As in Experiment 1a mice were then returned to their home cages and 24 hr spleens and trunk blood were collected for subsequent PFC and antibody determinations.

RESULTS AND DISCUSSION

Analysis of mononuclear cells revealed that none of the manipulations influenced the total number of cells. As expected the analysis of variance revealed that the stressor manipulation influenced both the PFC response, $F(3,26)=4.33$, $p<.05$, and antibody titers, $F(3,26)=5.10$, $p<.01$. Newman-Keuls multiple comparisons confirmed that footshock provoked a marked reduction of both these immunological responses (see Figure 1 and Table 1) relative to the remaining groups, which were found not to differ from one another.

In Experiment 1b several blood samples were lost, and hence the degrees of freedom for the analysis of variance of the PFC response differed from that in the analysis of the antibody titers. The scores for each of these measures are shown in Figure 2 and Table 2, respectively. Unexpectedly, the analysis of variance revealed that exposure to the light resulted in a marked reduction of the PFC response, $F(1, 32)= 16.79$, $p<.01$, and of antibody titers, $F(1,27)=9.04$, $p<.05$. The number of light bulbs present within the cage was without effect. While unexpected, these data indicate that simply presenting animals with a series of light presentations, albeit of very low intensity, provoked a suppression of the immune response just as the more powerful shock stimulus did. In fact, the magnitude of the suppression was about 50% in both instances.

While exposure to the weak light stimulus was not expected to represent a stressor and hence not affect immune activity, casual observation of the animals suggested that this treatment was, in fact stressful. When mice were removed from the apparatus virtually all of the light exposed subjects exhibited piloerection and increased boli were present in the chambers relative to that of the animals that had not been exposed to the light. This is not to say that exposure to light was as

stressful as footshock nor that it had similar consequences in all respects. Indeed, we have observed that the reduction of a palatable diet provoked by the footshock exposure was far more pronounced than that elicited by light exposure (Griffiths, Shanks, & Anisman, 1989).

Experiment 2

It is clear from the preceding experiment that presentation of weak lights may engender a suppression of the PFC response and antibody titers. Experiment 2 was undertaken to repeat this unexpected finding, and also to determine whether the effectiveness of the light in provoking an immunosuppression was related to the fact that the light stimulus was presented to animals in a novel environment. Accordingly, in Experiment 2 the light was presented to animals either in a novel environment or in their home cage.

METHOD

Subjects and Apparatus. Subject characteristics and apparatus specifications were the same as in Experiment 1b.

Procedure. Mice were received ip injection of SRBC (10^6 cells) as described earlier and then returned to their home cages. Seventy-two hr afterward mice received either light presentations (165 trials of 5 sec) as described earlier, apparatus exposure without light being presented, or handling (lifted by the tail and put down) but without either apparatus exposure or handling. Some of the animals received the treatments in a novel environment (the shock chambers described in Experiment 1), while others received the treatment in their home cages ($n=5-7/\text{group}$). Trunk blood and spleens were collected 24 hr after the treatment as described in Experiment 1a.

RESULTS AND DISCUSSION

Figure 3 and Table 3 show the PFC responses and antibody titers for each of the groups. Analysis of variance of the PFC values yielded only a significant main effect of the Treatment, $F(2,32)=10.96$, $p<.01$. Multiple comparisons confirmed that presentation of the light produced a reduction of the PFC response irrespective of the environment in which the stimulus was applied. The mild stress of transporting mice from the vivarium to the laboratory and placing them in a novel environment did not influence the immune response. Thus, it seems that the immunosuppressive effects of the light were independent of the context in which the light was applied.

It is interesting that when light was presented to animals in their home cage about half the animals buried the stimulus that was on the floor using the available bedding. If the bulb was not illuminated this type of burying was not evident. It was previously reported that rats will similarly bury a probe that was used to deliver shock, as well as other novel objects introduced to their environment (Pinel & Treit, 1978). It is believed that this behavior represents a defensive style, and as such supports the contention that presentation of the light was indeed stressful.

Experiment 3

It has been reported that uncontrollable stressors may induce several behavioral disturbances and may engender a variety of central neurochemical changes (see Ansman & Zacharko, 1989). It was demonstrated that the effectiveness of stressors in provoking later behavioral disturbances may be dependent on the presence of odors associated with the original stressor experience (Minor & Lolordo, 1984). Along the same line it has been reported that the odor of animals exposed to a stressor may be sufficient to elicit variations of central amine activity

(Iimori, Tanaka, Kohno, et al., 1982). In Experiment 3 we assessed whether the odor of a conspecific that had been exposed to a stressor (that of being exposed to light) would influence the immune response.

METHOD

Subjects. A total of 22, male, CD-1 mice as described in the preceding experiments served as subjects. Mice were immunized ip with SRBC (10^6 cells) as previously described and housed individually and assigned to one of three treatment conditions. Seventy-two hours later mice that were to receive the odor of stressed animals were placed (while in their own cages) into the sound attenuating chamber which contained the test cage of a mouse that had been exposed to the light stressor ($n=7$). A second group of mice were similarly placed into a sound attenuating chamber. This chamber, however, contained the test apparatus of a mouse that had not been stressed ($n=8$). Mice of the third group were left undisturbed ($n=7$). Spleens and trunk blood were taken 24 hr after the experimental treatments were applied.

RESULTS AND DISCUSSION

Figure 4 and Table 4 show the PFC responses and antibody titers of each of the groups. Analysis of variance indicated that the odor condition influenced antibody titers, $F(2,19)=5.73$, $p<.02$. Newman-Keuls multiple comparisons ($\alpha=.05$) confirmed that antibody titers were reduced in mice exposed to the stress-related odors relative to either of the other groups. Likewise, as seen in Figure 4, the PFC response was somewhat reduced, $F(2,19)=2.78$, $p<.10$, by exposure to the odors of stressed animals. Although not statistically significant, the PFC response in these animals paralleled the changes in antibody titer, and the magnitude of the PFC reduction is similar to that evident in mice exposed to footshock or light (Experiments 1 and 2).

Figure 1. Male, CD-1 mice (3 months of age) received intraperitoneal SRBC (10^6 cells) administration and were exposed to one of four experimental treatments 72 hr afterward. Mice of one group were placed in the shock boxes and exposed to 165 shocks (5 sec duration, 150 uA) at intervals of 30 sec (S). Mice of a second group were likewise placed in thoroughly cleaned chambers, but shock was not delivered (A). Animals in a third group were handled (lifted by the tail for approximately 2 sec), and immediately returned to their home cages (i.e., these animals were neither exposed to the apparatus nor shocked) (H), while animals in the fourth group were left undisturbed in the cages (N). All mice were then returned to their cages and 24 hr afterward sacrificed, spleens and trunk blood collected, for subsequent PFC and antibody determinations.

PFC/10⁶ MONONUCLEAR CELLS

Table 1
Serum Antibody Titer ($\log_2 \pm$ S.E.M.) to Sheep Red Blood Cells in Mice
Exposed to Either No Treatment, Handling, Apparatus or Footshock 72 hr
Following SRBC Inoculation

Treatment	Antibody Titer
No Treatment	4.86 ± 0.55
Handling	5.38 ± 0.20
Apparatus	4.57 ± 0.55
Footshock	$2.88 \pm 0.58^*$

$^*p < .05$ relative to each other group

Figure 2. Mice received the SRBC treatment as in Experiment 1 and 72 hr later were placed in the test apparatus. For half the mice 2 light bulbs were present in the chamber, while for half the animals 4 light bulbs were present. In the latter condition one light bulb was located on the outside of each end wall of the chamber and could diffuse into the chamber through a translucent disc. Two additional bulbs were located at either end of the chamber beneath the grid floor, approximately 1.0 cm from the end walls. For those groups for which two light bulbs were present one light was situated on the end wall and one beneath the grid floor at the same end of the chamber. For half the mice in each of these groups the lights were illuminated on 165 occasions for 5 sec at intervals of 30 sec between stimulus presentations, while for the remaining mice these bulbs were not illuminated. In chambers containing 2 light bulbs, approximately 5 footcandles of light were emitted. As in Experiment 1 mice were then returned to their home cages and 24 hr spleens and trunk blood were collected for subsequent PFC and antibody determinations.

Table 2
Serum Antibody Titer ($\log_2 \pm \text{S.E.M.}$) to Sheep Red Blood Cells in Mice
Exposed to No Treatment or Light 72 hr following SRBC Inoculation

Treatment	Antibody Titer
No Treatment	3.90 ± 0.41
2 Lights	$2.09 \pm 0.39^*$
No Treatment	4.71 ± 0.42
4 Lights	$2.50 \pm 0.71^*$

* $p < .05$ vs nontreated animals

Figure 3. Mice were inoculated with SRBC (10^6 cells; ip) as described earlier and then returned to their home cages. Seventy-two hr afterward mice received either light presentations (165 trials of 5 sec) (L) as described earlier, apparatus exposure without light being presented (A), or were left undisturbed in their home cages (N). Some of the animals received the treatments in a novel environment (N) (the shock chambers described in Experiment 1), while others received the treatment in their home cages (HC) (n=5-7/group). Trunk blood and spleens were collected 24 hr after the treatment as described in Experiment 1.

PFC/10⁶ MONONUCLEAR CELLS

Table 3
 Serum Antibody Titer ($\log_2 \pm \text{S.E.M.}$) to Sheep Red Blood Cells in Mice
 Exposed to Either No Treatment, Apparatus or Light in a Novel
 Environment or in Their Home Cages

	No Treatment	Apparatus	Light
Novel Environment	4.00 ± 0.41	4.33 ± 0.30	4.00 ± 0.63
Home-Cage	3.86 ± 0.62	4.00 ± 0.45	$2.29 \pm 0.56^*$

* $p < .05$ relative to mice in other home-cage groups and to mice receiving light in a novel environment

Figure 4. Mice were immunized with SRBC (10^6 cells; ip) as previously described, housed individually and assigned to one of three treatment conditions. Seventy-two hours later mice that were to receive the odor of stressed animals [Odor (Stressed)] were placed (while in their own cages) into the sound attenuating chamber which contained the test cage of a mouse that had been exposed to the light stressor (n=7). A second group of mice were similarly placed into a sound attenuating chamber. This chamber, however, contained the test apparatus of a mouse that had not been stressed (n=8) [Odor (Nonstressed)]. Mice of the third group were left undisturbed (n=7) [NT]. Spleens and trunk blood were taken 24 hr after the experimental treatments were applied.

PFC/10⁶ MONONUCLEAR CELLS

Table 4

Serum Antibody Titer ($\log_2 \pm \text{S.E.M.}$) to Sheep Red Blood Cells in Mice
Exposed to Either No treatment, Odors of Nonstressed Mice or Stressor-
Related Odors 72 hr Following SRBC Inoculation

Treatment	Antibody Titer
No Treatment	4.00 ± 0.57
Odors (Nonstressed Mice)	3.57 ± 0.37
Odors (Stressed Mice)	$1.86 \pm 0.46^*$

* $p < .05$ relative to each other group

Appendix C

Table 1a (Experiment 1a)
 Analysis of Variance of PFC Responses of Mice Exposed to One Session of
 Inescapable Footshock at 150 uA Applied at Various Intervals Following
 Antigen Administration

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Shock (S)	5	30807007.57	6161401.514	7.99	p=.000
Within Error	48	37002275.76	770880.745		

Table 1b (Experiment 1b)
 Analysis of Variance of PFC Responses of Mice Exposed to One Session of
 Inescapable Footshock at 300 uA Applied at Various Intervals Following
 Antigen Administration

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Shock (S)	5	23755718.70	4751143.739	3.73	p=.008
Within Error	38	48401351.19	1273719.768		

Table 1c (Experiment 1b)
Analysis of Variance of Antibody Titers of Mice Exposed to One Session of
Inescapable Footshock at 300 uA Applied at Various Intervals Following
Antigen Administration

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Shock (S)	5	43.8279	8.7656	2.77	p=.028
Within Error	47	148.7004	3.1638		

Table 2
Analysis of Variance of the PFC Responses Following Exposure to Varying
Number of Shocks

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Shock (S)	4	24680536.68	6170134.17	6.80	p=.000
Within Error	48	43569446.34	907696.7987		

Table 3 (Experiment 3)
 Analysis of Variance of the PFC Response as a Function of The Time
 Between Stressor Exposure and Sacrifice

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Day (D)	1	338165.239	338165.239	0.67	p=,422
Shock (S)	1	2898993.450	2898993.4550	5.78	p=,027
D x S	1	113181.211	113181.211	0.23	p=,640
Within Error	19	9526124.762	501374.987		

Table 4 (Experiment 4)
Analysis of Variance of Antibody Titers at Various Intervals Following
Stressor Exposure

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Day (D)	3	25.940	8.647	3.45	p=.022
Shock (S)	1	55.178	55.178	22.00	p=.000
D x S	3	3.229	1.076	0.43	p=.733
Within Error	59	147.986	2.508		

Table 5a (Experiment 5a)
 Analysis of Variance of the PFC Responses of Mice Exposed to
 Uncontrollable or Controllable Footshock (350 shocks)
 72 hr Following Immunization

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Shock	2	13918365.24	6959182.619	23.75	p=.000
Within Error	17	4982004.762	293059.1036		

Table 5b (Experiment 5b)
 Analysis of Variance of the PFC Responses of Mice Exposed to
 Uncontrollable or Controllable Footshock (60 shocks)
 72 hr Following Immunization

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Shock	2	796209.2112	398104.6056	0.38	p=.69
Within Error	24	25224314.86	1051013.119		

Table 6a (Experiment 6)
 Analysis of Variance of the PFC Responses of Mice Initially Exposed to
 Footshock 2 Weeks Prior to Immunization and Re-Exposed to Stressor-
 Related Environmental Cues 72 hr Afterward

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Initial					
Exposure (I)	1	753219.781	753219.781	6.27	p=.015
Reexposure (R)	1	16571718.245	6571718.245	13.80	p=.000
I x R	1	10422687.327	10422687.327	8.68	p=.005
Within Error	57	68425035.457	1200439.219		

Table 6b (Experiment 6)

Analysis of Variance of Antibody Titers of Mice Initially Exposed to
Footshock 2 Weeks Prior to Immunization and Re-Exposed to Stressor-
Related Environmental Cues 72 hr Afterward

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Initial					
Exposure (I)	1	20.643	20.643	15.75	p=.000
Reexposure (R)	1	25.786	25.786	19.68	p=.000
I x R	1	18.286	18.286	13.95	p=.000
Within Error	52	68.143	1.310		

Table 7a (Experiment 7a)
 Analysis of Variance of the PFC Responses of Mice Initially Exposed to
 Footshock Immediately Following Immunization and Re-exposed to
 Footshock or to Shock-Related Cues 72 hr Afterward

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Initial					
Exposure (I)	1	8129885.199	8129885.199	8.03	p=.006
Reexposure (R)	2	24057111.771	12028555.886	11.88	p=.000
I x R	2	8912767.098	4456383.549	4.400	p=.015
Within Error	84	85070232.242	1012740.860		

Table 7b (Experiment 7a)

Analysis of Variance of Antibody Titers of Mice Initially Exposed to
Footshock Immediately Following Immunization and Re-exposed to
Footshock or to Shock-Related Cues 72 hr Afterward

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Initial					
Exposure (I)	1	13.341	13.341	7.51	p=.008
Reexposure (R)	2	25.166	12.583	7.09	p=.002
I x R	2	12.458	6.229	3.51	p=.035
Within Error					

Table 8a (Experiment 8a)

Analysis of Variance of PFC Responses of Mice Exposed to Footshock on 5
Consecutive Days Commencing on the Day of SRBC Inoculation

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Shock	2	14822714.	7411357.1	8.07	p=.002
Within Error	22	2020352.187.	918341.903		

Table 8b (Experiment 8a)

Analysis of Variance of Antibody Titers of Mice Exposed to Footshock on 5
Consecutive Days Commencing on the Day of SRBC Inoculation

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Shock	2	113.506	6.753	3.68	p=.046
Within Error	18	33.065	1.837		

Table 8c (Experiment 8b)

Analysis of Variance of PFC Responses of Mice Exposed to Repeated
Footshock Prior to Inoculation

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Initial					
Exposure (I)	2	22783996.	11391998.	4.29	p=.018
Reexposure (R)	1	830482.81	830482.81	0.31	p=.578
I x R	2	33475044.	16737522.	6.31	p=.003
Within Error	65	172496450.2	26537915.42		

Table 8d (Experiment 8b)
 Analysis of Variance of Antibody Titers of Mice Exposed to Repeated
 Footshock Prior to Inoculation

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Initial					
Exposure (I)	2	22783996.	11391998.	4.29	p=.018
Reexposure (R)	1	830482.81	830482.81	0.31	p=.578
I x R	2	33475044.	16737522.	6.31	p=.003
Within Error	65	172496450.2	26537915.42		

Table 8e (Experiment 8c)

Analysis of Variance of PFC Responses of Mice Exposed to repeated
Footshock Prior to Inoculation

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Initial					
Exposure (I)	2	3511026.3	1755513.1	1.18	p=.314
Reexposure (R)	2	623602.38	311801.19	0.21	p=.811
I x R	4	21509023.	5377255.7	3.63	p=.011
Within Error	53	78586064.29	1482755.93		

Table 8f (Experiment 8c)

Analysis of Variance of Antibody Titers of Mice Exposed to repeated
Footshock Prior to Inoculation

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Initial					
Exposure (I)	2	36.113	18.056	7.55	p=.001
Reexposure (R)	2	10.612	5.306	2.22	p=.118
I x R	4	51.936	12.985	5.43	p=.001
Within Error	57	136.38	2.392		

Table 10b (Experiment 10)

Analysis of Variance of PFC Responses of 3- and 9- Month Old Mice
Exposed to Varying number of Shocks 72 hr Following Inoculation

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Age (A)	1	55956508.	55956508.	61.29	p=.000
Shock (S)	3	37061241.	12353747.	13.53	p=.000
A x S	3	7722548.9	2574183.	2.82	p=.042
Within Error	125	114124712.8	912997.703		

Table 10b (Experiment 10)
 Analysis of Variance of Antibody Titers of 3- and 9- Month Old Mice
 Exposed to Varying Number of Shocks 72 hr Following Inoculation

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Age (A)	1	1.001	1.001	60.44	p=.000
Shock (S)	3	44.515	14.838	8.95	p=.000
A x S	3	13.488	4.496	2.71	p=.048
Within Error	126	208.852	1.657		

Table 11a (Experiment 11)
 Analysis of Variance of PFC Responses of 3- and 9- Month Old Mice
 Exposed to No Treatment, Handling or Apparatus 72 hr Following
 Inoculation

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Age (A)	1	27404201.	27404201.	20.83	p=.000
Shock (S)	2	9290611.6	4645305.8	3.53	p=.034
A x S	2	2946657.2	1473328.6	1.12	p=.332
Within Error	76	99967962.46	1315367.927		

Table 11b (Experiment 11)
Analysis of Variance of Antibody Titers of 3- and 9- Month Old Mice
Exposed to No Treatment, Handling or Apparatus 72 hr Following
Inoculation

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Age (A)	1	50.370	50.370	21.75	p=.000
Shock (S)	2	21.755	10.877	4.70	p=.012
A x S	2	4.299	2.149	0.92	p=.399
Within Error	86	199.175	2.316		

Table 12a (Experiment 12)
Analysis of Variance of MHPG Accumulation in Hypothalamus

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day 4	1	206.134	206.134	9.56	p=.000
Within Error	126	127.609	1.013		

Table 12b (Experiment 12)

Analysis of Variance of NE Content in Hypothalamus

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day4	1	145.332	145.332	3.78	p=.054
Within Error	124	4761.606	3.84		

Table 12c (Experiment 12)

Analysis of Variance of MHPG Accumulation in Locus Coeruleus

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day 4	1	17.847	17.847	6.90	p=.010
Within Error	8	222.514	2.587		

Table 12d (Experiment 12)
 Analysis of Variance of NE Content in Locus Coeruleus

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day 4	1	257.674	51.534	1.22	p=.306
Within Error	84	3541.637	42.162		

Table 12e (Experiment 12)

Analysis of Variance of MHPG Accumulation in Hippocampus

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day 4	1	33.959	33.959	11.30	p=.001
Within Error	107	321.525	3.005		

Table 12f (Experiment 12)
Analysis of Variance of NE Content in Hippocampus

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day 4	1	0.305	0.305	0.03	p=.858
Within Error	11	1056.506	9.518		

Table 12 g (Experiment 12)
Analysis of Variance of MHPG Accumulation in Prefrontal Cortex

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day 4	1	6.514	6.514	2.08	p=.152
Within Error	123	385.074	3.130		

Table 12f (Experiment 12)

Analysis of Variance of NE Content in Prefrontal Cortex

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day 4	1	1.865	1.865	0.38	p=.536
Within Error	109	528.717	4.851		

Table 13a (Experiment 12)

Analysis of Variance of DOPAC Accumulation in Nucleus Accumbens

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day 3	1	280.026	280.026	7.67	p=.007
Within Error	100	3648.824	36.488		

Table 13b (Experiment 12)

Analysis of Variance of DOPAC Accumulation in Nucleus Accumbens

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day 4	1	26.579	26.579	0.73	p=.395
Within Error	100	3648.824	36.488		

Table 13c (Experiment 12)
Analysis of Variance of HVA Accumulation in Nucleus Accumbens

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day 3	1	4.708	4.708	0.11	p=.742
Within Error	82	354.446	43.225		

Table 13d (Experiment 12)

Analysis of Variance of HVA Accumulation in Nucleus Accumbens

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day 4	1	3.303	3.303	0.08	p=.783
Within Error	82	354.446	43.225		

Table 13e (Experiment 12)
Analysis of Variance of DA Accumulation in Nucleus Accumbens

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day 3	1	12408.847	12408.847	6.49	p=.012
Within erro	98	187299.880	1911.223		

Table 13f (Experiment 12)

Analysis of Variance of DA Accumulation in Nucleus Accumbens

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day 4	1	13265.417	13265.417	6.94	p=.010
Within Error	98	187299.880	1911.223		

Table 13g (Experiment 12)

Analysis of Variance of DOPAC Accumulation in Prefrontal Cortex

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day2	1	6.623	6.623	5.00	p=.027
Within Error	122	161.550	1.324		

Table 13h (Experiment 12)
Analysis of Variance of DOPAC Accumulation in Prefrontal Cortex

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day4	1	16.321	16.321	12.33	p=.001
Within Error	122	161.550	1.324		

Table 13i (Experiment 12)

Analysis of Variance of HVA Accumulation in Prefrontal Cortex

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day 2	1	2.427	2.427	0.68	p=.041
Within Error	111	398.315	3.588		

Table 13j (Experiment 12)

Analysis of Variance of HVA Accumulation in Prefrontal Cortex

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at day 4	1	4.861	4.861	1.35	p=.247
Within Error	111	398.315	3.588		

Table 13k (Experiment 12)

Analysis of Variance of DA Accumulation in Prefrontal Cortex

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day 2	1	2.637	2.637	1.39	p=.240
Within Error	124	234.593	1.892		

Table 131 (Experiment 12)

Analysis of Variance of DA Accumulation in Prefrontal Cortex

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day 4	1	0.002	0.002	0.00	p=.971
Within Error	124	234.593	1.892		

Table 13m (Experiment 12)

Analysis of Variance of DOPAC Accumulation in Caudate Nucleus

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day 4	1	14.635	14.635	0.32	p=.573
Within Error	109	5005.145	45.919		

Table 13n (Experiment 12)

Analysis of Variance of HVA Accumulation in Caudate Nucleus

<u>Source</u>	<u>df</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>SIG</u>
Antigen					
at Day 4	1	40.954	40.954	0.51	p=.448
Within Error	88	7083.913	80.499		

END

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