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**Alterations in the Responsiveness of the Immune System
Due to Strain and Stressor Parameters**

**A Thesis presented to the Faculty of Graduate Studies and Research
of Carleton University in partial fulfillment of the requirements of
the degree of Master of Science in Psychology**

**Leslie Kerr
1993**

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**The undersigned hereby recommend to
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Leslie Kerr

**in partial fulfilment of the requirements for the degree of
Master of Science**

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Chair, Department of Psychology

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June, 1993**

ABSTRACT

Stressors have been shown to provoke a suppression of the immune response engendered by antigen exposure. However, the development of the immune response and its subsequent modification by stressors may be dependent on organismic variables, stressor characteristics, and the timing of stressor application relative to the time of antigen inoculation. The first of this series of experiments assessed whether the development and the magnitude of the peak immune response to sheep red blood cells (SRBC) was species specific. The splenic plaque forming cell (PFC) response in the BALB/cByJ and the DBA/2J inbred mouse strains peaked 5 days following inoculation to SRBC (5×10^6 , ip). Both the magnitude and the time of the peak immune response observed in these strains differed from that previously seen in the outbred CD-1 strain, where the peak immune response occurred 4 days following inoculation. Although both the BALB/cByJ and the DBA/2J strains exhibited similar profiles with respect to the timing of the peak immune response, the developmental patterns as well as the magnitudes of the peak immune responses were significantly different. The magnitude of the peak immune response in the BALB/cByJ strain was significantly larger than that observed in the DBA/2J strain; however, the immune response mounted to SRBC developed sooner in the DBA/2J mice (4 as opposed to 5 days as in the BALB/cByJ strain). Experiment 2 demonstrated that stressor application influenced the magnitude of the peak immune response, but that this effect occurred only in the BALB/cByJ strain, and was dependent on

the timing and severity of the stressor experience. In BALB/cByJ mice, footshock of 150 μ A applied 4 days after antigen administration provoked a significant suppression of the peak PFC response. However, neither a mild stressor (novel apparatus exposure), nor a more severe stressor (footshock of 300 μ A), significantly altered the immune response in this strain. Interestingly, when applied just prior to the peak immune response, each of these stressors induced a significant immunosuppression. As observed previously in CD-1 mice, a stressor applied at earlier intervals did not influence the peak PFC response in the BALB/cByJ strain. There is reason to suspect that β -adrenergic receptors contributed to the immunosuppression induced by a stressor applied 4 days after SRBC inoculation, since propranolol antagonized the immunosuppressive effects of 150 μ A footshock. In contrast, propranolol did not influence the immunosuppression engendered by footshock applied 5 days after inoculation. It is provisionally suggested that stressors have multiple neurochemical and endocrine effects which might act to provoke an immunosuppression. Moreover, inasmuch as the expression of receptors present on lymphocytes may vary over time following activation, the relative contribution of these neurochemical and endocrine factors may differ over the course of the developing immune response. Likewise, it is suggested that the strain differences in the immunosuppressive effects of stressors may relate to differences in the neurochemical or neuroendocrine changes associated with aversive experiences in the BALB/cByJ and the DBA/2J strains.

ACKNOWLEDGEMENTS

I would like to thank Hymie Anisman for the considerable amount of assistance he has provided over the course of these studies.

My gratitude is extended to Anna Minkiewicz-Janda, who provided excellent tutelage in the art of the PFC and titer assays, among other lab procedures. Also, to all those who helped me in various ways around the lab; Bob R., Darlene, Leslie D., Marilee, Joanne, Rachel, and Glenda.

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Glossary

Adrenocorticotrophic Hormone (ACTH): a hormone secreted by the anterior pituitary gland that acts primarily on the adrenal cortex, stimulating its growth and its secretion of corticosteroids, which can effect immune functioning. The production of ACTH is increased during times of stress.²

Antibody: A protein formed in response to immunization. ¹

Antibody Titer: An immunological test that measures the relative amount of antibody in the serum of an animal.

Antigen: A substance which causes the induction of antibodies, and reacts in some specific manner with the antibodies so induced. Antigens may be soluble substances such as toxins and foreign proteins, or particulate such as bacteria and tissue cells.²

Cell Mediated Immunity: Specific immunity which is mediated by T lymphocytes.¹ Cell mediated immunity is responsible for reactions such as allograft rejection , delayed hypersensitivity, and is important in the defence against viral infections and against some bacteria.³

Complement: A series of serum proteins involved in the immune reaction. The complement cascade is triggered classically by the interaction of antibody with a specific antigen.¹

Corticotropin Releasing Factor (CRF): A neuropeptide produced by the hypothalamus which stimulates the immune system and the pituitary gland to produce adrenocorticotrophic hormone (ACTH). CRF is also known as corticotropin releasing hormone (CRH).²

Cytokine: A group of antigen-nonspecific glycoproteins produced by lymphocytes (**lymphokines**) or monocytes (**monokines**) which are involved in signalling between cells of the immune system. Those cytokines which participate in cellular communication between leukocytes are referred to as **interleukins**.¹

Humoral Immunity: Specific immunity mediated by antibodies which are present in plasma, lymph, and tissue fluids of the body, and which may also become attached to cells (cytophilic antibody).³

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.³

Interleukins: A group of antigen-nonspecific glycoproteins involved in lymphocyte activation and differentiation.

Interleukin 1 (IL-1) is produced by monocytes and macrophages; it promotes T cell proliferation.

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.⁴

Major Histocompatibility Complex (MHC): A cluster of genes important in immune regulation and signalling between cells of the immune system. The gene complex was originally identified as a locus of 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.⁴

Lymphocyte: A small, spherical cell with a large round nucleus, but virtually no 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.³

B cells: Cells that are derived from bone marrow without passing through the thymus. B lymphocytes play a major role in humoral immunity. On stimulation by an antigen they differentiate into antibody-forming plasma cells.³

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

T-Helper cells: A subset of T-lymphocytes which stimulate antibody production.

T-Cytotoxic cells: A subset of T-lymphocytes which directly lyse virus-infected cells, tumor cells, and transplanted tissue.

T-Suppressor cells: A subset of T-lymphocytes capable of regulating the magnitude of immune effector cell responding.¹

Macrophage: A large phagocytic cell of the mononuclear series. 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.⁴

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

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

Phytohaemagglutinin (PHA): A T cell mitogen which can induce cell functions and lymphocyte production.

Pokeweed Mitogen (PWM): Stimulates both T and B cells, and can induce IgM synthesis by B cells.⁴

Natural Killer Cells: Large, granular, lymphocyte-like killer cells which are capable of killing a number of virally-infected and transformed target cells with which they have had no prior interaction.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.⁴

Spleen: A secondary lymphoid organ, which lies in the peritoneum beneath the diaphragm, and behind the stomach. The spleen contains two main types of tissue termed the red pulp and the white pulp. It is a site of antibody production especially against particulate antigens (eg. SRBC).⁴

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.⁴

- ¹Benjamini E. & Leskowitz, s. Immunology: A Short Course. New York: A. John Wiley and Sons Publications Inc., 1991.
- ²Dorland's Illustrated Medical Dictionary. Twenty-fifth Edition. Philadelphia:W.B. Saunders Company, 1965.
- ³Herbert, W.J. & Wilkinson, P.C. A Dictionary of Immunology. Second Edition. Philadelphia: Blackwell Scientific Publications Ltd., 1977.
- ⁴Male, D. Immunology: An Illustrated Outline. St. Louis: The C.V. Mosby Company, 1987.

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Figure 1. Mean plaque forming cell (PFC) responses of BALB/cByJ and DBA/2J mice whose spleens were taken at various times (1,2,3,4, or 5 days) following intraperitoneal administration of SRBC (5×10^6 cells).

Figure 2a. Mean ($\pm$ S.E.M.) splenic plaque forming cell (PFC) responses in BALB/cByJ mice that were exposed to a single session of either apparatus (top frame), 150 μ A footshock (middle frame), or 300 μ A footshock (bottom frame) immediately following, 1, 2, 3, 4, or 5 days after SRBC (5×10^6) administration. All animals had their spleens removed 5 days after antigen inoculation.

* 150 μ A footshock applied 4 days after antigen administration in the BALB/cByJ strain had a significant ($p < 0.05$) immunosuppressive effect relative to the unstressed control animals. Taken together, all three stressor severities exhibited a significant ($p < 0.05$) immunosuppressive effect 5 days after antigen administration when compared to unstressed controls.

Figure 2b. Mean ($\pm$ S.E.M.) splenic plaque forming cell (PFC) responses in DBA/2J mice that were exposed to a single session of either apparatus (top frame), 150 μ A footshock (middle frame), or 300 μ A footshock (bottom frame) immediately following, 1, 2, 3, 4, or 5 days after SRBC (5×10^6) administration. All animals had their spleens removed 120 hours after antigen inoculation.

Figure 3. Mean PFC responses in BALB/cByJ mice that were exposed to either saline or propranolol treatment in conjunction with either no shock or footshock (150 μ A), 4 or 5 days after SRBC (5×10^6) injection. All mice had spleens removed 5 days post-SRBC administration.

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- Table 1.** Mean antibody titer to sheep red blood cells ($\text{Log}_2 \pm \text{S.E.M.}$) determined 3, 4, 5, or 6 days following antigen administration, titers determined 1 or 2 days after antigen administration for both strains were zeros and are not shown.
- Table 2a.** Mean antibody titer to sheep red blood cells ($\text{Log}_2 \pm \text{S.E.M.}$) in BALB/cByJ mice exposed to a single session of either apparatus, 150 μA , or 300 μA footshock at various intervals (0, 1, 2, 3, 4, or 5 days) following SRBC (5×10^6) inoculation. Trunk blood was collected 120 hours after antigen administration, the time of the peak immune response.
- Table 2b.** Mean antibody titer to sheep red blood cells ($\text{Log}_2 \pm \text{S.E.M.}$) in DBA/2J mice exposed to a single session of either apparatus, 150 μA , or 300 μA footshock at various intervals (0, 1, 2, 3, 4, or 5 days) following SRBC (5×10^6) inoculation. Trunk blood was collected 120 hours after antigen administration, the time of the peak immune response.

Alterations in the Responsiveness of the Immune System due to Strain and Stressor Parameters

Immune system activity had long been considered independent of central nervous system (CNS) influences. However, it has become increasingly evident that bidirectional communication exists between the immune system and the CNS, as well as between immune and endocrine systems (see reviews in Anisman, Zalcman & Zacharko,1993; Blalock,1992; Carr,1992; Dantzer & Kelley,1989; Dunn,1990; Rabin, Cohen, Ganguli, Lysle & Cunnick,1989; Rivier,1991). There is now ample evidence showing that CNS manipulations will modulate immune functioning, and conversely immunological manipulations can affect central neurotransmitter activity (Anisman et al.,1993; Dunn,1990; Rabin et al.,1989). Among other things, it appears that lymphoid cells secrete endocrine and neurotransmitter-like factors (broadly termed cytokines) which may not only be acting in a paracrine or autocrine fashion, but may also influence neurotransmission within the CNS (Besedovsky & Del Rey,1991; Blalock,1992; Carr,1992; Rivier,1991). Indeed, cytokines and cytokine receptors have been identified within the CNS, and it is thought that cytokines, such as interleukins, may be acting as central immunotransmitters (Dunn,1990; Imura, Fukata & Mori,1991; Lapchak,1992; Lapchak, Araujo, Quirion & Beaudet,1991; Merrill,1990; Nistico & De Sarro,1991). Additionally, lymphocytes bare receptors for several hormone and neurotransmitter substances (Blalock, Bost & Smith,1985; Felten & Felten,1991; Livnat, Felten, Carlson, Belinger & Felten,1985; Sanders & Munson,1985), and hence CNS, as well as

sympathetic neurons may influence the response of activated lymphocytes.

There still remains considerable uncertainty as to the processes underlying brain-immune communications. Several possible channels have been proposed through which the immune system may affect CNS activity, and conversely, through which the CNS may influence immune processes (see reviews in Anisman et al.,1993; Dunn,1990; Roszman & Carlson,1991). Among other things, communication between the CNS and immune system may occur via activation of the hypothalamic-pituitary-adrenal axis, hypothalamic-pituitary-gonadal axis, sympatho-adrenomedullary axis, peripheral catecholamine alterations, or directly through penetration of lymphocyte- or monocyte-derived substances (such as interleukins or endorphins) into the brain. Indeed, Blalock (1984,1992) proposed that the immune system acts as a sensory organ to inform the brain of an antigenic challenge. Subsequently, it was suggested that challenges to the immune system are interpreted by the brain much in the same way as stressors are interpreted (Anisman et al.,1993; Dunn,1990). In fact, alterations in immune functioning can lead to CNS variations that may engender behavioral changes comparable to those elicited by stressors (see reviews in Anisman et al.,1993; Dunn,1990). The bidirectional communication that is reported to exist between the CNS and the immune system may be necessary to provide the organism with different ways of recognizing challenges to homeostasis. This recognition would be essential so that an integrative-type of response (physiological and/or behavioral) to a physical or psychological stressor could be effectively initiated, and the impact of the stressor

reduced.

To a great extent the interest in bidirectional communication between the immune and central nervous systems occurred following numerous reports demonstrating that aversive environmental events can influence immune system changes. Curiously, despite the apparent acceptance of the supposition that stressors will affect immune activity, the literature is replete with inconsistent and even contradictory findings (reviewed in Anisman et al.,1993). Among other things, the incongruent findings may be related to the specific immune compartments examined (eg. splenic vs. circulating lymphocyte activity) or the immunological processes assessed (eg. type and number of activated immune cells, cytotoxicity of these cells, and cell proliferation in response to mitogens). Moreover, the immunological response to stressors may vary as a function of numerous organismic and experiential variables, as well as characteristics of the stressor procedures employed. Indeed, it has been demonstrated that different immunoregulating neural and neuroendocrine mechanisms (e.g., opioid dependent vs. opioid independent, corticosterone dependent vs. corticosterone independent) can be engendered by one type of stressor but not by another (Keller, Weiss, Schleifer, Miller, & Stein,1983; Shavit, Terman, Lewis, Zane, Gale & Liebskind,1986; Terman, Morgan & Liebskind,1986). Inasmuch as appreciable inter-laboratory differences exist with respect to the stressful procedures that have been employed, it is not surprising that apparently contradictory results have been reported.

The present investigation will be undertaken to assess the immunological impact of three variables that have been identified as

possibly being fundamental in determining the immunological changes associated with stressors. Specifically, the contribution of stressor severity, timing of stressor application relative to antigenic challenge, and the strain of animal used, will be assessed. The following sections provide an overview of the stress-immune literature, with particular attention devoted to the potential contribution of these three variables.

Acute Stressor Effects on Immune Functioning

Aversive stimulation may effectively compromise various immune processes in humans and may influence the course of some pathologies (see reviews in Daruna & Morgan,1990; Glaser, Kiecolt-Glaser, Bonneau, Malarkey, Kennedy & Hughes, 1992; Kiecolt-Glaser, Garner, Speicher, Penn, Holliday & Glaser,1984). Likewise, in infrahuman animals, acute stressors may suppress splenic and serum natural killer (NK) cell activity (Aarstad, Gaudernack & Seljelid,1983; Shavit, Lewis, Terman, Gale & Liebeskind,1984), the proliferative response to T and B cell mitogens (Cunnick, Lysle, Armfield & Rabin,1988; Keller, Weiss, Schleifer, Miller & Stein,1981; Lysle, Cunnick, Fowler, Rabin,1988; Lysle, Lyte, Fowler & Rabin,1987), the secretion of various cytokines (e.g., IL-1, IL-2, IL-6) (Abraham,1991; Dunn,1990; Rivier,1991), plasma IL-1 responses to endotoxin challenge (Berkenbosch, Wolvers & de Rijk,1991), as well as the splenic plaque forming cell (PFC) response, and plasma antibody titers (Esterling & Rabin,1987; Okimura & Nigo,1986). Additionally, stressful events have been shown to influence pathologies associated with viral challenge, tumorigenic processes, autoimmune-like disorders, and rejection of allogenic skin grafts (Grotta, Moynihan, Schmidt, Schactman, Cohen &

Ader,1989; Sklar & Anisman,1981; Wistar & Hildemann,1960) .

The preponderance of data has certainly been consistent with an immunosuppressive role for acute stressors. For instance, several investigators have demonstrated that various forms of aversive stimulation (e.g. footshock, cold water immersion, surgical trauma) can result in the suppression of NK cytotoxicity (Aarstad et al.,1983; Okimura, Ogawa & Yamauchi,1986; Zalcman, Irwin & Anisman,1991). Additionally, it has been demonstrated that suppression of NK cytotoxicity is dependent on stressor controllability (Shavit, Ryan, Lewis, Laudenslager, Terman, Maier et al.,1983). In particular, these investigators demonstrated that although escapable footshock did not influence the NK response, an identical amount of inescapable shock effectively reduced NK cytotoxicity. Moreover, it was found that treatment with the opioid antagonist, naltrexone, effectively eliminated the impact of stressors, leading to the suggestion that the suppression of the NK cell activity induced by a stressor was mediated through an opiate receptor (Shavit et al.,1984).

In addition to the effects of opiate peptides, it appears that corticotropin releasing factor (CRF) may also be fundamental to stressor-induced alterations of NK cell activity. In particular, it was demonstrated that pretreatment with a CRF antibody prevented the footshock-provoked NK-cell response (Irwin,Vale & Rivier,1990). Since such an effect was also evident in hypophysectomized animals (Jain, Audhya, Hutchison, & Hollander,1989; Jain, Zwickler, Hollander, Brand, Saperstein, Hutchison et al.,1991), it is likely that a pituitary-independent mechanism was responsible for the stressor induced suppression. In fact, it has been demonstrated that CRF activates

autonomic nervous system activity, as reflected by increased plasma concentrations of norepinephrine and epinephrine (Brown, Fisher, Rivier, Spiess, Rivier & Vale,1981; Fisher, Rivier, Rivier, Spiess, Vale & Brown,1982). The finding that sympathetic denervation of the spleen abolished the effects of CRF on NK cell activity is consistent with a role for autonomic mechanisms, involving CRF secretion, in stressor provoked NK cell suppression (Besedovsky, Del Rey, Sorkin, Da Prada & Keller,1979; Sundar, Cierpial, Kilts, Ritchie & Weiss,1990).

Apart from the suppression of NK activity, acute stressors have repeatedly been shown to reduce the proliferative response to mitogens in both spleen and blood derived lymphocytes (Cunnick et al.,1988; Lysle et al.,1987; Lysle et al.,1988). However, this particular effect, unlike the alterations of NK cell activity, was not always modifiable by opiate peptide manipulations. The differential effects of opioid manipulations on mitogen induced cell proliferation may be due to time-dependent expression of opioid receptors on lymphocytes, or the particular lymphocyte population tested (Carr, Radulescu, DeCosta, Rice & Blalock,1990; Kusnicov, Husband, King & Smith, 1989). Alternatively, the non-opioid effects that have been observed may be due to mechanisms other than the classical opioid receptor-ligand interaction, and consequently are not dependent on the lymphocyte subset tested or the expression of opioid receptors on the lymphocytes (Cunnick et al.,1988; Keller et al.,1983; Cunnick, Lysle, Armfield & Rabin,1991; Shavit et al.,1984). Indeed, since opiate peptides are present throughout the central and peripheral systems (Bhargava, Matwyshyn, Hanissian & Tejawani,1988; Morley, Kay, Solomon & Plotnikoff,1987), it is conceivable that opioids could be involved in the

modulation of the immune response via the CNS, autonomic nervous system, or the endocrine systems (thereby engendering a non-opioid type of response).

Whereas stressor-induced suppression of the NK cytotoxicity appears to be mediated by a pituitary-independent mechanism, this does not appear to be true for the the T cell proliferative response. Indeed, it has been demonstrated that the stressor engendered suppression of the T cell proliferative response to a mitogen was partially antagonized in adrenalectomized animals, and this affect could be reinstated with glucocorticoid replacement therapy (Keller, Weise, Schleifer, Miller & Stein, 1981). Given that glucocorticoids are known to act as immunosuppressants, and are secreted in response to stressors, it is tempting to suppose that the immunosuppression engendered by stressors involves adrenocorticoids (Anisman et al.,1993). Further studies, however, indicated that it was essential to differentiate stressor effects in splenic and blood immune responses. In particular, it appeared that stressor effects on blood lymphocytes could be influenced by glucocorticoids and were not subject to adaptation with repeated environmental insults, whereas the splenic proliferative response appeared to be glucocorticoid-independent, subject to an adaptation with repeated stressors, and modifiable by epinephrine manipulations (Cunnick et al.,1988; Cunnick, Lysle, Kucinski & Rabin,1990; Lysle et al.,1987). Additionally the time course for the stressor effects in these two compartments could be differentiated from one another (Cunnick et al.,1988; Cunnick et al.,1990; Laudenslager, Fleshner, Hofstader, Held, Simons & Maier,1988; Lysle et al.,1987). While the suppression of the splenic

lymphocyte response to a stressor is only evident within a 24 hour period following stressor exposure, the effects of stressors on the blood-lymphocyte response could be measured up to 48 hours after stressor application (Cunnick et al.,1988; Lysle et al.,1987). The possibility was thus entertained that the immunosuppressive effects of stressors could not only be differentiated on the basis of whether they involved modulation by adrenocortical or sympathetic mechanisms, but whether they were compartment-specific.

Although there is evidence suggesting that opioid manipulations more readily influence the NK cell response than the proliferative response to mitogens, Carr (1991) indicated that this differentiation may have been related to the specific type of opioid receptors that had been activated. At least four opiate receptors have been identified on lymphocytes (δ , ϵ , κ , μ). The δ - (preferring enkephalins), and ϵ - (preferring β -endorphin), receptors appear to be primarily responsible for augmenting the mitogen cell proliferative response (Fischer,1988; Millar, Hough, Mazorow, Gootenberg,1990), while μ - (preferring morphine), κ - and δ - receptors have been associated with the enhanced activity of NK cells (Carr,1990; Shavit et al.,1986; Sibinga & Goldstein,1988). The role that opiate peptides play in immunomodulation, however, is difficult to interpret since these peptides may also interact with the CNS (e.g. stimulating sympathetic outflow) and neuroendocrine systems (e.g. mediation of CRF production), besides directly acting on the immune system. Furthermore, the effects of opioids (endogenous or other) on the immune response have been shown to depend on the opiate peptide concentrations, the types of receptors stimulated, the type of immune

response examined, and the strain of the animal used (Shavit et al.,1984; Sibinga & Goldstein,1988; Van den Bergh, Rozing & Nagelkerken,1991). However, considering the immunosuppressive effects of stress, coupled with the finding that stressors influence circulating opiate peptides concentrations, it is tempting to suppose that opiate peptides may contribute to stressor-induced immunomodulation.

Time Course of the Immune Response

In addition to variations of NK cytotoxicity and the proliferative response to T and B cell mitogens, several investigators reported that stressors may affect the number of antibody forming cells, as well as antibody titers. For instance, the splenic plaque forming cell response and serum antibody titers in response to sheep red blood cells (SRBC) was markedly reduced in mice that had been exposed to a stressor (see Anisman et al.,1993). However, it appeared that this effect was dependent upon the timing of stressor application relative to SRBC immunization. For example, if CD-1 mice were stressed 72 hours following immunization (the peak PFC response being 96 hours after immunization in this strain), the PFC response was appreciably suppressed. At other times following immunization no such effect was evident. Okimura and Nigo (1986) also noted time-dependent effects, in that restraint applied two days following antigen administration did not produce an immunosuppressive effect, whereas, stressor exposure on two days prior to antigen administration did provoke an immunosuppression.

Interestingly, in older mice (nine- as opposed to three-months)

the critical period for stressor induced suppression of the immune response has been shown not to be comparable to that seen in younger mice, although the immunosuppressant effects could be more readily induced (Zalcman, Minkiewicz-Janda, Richter & Anisman,1988; Zalcman, Richter & Anisman,1989). Curiously, in older animals that had been stressed within 24 hours of SRBC treatment a marked immunoenhancement was evident. Inasmuch as it is unlikely that a stressor could provoke a genuine immunofacilatory action in older animals, it was suggested that the stressor disrupted immunoregulatory processes (e.g., attenuation of T suppressor cells), thereby provoking the apparent immunoenhancement (Zalcman, Henderson, Richter & Anisman,1989). Whether this explanation is viable still remains to be determined, but these data serve to illustrate that the effects of stressors may be influenced by organismic variables, such as the age of the organism, and that under some conditions stressors may provoke immunoenhancing rather than immunosuppressive effects.

Although a stressor applied soon after SRBC inoculation in young adult mice did not significantly affect the peak immune response, this treatment proactively influenced the immune response to a subsequent stressor applied 72 hours after immunization. In particular, the immunosuppression ordinarily provoked by a stressor applied 72 hour following inoculation was absent in mice that had previously been stressed soon after SRBC treatment. Moreover, in mice that had been stressed soon after inoculation subsequent exposure (at the 72 hour interval) to stressor-related cues resulted in a significant immunoenhancement (Zalcman et al.,1989). It is unclear

whether the immunoenhancement was due to a compensatory conditioned immune response stemming from the initial stressor experience, or was simply a reflection of the impact of a mild stressor experience (consider that a CS is typically less effective than the UCS) provoking an immunoenhancing effect.

Effects of Stressor Parameters on Immune System Responsiveness

Commensurate with the proposition that weak stressors may elicit immunofacilitory actions, several investigators reported that mild environmental challenges may augment the splenic mitogen response (Croiset, Heijnen, Veldhuis, de Weid & Ballieux, 1987), the SRBC elicited PFC response (Croiset, Ballieux, de Weid & Heijnen, 1989; Fujiwara & Orita, 1987), as well as NK cell activity and IL-2 production (Weiss, Sundar, Becker & Cierpial, 1989). For instance, whereas 2-19 hours of intermittent tail shock provoked an exceptionally pronounced suppression of NK cell cytotoxicity in rats, three shock presentations had an immunoenhancing effect (Weiss et al., 1989). It was similarly reported that while handling rats for 1 minute provoked an enhancement of both the T and B cell proliferative response in blood, the stress of supine restraint for 1 minute, provoked an immunosuppression (Rinner, Schauenstein, Mangge, Porta & Kvetnansky, 1992). Likewise, the PFC response to SRBC, which is typically suppressed by relatively strong stressors was enhanced by five minutes of restraint (Berkenbosch et al., 1991). Croiset et al. (1987) similarly demonstrated an enhanced immune response in mice that received a single foot shock or exposure to a novel environment prior to SRBC inoculation. Interestingly, in this experiment a single,

mild stressor session followed by re-exposure to fear eliciting cues did not produce the immunoenhancement. These investigators (Croiset et al.,1987) noted that the immunoenhancing effects of a mild stressor were evident if SRPC was administered within 15 minutes of the stressor; however, if longer periods (1,4 or 8 hours) intervened between the two treatments, the immune enhancement was not evident (Croiset et al.,1989).

The mechanisms operative in eliciting immunofacilatory and immunosuppressive effects of mild and strong stressors, respectively, remain to be identified. It is interesting, however, that Fujiwara and Orita, (1987) found that the PFC response (measured *in vitro*) in untreated mice was enhanced by administration of sera from stressed (tail pinch) mice. Pretreatment of mice with the β -NE receptor antagonist, propranolol, antagonized the effects of the stressor, where as an α -NE receptor antagonist was without effect. These investigators offered the proposition that mild stressors promote T-helper cell activation through epinephrine receptor stimulation, hence leading to immunoenhancement. As stressor severity increases, the secretion of adrenocorticoids ordinarily engendered by such treatment will serve to provoke an immunosuppression. In effect then, various stressor severities may differentially activate "stressor-specific" receptors present on lymphocytes, which may be responsible for the opposite immunological changes that have been observed.

The time-dependent effects of stressors (i.e., suppressing the PFC response when applied at 72 hr after immunization but not at earlier intervals) may also be due to, among other things, the differential expression of α - and β -epinephrine receptors on lymphocytes. In

particular, Sanders and Munson (1985) demonstrated that the effects of β -NE receptors antagonist on the *in vitro* generated PFC response varied over time following SRBC administration, and suggested that the expression or responsivity of adrenergic receptors varied over the course of the immune response being mounted. At this juncture, it should be noted, however, that Rinner et al. (1992) found that the time course for the immunoenhancement associated with a mild stressor did not correspond with variations of plasma epinephrine, norepinephrine or prolactin. Moreover, analyses of peripheral hormone levels among individual animals did not correspond with the immune response. Thus, these investigators provisionally dismissed a role for peripheral catecholamines in determining the immunoenhancement. However, it is important to recognize that the mechanisms that support an immunoenhancement in blood lymphocytes may be different from those subserving this effect in splenic lymphocytes. Furthermore, it may ultimately be important to distinguish between effects seen following *in vivo* lymphocyte activation from the *in vitro* generated immune response. Indeed, a correlation between epinephrine and norepinephrine levels in blood and alterations of the immune response, as gauged by the PFC response, need not be expected since the critical feature in determining the nature of the immune response may be the expression of α and β receptors on lymphocytes.

Genetic Influences on the Immune Response

Although it is well established that profound genetic differences exist across strains of mice and rats with respect to basal hormonal, neurochemical, and immunological activities, there are a paucity of

data evaluating the impact of stressors as a function of the genetic variable. This is all the more surprising when it is considered that the response to stressors in humans and in infrahuman animals is, other things being equal, exceedingly variable. It is significant that the few studies which have been reported with respect to stressor effects on immune activity have revealed several paradoxical findings. For instance, in contrast to the suppression of the mitogen response typically observed in rats, Lysle, Cunnick and Rabin, (1990) reported that stressor exposure increased the mitogen response in three of four strains of mice (C3H/HeJ, C57BL/6N, HLA-SW/ICR) and had no effect in a fourth strain, C3H/HeN. The extent of the immunoenhancement was directly related to stressor severity. In a subsequent study using the HLA-SW-ICR strain, it was observed that with repeated stressor exposure an adaptation developed such that the enhanced mitogen response was no longer evident. In a sense, the latter finding is reminiscent of studies conducted in rats, where the immunosuppressive effects of an acute stressor are abated with repeated environmental insults (Cunnick et al.,1988; Lysle et al.,1987). Unfortunately, it is entirely unclear why these researchers observed acute stressors to provoke an enhancement of the mitogen response in mice, rather than the suppression they typically observed in rats (see Rabin et al.,1989). At this juncture, the possibility must be considered that these results reflect species differences in the mitogen response following stressor application. Alternatively, in light of the findings that stressors may suppress the PFC response in non-inbred mice, the possibility exists that the immunoenhancement reported by Lysle et al. (1990) was exclusive to inbred mice or to the particular strains

employed by those investigators.

In contrast to the stressor induced enhancement of the mitogen response in mice, it has been reported that footshock will produce a reduction of NK cytotoxicity. This effect was evident in three inbred strains of mice, including C3H/HeJ, which Lysle et al. (1990), had shown to exhibit enhancement of a mitogen response (Zalcman et al., 1991). It might be noted that in this particular study the time course for the NK cell alterations varied across the three strains of mice. In BALB/cByJ mice, the suppression of NK cell cytotoxicity lasted only 24 hours, in C57BL/6J it was still apparent up to 48 hours after stressor exposure, while in C3H/HeJ, the suppression was present at the 48 hour interval but took longer to develop following stressor exposure (Zalcman et al., 1991). Livnat, Irwin, Ader, Gallo, and Anisman (1985) similarly observed time- dependent variations in the development of stressor induced suppression of NK cell cytotoxicity in C57BL/6J and DBA/2J mice.

Genetic analyses which have been conducted to assess the neurochemical and hormonal changes associated with stressors have indicated that the basal level or turnover of a neurotransmitter or hormone is not necessarily indicative of the neurochemical/hormonal response to a stressor (Anisman, Irwin, Bowers, Ahluwalia & Zacharko, 1986; Anisman, Zalcman, Shanks & Zacharko, 1991; Molina, Volosin, Cancela, Keller, Muraua & Basso, 1990). That is, in the absence of a stressor, concentrations of circulating steroids in a particular strain were not indicative of its reactivity to a stressor. Furthermore, marked strain differences in neurotransmitter lability exist in response to stressors, such that the appearance of a particular

neurotransmitter change in a given brain region is typically not predictive of the same (or other) transmitter alterations in other brain regions. These caveats aside, behavioral and biochemical analyses have indicated that the BALB/cByJ strain of mouse is particularly vulnerable to stressor-induced behavioral and neurochemical alterations, and also exhibits particularly marked alterations of plasma corticosterone in response to environmental challenges (Shanks & Anisman,1989; Shanks, Griffiths, Zalcman, Zacharko & Anisman,1990; Shanks, Zalcman, Zacharko & Anisman,1991). Thus, this strain, together with somewhat more hardy animals such as the C57BL/6J or DBA/2J, may serve as an ideal preparation to evaluate the impact of stressors on immune system activity. Additionally, since considerable information is already available in these strains concerning corticosterone and central amine variations engendered by stressors it may be possible to ascertain whether correspondence exists between the immunological, neurochemical and adrenocortical alterations engendered by stressors.

Summary

The available data suggest that several fundamental variables may influence the nature of the immunological alterations provoked by stressors. Of course, the nature of the stressor effect will be dependent upon the immune compartment being examined (eg., in blood vs. lymphoid organs, such as spleen). Additionally, it seems highly likely that while acute stressors tend to provoke immunosuppressant effects, chronic stressors may either have no effect or actually enhance the immune response (see review in

Anisman et al.,1993). Likewise, there is reason to suppose that the impact of acute stressors will vary with the species or strain being examined, the severity of the insult, and the timing of its application relative antigenic challenge. The purpose of the present investigation was to assess the contribution of the latter three variables in determining the impact of stressors on the PFC response.

EXPERIMENT 1

Determination of the Peak Immune Response in the BALB/cByJ and DBA/2J Mouse Strains

It will be recalled that in the CD-1 mice there exists a critical period following antigen administration during which a stressor will come to affect the peak splenic PFC response. This particular effect did not appear to be related to the time between stressor exposure and spleens being excised, since the suppression was only evident if the stressor was applied 3 days after the antigenic challenge irrespective of whether a 1 or a 2 day period intervened between stressor exposure and the immune assay (Zalcman et al.,1988). Inasmuch as this time-dependent effect has as yet only been demonstrated in the CD-1 mouse, it is unclear whether it is, in fact, unique to this strain. Moreover, if critical periods exist in other strains they may well differ from that seen in CD-1 mice, particularly since the development of the peak splenic PFC response might be strain-dependent. Accordingly, prior to evaluating the effects of stressor exposure on the PFC response a preliminary study was conducted in two strains of mice, BALB/cByJ

and DBA/2J, to evaluate the time course and magnitude of the PFC response to sheep red blood cells.

METHOD

Subjects. A total of 60 male mice each of the BALB/cByJ and DBA/2J strain, approximately 6 weeks of age, were obtained from the Jackson Laboratory, Bar Harbor, Maine. Mice were housed, by strain, in groups of 4-5 in standard polypropylene cages, and permitted to acclimatize to the laboratory for 4-6 weeks before being used as experimental subjects. Mice were permitted free access to food and water and maintained on a 12 hour light-dark cycle (light 0700-1900). Testing procedures were conducted between 0800-1200.

Immunization. Sheep red blood cells were prepared by washing citrated sheep's blood 3 times in sterile saline. Animals were immunized intraperitoneally with 5×10^6 cells in a volume of approximately 0.18 ml. It was previously observed that a dosage of 10^6 - 10^7 cells resulted in a PFC response such that experimental manipulations could provoke either a decrease or increase 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 HBSS medium (GIBCO) supplemented with Hepes buffer (1.0 M solution), Gentamycin sulphate (40 mg/ml) and Penicillin G (100 units/ml: Sigma). The spleen cells were washed by centrifugation at 400 g for 10 minutes and resuspended in 2 ml of HBSS medium. The cells were then layered on a Ficoll-Hypaque gradient (density=1.1) and centrifuged at 700g for 30 minutes. After centrifugation, the

mononuclear cells at the interface were removed, resuspended in 4 ml HBSS and washed 3 times at 400g. Twenty microliters of the cell suspension (at 10^7 cells/ml) were combined with 20 μ l of guinea pig complement (absorbed with SRBC at a ratio of 2:1), 20 μ l of HBSS medium, and 20 μ l of SRBC (approximately 2.5×10^8 cells/ml). 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 μ l. 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 15 minutes. Plaques were counted by microscopic examination of the microslides at a magnification of x10. 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 400g for 10 min and the supernatant collected and spun at 600g. Serum complement was then inactivated at 56°C for 30 minutes. Two fold serial dilutions of inactivated serum, saline, and a 1% SRBC solution was then made in glass microwells. The dilution at which aggregation of SRBC was still evident was considered to be the antibody titer and expressed in $\log_2$ units.

Procedure. Mice of each strain of Experiment 1 were individually housed and assigned to 6 treatment conditions. Mice were inoculated intraperitoneally (ip) with SRBC (5×10^6 cells) and at various times thereafter (1, 2, 3, 4, or 5 days) decapitated, and spleens and trunk blood, collected for subsequent determination of the PFC response and antibody titers.

RESULTS AND DISCUSSION

Figure 1 depicts the PFC responses of the BALB/cByJ and DBA/2J mouse strains at various times following SRBC administration.

Analysis of variance confirmed that the splenic PFC response varied as a function of the interaction between Strains x Days $F(5,107) = 4.782$, $p < 0.01$. Further, Newman Keuls multiple comparison ($\alpha = 0.05$) of the simple main effects indicated that the immune response measured 5 days following SRBC inoculation, in both the BALB/cByJ and DBA/2J mice was significantly larger than at any other time period.

Interestingly, the peak immune response of the BALB/cByJ strain (5 days after inoculation) was significantly larger than that of the DBA/2J strain. However, the immune response of the BALB/cByJ mice measured 4 days was significantly smaller than the DBA/2J mice at this time period. Thus, although these two strains of mice exhibited a peak in their immune response to SRBC 5 days after inoculation, the BALB/cByJ immune response was larger in magnitude compared to the DBA/2J immune response. In contrast, the DBA response showed a somewhat earlier immune activation to SRBC (see Figure 1, 4 days), although the ultimate magnitude of responding did not reach that seen in the BALB/cByJ strain of mouse.

 Insert Figure 1 about here

Analysis of antibody titers revealed a significant Days x Strains interaction $F(1,108) = 3.122$, $p = 0.01$ (see Table 1). In agreement with the PFC response, Newman Keuls multiple comparison ($\alpha = 0.05$) demonstrated that antibody titers in the DBA/2J and BALB/cByJ

strains were significantly higher 5 days after SRBC inoculation than at earlier periods. Additionally, in the DBA/2J strain, the antibody titers, 4 days following SRBC administration, were significantly higher when compared to the titers observed in the BALB/cByJ strain, mimicking the differences observed in the simple main effects of the PFC response between Strains x Days. Likewise, there was no difference between the antibody titers between the two strains, measured 5 days post-SRBC.

Insert Table 1 about here

Figure 1. Mean plaque forming cell (PFC) responses of BALB/cByJ and DBA/2J mice whose spleens were taken at various times (1,2,3,4, or 5 days) following intraperitoneal administration of SRBC (5×10^6 cells).

Peak Splenic PFC Response in BALB/cByJ and DBA/2J Mice

Table 1.

Mean antibody titer to sheep red blood cells ($\text{Log}_2 \pm \text{S.E.M.}$) determined 3, 4, 5, or 6 days following antigen administration, titers determined 1 or 2 days after antigen administration for both strains were zeros and are not shown.

Days:	3	4	5	6
BALB/cByJ	0	0.455 ± 0.455	$3.455^* \pm 0.545$	2 ± 0.931
DBA/2J	0.273 ± 0.195	$2.636^{**} \pm 0.411$	$3.636^* \pm 0.527$	2 ± 0.258

* serum antibody titers were significantly higher ($p < 0.05$) 5 days following SRBC inoculation than at any other time in the development of the immune response measured days 1-6.

**serum antibody titers of the DBA/2J strain, determined 4 days after SRBC injection, were found to be significantly higher than those of the BALB/cByJ strain at the same time period.

EXPERIMENT 2

The Effects of Different Stressor Severities on the Peak Splenic PFC Response in BALB/cByJ and DBA/2J Mice

Contrary to the CD-1 mouse, the peak PFC response in both the BALB/cByJ and DBA/2J mice occurred 5 days following SRBC inoculation. As such, it was considered that a critical period for a stressor effect might differ from that previously reported in the CD-1 strain. Thus, the second experiment assessed the effects of stressors, applied at various intervals after immunization, on the peak splenic PFC response. Given that the immune response may be differentially affected as a function of stressor severity, in this experiment several stressor intensities were assessed (150 μ A, 300 μ A, and apparatus exposure) at various times following SRBC inoculation.

METHOD

Subjects. A total of 190 mice each of the BALB/cByJ and DBA/2J strains were employed. The subject characteristics and housing procedures were the same as those of Experiment 1.

Apparatus. Five identical black Plexiglas chambers, measuring 30x14x15 cm, were employed to deliver inescapable footshock. 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 μ A, 60 Hz, AC) was delivered through a 3000-V source, thereby providing a relatively constant

current.

Procedure. Mice of each strain will receive SRBC (5×10^6) injection as described in Experiment 1. At various times afterward (0, 1, 2, 3, 4, or 5 days) independent groups of each strain were exposed to one of three treatments. Mice were individually placed in the shock apparatus for a 1.1 hour period. During this time mice of one group received 360 shocks of $150 \mu\text{A}$ and 2 second duration at intervals of 9 seconds. The second group of mice received the same treatment but at a shock intensity of $300 \mu\text{A}$, while mice of the third group were placed in the shock boxes, but did not receive the footshock treatment. One additional group of mice were not exposed to the apparatus and served as an external control condition. Five days following inoculation, this being the time of the peak immune response in both strains, mice were decapitated and spleens and trunk blood collected for immune assay as described earlier.

RESULTS AND DISCUSSION

A 2 (strain) x 3 (stressor) x 6 (days) between group analysis of variance was performed to assess the effects of the stressor treatments applied at various times following SRBC inoculation. Dunnett's tests were used to compare specific treatments to the outside control groups (i.e., animals that did not receive any of the stressor treatments). In order to compare the relative effects of the stressor treatments in the two strains of mice, a separate analysis of variance was conducted comparing individual scores as a proportion of the mean nonstressed PFC scores for each of the strains.

The mean PFC scores for each of the groups in Experiment 2 are shown in Figures 2a and 2b. Analysis of variance of the PFC scores

among nonstressed BALB/cByJ and DBA/2J mice revealed that the two groups did not differ from one another, although as in Experiment 1, the PFC values were somewhat higher in the BALB/cByJ mice.

Analysis of the PFC scores as a function of Strain, Stressor, and Days revealed only a significant main effect of the Day of treatment, $F(5,521) = 2.48$, $p < 0.05$. Newman-Keuls multiple comparisons ($\alpha = 0.05$) revealed that this main effect was attributable to the PFC scores of mice stressed on Day 5 being significantly lower than those of mice exposed to a stressor on Day 2. Likewise, mice exposed to a stressor on Day 4 exhibited PFC scores somewhat lower than mice treated on Day 2. However, this difference did not reach statistical significance ($p > 0.05$). None of the other comparisons approached significance.

 Insert Figure 2a & 2b about here

Dunnett's tests revealed that in the BALB/cByJ mice, a stressor applied on Day 5 provoked a significant reduction of the PFC response relative to nonstressed control mice. In addition, shock of 150 μ A applied on Day 4 provoked a significant PFC reduction relative to nonstressed animals. None of the other groups were found to differ significantly from mice that had not been treated. In contrast to the effects seen in the BALB/cByJ mice, in the DBA/2J strain, a stressor applied on Day 0 reduced the PFC response relative to nonstressed mice. While there was a marginal reduction of the PFC response on Day 3 and 4 in some of the stressed animals, these differences were not significant.

Figure 2a. Mean ($\pm$ S.E.M.) splenic plaque forming cell (PFC) responses in BALB/cByJ mice that were exposed to a single session of either apparatus (top frame), 150 μ A footshock (middle frame), or 300 μ A footshock (bottom frame) immediately following, 1, 2, 3, 4, or 5 days after SRBC (5×10^6) administration. All animals had their spleens removed 5 days after antigen inoculation.

* 150 μ A footshock applied 4 days after antigen administration in the BALB/cByJ strain had a significant ($p < 0.05$) immunosuppressive effect relative to the unstressed control animals. Taken together, all three stressor severities exhibited a significant ($p < 0.05$) immunosuppressive effect 5 days after antigen administration when compared to unstressed controls.

Critical Periods of Stressor Effects on the Peak Splenic PFC Response in BALB/cByJ Mice

Figure 2b. Mean ($\pm$ S.E.M.) splenic plaque forming cell (PFC) responses in DBA/2J mice that were exposed to a single session of either apparatus (top frame), 150 μ A footshock (middle frame), or 300 μ A footshock (bottom frame) immediately following, 1, 2, 3, 4, or 5 days after SRBC (5×10^6) administration. All animals had their spleens removed 120 hours after antigen inoculation.

Critical Periods of Stressor Effects on the Peak Splenic PFC Response in DBA/2J Mice

The analysis of variance of the proportion of stressed to nonstressed scores revealed a significant main effect of Strains $F(1,521)=4.65$, $p<0.05$, indicating that relative to nonstressed mice, the BALB/cByJ mice that were exposed to a stressor exhibited a greater reduction of the PFC response (proportion = 0.917) than similarly treated DBA/2J mice (proportion = 0.978). In addition, the analysis indicated that the proportion scores varied over the days following inoculation, $F(5,521)=2.48$, $p<0.05$.

Tables 2a and 2b show the antibody titers of the BALB/cByJ and DBA/2J exposed to the three stressor severities, respectively. Analysis of antibody titers revealed a significant Treatment x Strain interaction $F(2,379) = 4.901$, $p<0.01$ (see Tables 2a & 2b). Newman Keuls multiple comparisons ($\alpha = 0.05$) of the simple effects of this interaction confirmed that in the presence of a stressor, antibody titers of the BALB/cByJ mice were lower than those of comparably treated DBA/2J mice. However, this effect was only evident in those mice exposed to apparatus or 150 μ A footshock, whereas in mice that received the more severe stressor, 300 μ A footshock, titers were equivalent in the two strains.

 Insert Tables 2a & 2b about here

Although the Strain x Day interaction was not significant ($p=0.058$), multiple comparisons were conducted of the simple effects of this interaction, since an an apriori assumption had been made that

a significant immunosuppressive effect would be observed around the time of the peak immune response. Interestingly, comparisons of the simple effects of this interaction also revealed that a stressor (regardless of its severity) applied at any time during the development of the immune response in BALB/cByJ mice, had more of an immunosuppressive than any other stressor severity. It was observed, however, that the most severe stressor, 300 μ A, applied at the time of the peak immune response (5 days post-SRBC) caused the antibody titers to be reduced in the more hardy DBA/2J strain, compared to those of the BALB/cByJ mice. Although Newman Keuls multiple comparisons of the simple effects revealed no significant immunosuppressive effects of a stressor, regardless of the time of application following SRBC injection, a significant main effect of the Day of stressor exposure was observed, $F(5,379) = 3.33$, $p < 0.05$. This effect however, was due to the serum antibody titers of mice exposed to a stressor on Day 5 being higher than the antibody titers of mice exposed to a stressor on Day 0. This result was opposite to the main effect of the Day of stressor exposure observed in the PFC response, such that the PFC responses of mice exposed to a stressor on Day 5 was lower than those of mice exposed to a stressor on Day 2. This disparate effect of stressors on the PFC and titer responses may be provisionally attributed to the cycling of lymphocytes during the development of the immune response, such that a stressor applied at a specific times during this cycling may have differential effects on the lymphocytes in the blood than those residing in the spleen (Cohen & Crnic, 1984; Lysle, et al., 1987; Teshima, Sogawa, Kihara, Nagata, Ago, & Nakagawa, 1987).

Table 2a.

Mean antibody titer to sheep red blood cells ($\text{Log}_2 \pm \text{S.E.M.}$) in BALB/cByJ mice exposed to a single session of either apparatus, 150 μA , or 300 μA footshock at various intervals (0, 1, 2, 3, 4, or 5 days) following SRBC (5×10^6) inoculation. Trunk blood was collected 120 hours after antigen administration, the time of the peak immune response.

<u>APPARATUS</u>					
<u>Day 0</u>	<u>Day 1</u>	<u>Day 2</u>	<u>Day 3</u>	<u>Day 4</u>	<u>Day 5</u>
3.07 $\pm$ 0.31	3.54 $\pm$ 0.24	3.79 $\pm$ 0.33	3.31 $\pm$ 0.33	3.07 $\pm$ 0.34	3.64 $\pm$ 0.25

<u>150 μA FOOTSHOCK</u>					
<u>Day 0</u>	<u>Day 1</u>	<u>Day 2</u>	<u>Day 3</u>	<u>Day 4</u>	<u>Day 5</u>
3.06 $\pm$ 0.30	3.41 $\pm$ 0.29	3.35 $\pm$ 0.32	3.73 $\pm$ 0.23	3.47 $\pm$ 0.23	3.93 $\pm$ 0.34

<u>300 μA FOOTSHOCK</u>					
<u>Day 0</u>	<u>Day 1</u>	<u>Day 2</u>	<u>Day 3</u>	<u>Day 4</u>	<u>Day 5</u>
3.67 $\pm$ 0.20	3.29 $\pm$ 0.42	3.75 $\pm$ 0.37	3.53 $\pm$ 0.30	3.35 $\pm$ 0.17	3.56 $\pm$ 0.35

Table 2b.

Mean antibody titer to sheep red blood cells ($\text{Log}_2 \pm \text{S.E.M.}$) in DBA/2J mice exposed to a single session of either apparatus, 150 μA , or 300 μA footshock at various intervals (0, 1, 2, 3, 4, or 5 days) following SRBC (5×10^6) inoculation. Trunk blood was collected 120 hours after antigen administration, the time of the peak immune response.

<u>APPARATUS</u>					
<u>Day 0</u>	<u>Day 1</u>	<u>Day 2</u>	<u>Day 3</u>	<u>Day 4</u>	<u>Day 5</u>
3.75 $\pm$ 0.41	3.86 $\pm$ 0.26	3.67 $\pm$ 0.42	4.17 $\pm$ 0.40	4.17 $\pm$ 0.48	5.00 $\pm$ 0.31

<u>150 μA FOOTSHOCK</u>					
<u>Day 0</u>	<u>Day 1</u>	<u>Day 2</u>	<u>Day 3</u>	<u>Day 4</u>	<u>Day 5</u>
5.00 $\pm$ 0.59	4.63 $\pm$ 0.63	3.71 $\pm$ 0.57	4.13 $\pm$ 0.48	3.86 $\pm$ 0.52	5.00 $\pm$ 0.61

<u>300 μA FOOTSHOCK</u>					
<u>Day 0</u>	<u>Day 1</u>	<u>Day 2</u>	<u>Day 3</u>	<u>Day 4</u>	<u>Day 5</u>
3.00 $\pm$ 0.44	3.38 $\pm$ 0.42	3.13 $\pm$ 0.52	2.71 $\pm$ 0.57	3.75 $\pm$ 0.65	4.88 $\pm$ 0.30

Dunnett's tests revealed that in the DBA/2J mice, a stressor applied on Day 5 provoked a significant increase in antibody titers relative to nonstressed control mice. In contrast, antibody titers in BALB/cByJ mice were unaffected by the stressor treatments. In effect then, the consequences of a stressor treatment on antibody titers did not parallel the effects observed with respect to the splenic PFC response. Whether these differential results reflect involvement of different mechanisms (e.g., corticoids vs catecholamines in blood and spleen, respectively), or whether they represent differences in lymphocyte trafficking patterns (i.e., changes in lymphocyte populations in different organs at various times after cell activation or stressor application) remains to be determined.

EXPERIMENT 3

Lymphocyte Catecholamine Receptors Mediate Stressor Induced Immunosuppression

It is not necessarily the case that the effects of stressors on immune activity in different strains of mice, or at various times following immunization, involve comparable mechanisms. For example, stressor exposure has been shown to influence activity within the IL-1 and CRF interface (Saperstein, Brand, Auhya, Nabriski, Hutchinson, Rosenzweig & Hollander, 1992). Additionally, following antigenic challenge, central IL-1 excites CRF release from the paraventricular nucleus, which in turn provokes both the secretion of ACTH from the pituitary, as well as sympathetic innervation of splenic lymphocytes (Dunn, 1990; Felten, Cohen, Ader, Felten, Carlson &

Roszman,1991; Felten, Felten, Carlson, Olschowka & Livnat,1985; Saperstein et al.,1992). Accordingly, changes in immune functioning associated with stressors may stem from either CRF effects on pituitary, and hence adrenocortical functioning, or alterations at the level of the spleen stemming from sympathetic innervation. Thus, it is conceivable that interindividual variability in physiological responding to a stressor will alter the observed effects of a stressor on the immune response. Given the particular responsiveness to stressors exhibited by BALB/cByJ mice, as reflected by hypersecretion of corticosterone, as well as central catecholamine alterations (Anisman, Zalcman, Shanks & Zacharko,1991), the possibility exists that stressful events would be more effective in provoking an immunosuppressant effect in this strain than in DBA/2J mice. It will be recalled, however, that the expression of different types of receptors on activated lymphocytes may vary over the course of the immune response. Therefore, the effects of corticoids, as well as other lymphocyte modulating compounds, such as catecholamines, may also vary with the development of an immune response. Accordingly, Experiment 3 was conducted to assess the impact of epinephrine receptor blockade by propranolol on the stressor-provoked alterations of the PFC response in BALB/cByJ mice stressed either 4 or 5 days following SRBC inoculation.

METHODS

Subjects and Apparatus. A total of 160 mice of the BALB/cByJ strain served as subjects. The subject characteristics and the apparatus were the same as that of Experiments 1 and 2.

Procedure Mice of Experiment 3 received i.p. administration of

SRBC (5×10^6) cells, and either 4 or 5 days later (times at which stressor exposure provoked a suppression of the peak splenic PFC response) exposed to either 150 μ A footshock or no treatment. The footshock treatment was the same as that of Experiment 2. Twenty minutes prior to the treatment (shock or no treatment) mice received either propranolol hydrochloride (10 mg/kg in a volume of 10 ml/kg) or an equivalent volume of saline. Five days following SRBC inoculation, mice were decapitated for spleen removal and trunk blood collection. As in the preceding experiments the splenic PFC response and serum antibody titers were determined.

RESULTS AND DISCUSSION

The mean PFC response of the BALB/cByJ strain across days, stressor, and drug treatments is shown in Figure 3. The analysis of variance confirmed that the PFC response varied as a function of the Drug x Stressor treatment interaction $F(1,112) = 8.69$, $p < 0.01$. Newman Keuls multiple comparison ($\alpha = 0.05$) of the simple effects of this interaction confirmed the presence of a shock-induced immunosuppression in saline treated animals. Moreover, these comparisons demonstrated that propranolol alone (in the absence of any stressor treatment) provoked a suppression of the immune response, compared to saline treated controls. Analysis of variance of the main effects of stressor treatment $F(1,112) = 5.151$, $p < 0.05$, and day of stressor application $F(1,112) = 4.024$, $p < 0.05$, demonstrated that stressors applied either 4 or 5 days after SRBC administration resulted in a suppression of the immune response, compared to non-stressed controls.

Although the Shock Treatment x Days x Drug Treatment

interaction was not significant, Newman Keuls multiple comparison of the simple effects for this interaction were conducted since an apriori prediction had been made concerning specific comparisons between stressor treatment and the timing of the stressor treatment. These comparisons confirmed the immunosuppressive effects of a stressor applied on Days 4 and 5 following inoculation, as observed in Experiment 2. Additionally, these comparisons revealed that four days after SRBC inoculation, propranolol, when combined with a stressor (footshock, 150 μ A) effectively blocked the immunosuppressive effects normally induced by footshock, such that no difference in the PFC response was observed when this treatment group was compared to non-shocked saline or propranolol controls.

 Insert Figure 3 about here

In contrast to the effects of propranolol on Day 4, Newman Keuls comparisons revealed that five days after SRBC inoculation, propranolol alone had immunosuppressive effects. Additionally, the effects of a stressor on the immune response was not alleviated with the administration of propranolol, as observed on Day 4. Thus, propranolol was shown to protect against the immunosuppressive effects of a stressor treatment applied four days after inoculation, but not five days after SRBC administration.

The initial apriori assumption that propranolol would alleviate the immunosuppressive effects of footshock applied four days after SRBC inoculation was observed. Moreover, it appears that any effects that propranolol has on the PFC response four days after SRBC

inoculation is dependent upon different mechanisms than those effects observed five days after inoculation. Indeed, it is well known that T-lymphocyte receptors for β -NE (among others) are differentially activated over the time course of the immune response (Sanders & Munson, 1985). Thus, the disparate effects observed with propranolol treatment between Days 4 and 5, could be due to the differential activation of various receptors of the immune system.

Figure 3. Mean PFC responses in BALB/cByJ mice that were exposed to either saline or propranolol treatment in conjunction with either no shock or footshock (150 μ A), 4 or 5 days after SRBC (5×10^6) injection. All mice had spleens removed 5 days post-SRBC administration.

**Effects of the β -NE Antagonist Propranolol
on the Critical Periods of the Splenic PFC
Response of BALB/cByJ Mice**

GENERAL DISCUSSION

Commensurate with earlier reports (Zalcman et al., 1988), exposure to footshock in the present investigation was found to elicit a reduction of the peak splenic PFC response to SRBC. This effect, however, appeared to be dependent upon the strain of mouse used, the stressor severity to which mice had been exposed, as well as the timing of stressor application relative to SRBC inoculation. The generation of the immune response is a complex process comprising a number of cell interactions which may involve several possible sites of genetic regulation (each potentially unique to a given species) (Anisman & Zacharko, 1992; Krehbiel, Bartel, Dirks & Wiens, 1986; Lysle et al., 1990; Shanks et al., 1990; Trullas, Jackson & Skolnick, 1989; Zalcman et al., 1991). As such, it is not surprising that the results of the present investigation using BALB/cByJ and DBA/2J mice were, in some respects, not entirely congruent with earlier reports involving the CD-1 strain (Zalcman et al., 1988). The first experiment of this series, in fact, confirmed that genetic variability exists in the development of the immune response mounted to SRBC. In particular, both the BALB/cByJ and the DBA/2J inbred strains exhibited a peak splenic immune response 5 days after SRBC administration, whereas in the outbred CD-1 mouse (Zalcman et al., 1988) the peak immune response occurred 4 days after inoculation.

Despite the finding that both the BALB/cByJ and DBA/2J strains exhibited a peak immune response 5 days following antigen administration, the developmental profile of the immune response over days following inoculation could be differentiated from one another. That is, although the emergence of the T cell dependent

immune response was apparent earlier in DBA/2J mice, the peak response ultimately attained was greater in the BALB/cByJ strain. The factors governing these differences remain to be identified, but it should be underscored that these strains are also known to differ in numerous respects, including basal and stressor provoked activity of endocrine and neurochemical systems (e.g. differential central catecholamine and corticosterone alterations) (Anisman & Zacharko, 1992; Rabin, Lyte & Hamill, 1987). Thus, it should be considered that the developmental course and the magnitude of the peak immune response to SRBC could involve diverse mechanisms.

There are several reports available suggesting that the magnitude of immunological changes observed with stressor application may be attributable to, among other things, genetic differences (Lysle et al., 1990; Shanks & Anisman, 1988; Trullas et al., 1989), stressor severity (Jessop, Gale & Bayer, 1988; Laudenslager et al., 1988; Mormede, Dantzer, Michaud, Kelley & Le Moal, 1988; Sandi, Borrell & Guaza, 1992), the type of stressor regimen employed (acute vs chronic) (Cunnick, Cohen, Rabin, Carpenter et al., 1991; Rinner et al., 1992; Terman et al., 1986), and as indicated earlier, the timing of the stressor application relative to the development of the immune response (Okimura & Nigo, 1986; Zalcman et al., 1988). The present investigation revealed distinct variations in immune responding to the effects of different stressor severities (applied at various times after antigen exposure) in BALB/cByJ and DBA/2J mice. In the BALB/cByJ strain, a single session of footshock (150 μ A) applied 4 days post-antigen administration elicited a significant immunosuppression relative to nonstressed animals. Interestingly, neither 300 μ A nor

apparatus exposure, caused a similar immunosuppressive effect in this strain. However, when a stressor was applied 5 days following SRBC inoculation, a significant suppression of the immune response was consistently observed irrespective of the stressor severity employed. At earlier times, most notably 1-3 days after inoculation, there was no evidence of the stressor treatments influencing the magnitude of the peak immune response in the BALB/cByJ strain.

In contrast to the immunosuppressive effects of a stressor in the BALB/cByJ mice, the immune alterations apparent in the DBA/2J strain were modest. Indeed, the limited stressor-induced immune alterations that were observed were inconsistent in terms of the timing of their appearance relative to antigen exposure. In general, the DBA/2J mice have been shown to be more resilient to stressor effects than the BALB/cByJ mice, exhibiting less profound behavioral impairments, and smaller alterations of neuroendocrine functioning after acute exposure to various stressors (e.g. footshock, restraint, forced swim, tail suspension) (Anisman & Zacharko, 1992; Shanks & Anisman, 1988,1989; Trullas et al., 1989). Thus, the lack of a stressor effect on the immune response of this particular strain may provisionally be attributed to limited stressor responsivity of DBA/2J mice.

Numerous mechanisms could potentially subserve the severity-specific effects of stressors (each possibly dependent on a particular species of animal) (see reviews in Anisman et al., 1993; Rabin et al., 1989). For example, a comparison of stressor severities on cell proliferation in response to Con A in mice revealed that the effects observed were incongruent with those previously seen in rats (Lysle et al., 1990). In particular, whereas stressors typically suppressed the

splenic mitogen response in rats (Cunnick et al., 1988; Lysle et al., 1987), footshock induced an immunoenhancement in mice, even though stressors reliably increase adrenal corticoid secretion in both species. Moreover, the extent of the immunoenhancement in mice was directly related to the severity of the stressor. Thus, although the immunosuppression associated with severe acute stressors has been, in many instances, attributed to HPA activation, resulting in an increase in circulating corticosterone concentrations (a factor shown to have immunosuppressive effects), this mechanism does not appear to be the sole cause of immunomodulation (as demonstrated by the dissimilar effects of severe stressor application in rats and in mice). In contrast to the stressor-induced immunoenhancement in mice observed by Lysle et al. (1990), the present investigation demonstrated no evidence of an enhancement of the PFC response in stressed mice. These inconsistent results may be attributed to the various neuroendocrine, neurochemical, and behavioral differences in response to stressors that exist between various strains of mice; however, there is no reason to expect that the PFC and mitogen responses would parallel one another given that the immune response that is measured via the splenic PFC assay is generated *in vivo*, whereas the immune response to a mitogen is generated *in vitro*.

Contrary to the immunosuppressive (or immunoenhancing) effects of severe stressors, several investigators have proposed that exposure to mild stressors may elicit an immunoenhancement (Fujiwara & Orita, 1987; Lysle et al., 1990). Such effects were not only observed with respect to NK cell activity, but also in the analysis of the mitogen or splenic PFC responses (Berkenbosch et al., 1991; Rinner et

al., 1992). Indeed, Fujiwara and Orita (1987) suggested that mild stressors provoke epinephrine-stimulated T-cell activation, thus leading to an immunoenhancement, whereas severe stressors lead to immunosuppression owing to involvement of alternative systems, such as pituitary-adrenal activation. It is interesting, however, that in the present investigation, a mild stressor in the form of apparatus exposure did not lead to a noticeable increase of the PFC response, in fact a slight reduction in the immune response was observed. Several investigators (Croiset et al., 1987; Cunnick et al., 1988), however, have demonstrated differential immunoreactivity to mild environmental challenges, which were dependent upon several organismic and experiential factors. Thus, despite the fact that an immunoenhancement was not apparent in the present investigation, it would be premature to assume that such an outcome would not be evident under appropriate conditions (e.g., upon stressor reexposure, employing different stressor parameters, as a function of the backdrop upon which the stressor was superimposed, as well as the particular aspect of the immune response being monitored).

As indicated earlier, the disparate effects of stressor application in the BALB/cByJ and the DBA/2J strains were not unexpected given the differences in the behavioral and neurochemical alterations elicited by stressors in these strains. These factors have been shown to differentially affect various compartments of the immune response (Lysle et al., 1987; Rinner et al., 1992; Cunnick et al., 1988; Teshima Sogawa, Kihara, & Nakagawa, 1991) which, in turn, may involve modulation by different mechanisms, e.g., opiate peptides, endorphins, norepinephrine, epinephrine, ACTH, CRF, or glucocorticoids (Cunnick et

al., 1988; Sandi et al., 1992; Weiss et al., 1989; Dantzer & Kelley, 1989). In effect, multiple physiological pathways that are capable of mediating the stressor-induced alteration of the immune response may exist. Indeed, it is conceivable that in the BALB/cByJ and DBA/2J strains the SRBC inoculation may have differentially influenced endocrine and neurotransmitter activities, since the antigen itself, it will be recalled, may be perceived (or at least interpreted) as a stressor (Anisman et al., 1993; Dunn, 1990). Whether an immunosuppression is observed within a species of animal may depend on the reactivity of specific neurochemical and endocrine systems, which may be related to, among other things, the perceived severity of the stressor employed.

As previously mentioned, footshock of 150 μ A affected the PFC response of the BALB/cByJ strain irrespective of whether the treatments were applied immediately or 24 hours prior to the spleens being taken. Surprisingly, in this strain the most intense treatment, footshock of 300 μ A, as well as the mildest stressor, apparatus exposure, only influenced the PFC response when applied on the day of the peak immune response. Inasmuch as such an outcome was apparent on successive replications of the study, it is unlikely that this finding was a spurious one. A simple explanation for these findings is not readily available, at least not one that attempts to account for the data using a single mechanism. There is, however, no a priori reason to believe that only a single mechanism is operative in determining the impact of stressors on the PFC response. To be sure, the processes involved in generating the immune response may vary over time following activation of the immune system, and therefore these

developmental processes may be differentially influenced by stressors applied at various times over the course of the immune response being mounted.

It is now well documented that a variety of receptors (e.g., catecholamine, serotonin, opioid peptides, ACTH, corticoid) present on lymphocytes are involved in intercellular communication, and can contribute to the activity of the immune system. In their reviews of the literature Sanders and Munson (1985), and Roszman and Carlson (1991) both indicated that norepinephrine receptors present on the lymphocyte cell surface are particularly amenable to neurotransmitter manipulations, but that the effectiveness of catecholamine stimulant or blocking agents varies the course of the immune response being mounted. At this juncture, however, it is important to note that these investigators did not address the propensity of these agents to elicit immunosuppressive or immunoenhancing effects in conjunction with stressor application. Given the varied neuroendocrine and neurochemical effects that can occur after exposure to an environmental insult, it would not be surprising to observe dissimilar effects of catecholamine agonists or antagonists on the functioning of the immune system as compared with that of nonstressed animals. At any rate, these investigators have suggested that in the absence of stressor-induced modulation of immune functioning, the effectiveness of catecholamine manipulations were dependent on the specific immune response examined (e.g. T or B cell dependent immune response; mitogen or PFC assays), as well as the developmental phase at which the immune response was manipulated. For example, Sanders and Munson (1984) demonstrated that *in vitro* treatment

with the β -NE antagonist, propranolol, inhibited the enhanced PFC response to SRBC in splenic cell cultures ordinarily elicited by norepinephrine. However, this effect was only evident up to 5 hours following exposure of these cells to SRBC, after which, propranolol had no suppressive effect on the maximal antibody response (i.e., determined 5 days after SRBC introduction to the culture medium). Interestingly, the addition of catecholamine agonists (as opposed to antagonists effects observed by Sanders and Munson (1984)), such as isoproterenol and norepinephrine, directly inhibited the *in vitro* generated lymphocyte response to a T-cell dependent mitogen (Goodwin, Messner & Williams, 1979). It appears that the addition of an endogenous or exogenous adrenergic agonist directly before or in conjunction with an antigen will suppress the lymphocyte response to a mitogen (Roszman & Carlson, 1991). However, the introduction of the agonists at other times after the initiation of the immune response, has been shown to produce either a suppressive (Besedovsky et al., 1979; Carlson, Brooks & Roszman, 1989) or enhancing (Burchiel & Melmon, 1979; Khan, Silverman, Engelman & Melmon, 1985) effect.

The mechanisms governing the various effects of agonists on the development and functioning of the immune response remain to be fully examined. However, studies attempting to quantify the effects of adrenergic agonists *in vitro* suggest that, in general, catecholamine receptor agonists enhance the generation of B cells and cytotoxic T cells, but tend to inhibit the functional effectiveness of these effector cells. Conversely, depleting the concentration of peripheral norepinephrine, through chemical (6-hydroxydopamine) sympathectomy, either produced no change in the primary murine

antibody response to a T-dependent antigen (Miles, Quintans, Chelmicka-Schon, & Arnason, 1981), or suppressed the immune response in mice (Hall, McClure, Hu, Tare, Selas, & Goldstein, 1982; Livnat, Felten, Carlson, Bellinger, & Felten, 1985). It is not known at this time whether the reduction of immunoresponsiveness to the antigen as a result of 6-OHDA lesioning of the sympathetic nervous system was a function of the reduction of the total number of lymphocytes generated in response to antigenic stimulation or the reduction of the functional effectiveness of the lymphocytes toward the antigen.

Although *in vitro* systems are easily manipulated and controlled, they unfortunately do not provide an index of the interactive effects that naturally occur in an intact system. Interactions between immune factors and cytokines, neurohormones, hormones, or neurotransmitters (among other things) are possible, and may contribute to the contradictory effects of catecholamines on *in vitro* and *in vivo* generated immune responses (Roszman & Carlson, 1991; Sanders & Munson, 1985). However, these investigators and others (Fuchs, Campbell & Munson, 1988) cautioned that the effects of agonists (or antagonists) on adrenergic receptors are not only dependent on the type of immune response being examined, but also on the concentration of the agent used during the course of development of the immune response. For instance, the addition of a β -NE receptor agonist, isoproterenol (in high concentrations), to splenic cell cultures lead to an enhancement of the *in vitro*-generated murine antibody response to SRBC, whereas exposure to approximately the same concentration of drug suppressed the *in vivo*-generated antibody

response (see reviews in Sanders & Munson, 1985). Interestingly, isoproterenol applied in lower concentrations enhanced the *in vivo*-generated antibody response (Braun & Rega, 1972); however, this effect was suggested to be dependent on the timing of agonist exposure with respect to antigen administration (Besedovsky, Da Prada, Del Rey, & Sorkin, 1981). The seemingly inconsistent observations of adrenergic receptor manipulations (with either endogenous or exogenous agents) has been suggested to be due, in part, to the large variations in β -adrenergic receptor densities that occur over the course of the proliferation of different cell types (macrophage, T and B cells) (Fuchs, Albright & Albright, 1988). Roszman and Carlson (1991) indicated that the most consistent effects of propranolol on immune functioning were observed when a T cell dependent antibody response was assessed (e.g. SRBC or Con A). This may simply have been due to T cells having fewer β -adrenergic receptors than do B cells (Fuchs et al., 1988), and therefore were less likely to have exhibited fluctuations in β -NE receptor density and consequently did not exhibit large variability in the response to β -adrenergic manipulations (Fuchs et al., 1988). Alternatively, relative to B cells, T cells residing in the spleen have the capacity for a more direct interaction with the sympathetic nervous system (Felten, 1991; Felten & Felten, 1991), and hence may be more susceptible to fluctuations associated with NE alterations. These possibilities still remain to be more thoroughly demonstrated in an *in vivo* generated antibody response. Nevertheless, they should be considered when examining the impact of stressors on *in vivo* assessments of immune functioning.

Although contradictory results exist concerning the effects of β -NE manipulations on immune activity, there are some unexpected similarities, as well. For instance, Carr, Woolley, and Blalock (1992) demonstrated that cold stress induced an enhancement of SRBC-specific murine IgM production by splenocytes. Interestingly, four consecutive days of treatment with the β -NE antagonist, propranolol, augmented the immunoenhancing effects of the cold exposure (parenthetically, propranolol alone was shown to significantly increase the IgM response to SRBC). In another study, a single administration of propranolol to rats, 30 minutes before a foot shock stressor, protected against the stressor-induced suppression of cell proliferation in response to a T-cell mitogen (ConA) (Cunnick et al., 1990). Taken together, these studies suggest that β -NE receptor antagonism may act against the immunosuppressive consequences of stressors or augment the immunoenhancing actions of some forms of environmental insult. The specific mechanisms that may be involved in such outcomes, and whether these reflect changes of central or peripheral (sympathetic) NE activity, or variations of receptor activity on lymphocytes themselves remain to be determined.

In support of a role for NE in mediating the footshock (150 μ A) induced suppression of the immune response (4 days following SRBC injection) in the BALB/cByJ mouse, it was demonstrated in the present investigation that the suppression of the PFC response was attenuated by propranolol. Interestingly, propranolol administered 5 days after SRBC inoculation (the time of the peak immune response) did not prevent the immunosuppression observed after 150 μ A footshock. In fact, in the absence of a stressor, propranolol administered at this time

caused suppression of the splenic PFC response. Thus, these data suggest that while β -NE stimulation might play a protective role in the immunosuppression provoked by a stressor applied 4 days after inoculation, it was not pertinent in alleviating the suppression elicited by a stressor applied 5 days after inoculation.

The finding that propranolol reduced the stressor effects on the splenic PFC response is commensurate with reports demonstrating that the administration of the drug 20-30 minutes prior to acute stressor exposure, attenuated the suppression of cell proliferation elicited by a T cell mitogen (Cunnick et al.,1990; Lysle, Cunnick & Maslonek,1991; Teshima et al.,1991). These data, taken together with the views expressed by Sanders and Munson (1985) lead to the suggestion that stressors may cause an immunosuppression by virtue of their effects on β -NE receptors present on lymphocytes. Inasmuch as the receptor array may vary over the course of an immune response being mounted, it would be expected that the impact of stressors on immune functioning would likewise vary as a function of the time of application relative to development of an immune response. In effect, at specific times during the development of the immune response (eg. 24 hours prior to the peak immune response) stressor-induced alterations of catecholamines may influence receptors present on lymphocytes that normally would not be present at other times in the development of the immune response (eg. on the day of the peak immune response).

In assessing the impact of stressors on immune functioning, it is essential to consider the specific immune compartment being examined. Cunnick et al. (1988) indicated that it is important to distinguish between the mitogen response in spleen from that seen in

serum. While propranolol antagonized the splenic mitogen response, this β -NE antagonist did not affect the mitogen response in serum. Interestingly, administration of the β -NE agonist, epinephrine, has been shown to result in a decrease in the mitogen responsiveness of both splenic (Carlson et al., 1989; Johnson, Ashmore & Gordon, 1981) and peripheral blood (Crary, Borysenko, Sutherland, Kutz, Borysenko & Benson, 1983) lymphocytes. Furthermore, adrenocortical manipulations influenced the stress-induced immunomodulation of lymphocytes in blood but not spleen. Thus, although the data in the present investigation suggest a time-dependent role for NE modulation of stressor effects on the formation of antibody forming cells, such an outcome could be unique to immune cells in spleen and not in other tissues.

Apart from β -adrenergic receptors, α -adrenergic receptors have also been isolated on some activated T cell subsets, as well as on macrophages and B cells (Besedovsky et al., 1979; Sanders & Munson, 1984). However, the role of α -NE receptor activation in antibody formation is less clear and remains to be thoroughly investigated (see reviews in Roszman & Carlson, 1991; Sanders & Munson, 1985). To be sure, stimulation of alpha 2-adrenergic receptors in the paraventricular nucleus, just like IL-1 stimulation of CRF secretion, has been shown to cause a dose-dependent increase in corticosterone secretion (Daniels, Jaffer, Russell & Taljaard, 1993). In view of the immunosuppressive action of adrenal steroids, coupled with the impact of stressors on central α -NE receptors (Stone, 1987; Stone & Platt, 1982), much greater attention should be devoted to the potential role for these receptors in determining immunological

changes associated with aversive events.

As already alluded to, there are a number of reports demonstrating that pituitary-derived hormones play an important part in the development and initiation of the immune response to an antigen. Several studies have revealed that hypophysectomy may influence humoral and cell-mediated immunity (Cross, Markesbury, Brooks & Roszman, 1984). Stressful events have been shown to cause an increase the circulating levels of ACTH which are able to bind to ligand-specific receptors on lymphocytes, thereby causing an intracellular increase of second messenger levels (eg. cAMP, calcium). The specific second messenger produced is dependent upon the timing of the increased ACTH levels with respect to the development of the immune response (Roszman & Brooks, 1992). If ACTH concentrations increase during the initial stage of the immune response being mounted, then a decrease in the proliferative capacity of the lymphocytes is usually observed (Krammer, 1988). This immunosuppressive effect is dependent upon the interaction of at least two receptors on an activated lymphocyte; the receptor for the neurohormone, ACTH, and the receptor for either the antigen itself or a specific lymphokine (in particular IL-1 or IL-2) produced by the binding of an antigen to a lymphocyte (Roszman & Brooks, 1992). The interactive effects of these two receptors can potentially affect the biochemical and molecular processes of the cell via second messenger systems, thus altering the functioning of the lymphocyte (Carlson et al., 1989; Roszman & Brooks, 1992).

In addition to the direct effects of ACTH on lymphocytes, this hormone may affect immune functioning by provoking the secretion of

circulating levels of glucocorticoids. As discussed earlier, lymphocytes possess receptors for many different neurohormones, including adrenal steroids (such as corticosterone in rats and mice). Corticoids could potentially influence immune functioning by directly affecting receptors present on lymphocytes, or could do so through an indirect action. For example, glucocorticoids have been shown to regulate the activation and density of β -adrenergic receptors (Carr et al., 1992; Davies & Lefkowitz, 1974). Accordingly, stressor-induced elevation of corticosteroids may alter the expression not only of glucocorticoid receptors on lymphocytes, but also β -adrenergic receptors, thereby altering time-dependent effects of regulatory factors on lymphocytes.

Glucocorticoid receptor activation has been implicated as one factor that might contribute to stressor induced suppression of the immune response (Jacobson & Sapolsky, 1991; Munck & Guyre, 1991). While there are numerous studies inconsistent with such an hypothesis, it may be the case that these studies have not adequately considered that the impact of stressors may involve multiple factors, the contribution of which could vary over the course of the immune response being mounted. For example, while propranolol treatment was effective in antagonizing the effect of a stressor applied 4 days after inoculation, it was ineffective in antagonizing the immunosuppression induced by a stressor applied 5 days after SRBC inoculation. Thus, these data implicate some mechanism other than catecholamines in accounting for the immunosuppression observed 5 days following antigen administration. A plausible candidate in this respect is corticosterone, since stressors reliably increase adrenal corticoid concentrations, which are known to have immunosuppressive

properties. Inasmuch as the stressor-induced increase of corticosterone is particularly marked in the BALB/cByJ mouse, the immunosuppression associated with footshock applied just prior to the peak immune response in the present investigation may have been subserved by glucocorticoid release. We are presently assessing the possibility that the glucocorticoid antagonist, RU-38486, will effectively eliminate the immunosuppression associated with a stressor applied 5 days after inoculation, but not at earlier time periods. Of course, the possibility is not being dismissed that the interactions of other mechanisms with glucocorticoids, including alterations of sympathetic activity, may better account for the immunosuppressive effects of stressors in the BALB/cByJ strain of mouse.

The findings that HPA axis may influence immune functioning gave rise to the supposition that corticotropin releasing factor (CRF), secreted from the paraventricular nucleus of the hypothalamus to provoke ACTH secretion, may play an integral role in subserving the stressor-induced immunosuppression (Jain et al., 1989, 1991; Kavelaars et al., 1989). Moreover, the findings that (a) IL-1 provoked the release of CRF from the paraventricular nucleus, could induce an immunosuppression much like a stressor (Breder, Cinarello, & Saper, 1988; Sapolsky, Rivier, Yamamoto, Plotsky, & Vale, 1987), and (b) antibodies for IL-1 antagonized the effects of stressors (Berkenbosch et al., 1991), raised the possibility that both IL-1 and CRF were part of a complex loop regulating stressor effects on immune functioning (Rothwell, 1989; Sapolsky et al., 1987). However, given that the immunosuppressive effects of stressors were present in

hypophysectomized (Jain, et al.,1989), and in adrenalectomized animals (Andreis, Neri, Belloni, Mazzocchi, Kasprzak, & Nussdorfer, 1991) indicated that the contribution of IL-1 and CRF were not part of a regulatory loop directly involving the HPA axis . It is known that CRF may influence the activity of sympathetic fibers that terminate in or about the regions of lymphoid tissues, such as the spleen. Thus it is possible that the effects of stressors on immune functioning may involve sympathetic (catecholamine) innervation secondary to variations of central CRF and IL-1 activity. Of course, IL-1 and CRF are not restricted to the paraventricular nucleus of the hypothalamus, and their contribution may involve pathways other than sympathetic systems. Indeed, administration of CRF to regions such as the locus coeruleus, as well as the amygdala, will engender "fear-like" behavioral responses (Koob, Heinrichs, Pich, Menzaghi, Balwin, Miczek & Britton, 1993). Thus, the release of CRF in the face of environmental challenge may come to provoke fear and/or anxiety, which ultimately give rise to the immunosuppression. As discussed earlier, the differential effects of a stressor observed between the BALB/cByJ and DBA/2J mouse strains, may be due to, among other things, differences between the strains in the perception of or reactivity to stressful events. This variation in the perception of the stressor could engender potentially divergent central neurochemical (eg. NE and 5-HT) and neurohormonal (eg. CRF and ACTH) reactions, ultimately causing variations in the profiles of immune responses to a stressor application.

In summary, numerous possible mechanisms such as opioids (Murgio, Faith, & Plotnikoff, 1986; Shavit, 1991; Sibinga & Goldstein,

1988), cytokines (delRey, Besedovsky, Sorkin, & Dinarello, 1987), catecholamines (Felten, 1991; Felten, Ackerman, Wiegand, & Felten, 1987; Walker & Codd, 1985), and hormones, including ACTH, CRF, and glucocorticoids (Berkenbosch et al., 1991; Besedovsky, delRey, Sorkin, & Dinarello, 1986; Coffey & Hadden, 1985; Cunnick et al., 1990; Munck, Guyer, & Holbrook, 1984; Su, London, & Jaffe, 1988; Weigent & Blalock, 1987), have been identified that can potentially influence the development of the immune response via lymphocyte receptor activation (Carr, 1992; Felten, Felten, Bellinger, Carlson, et al., 1987; Roszman & Brooks, 1992). These various mechanisms may contribute to the interspecies differences in behavioral and physiological impact of varying degrees of stressful events, including divergent responding to a given stressor (e.g. footshock stressor (Gamzu, Vincent, Tare, Benjamin, Farrar, & Sullivan, 1984; Lysle et al., 1990; Mormede et al., 1988; Shanks & Anisman, 1988; Wahlsten, 1972), or restraint (Cabib, Kempf, Schleef, Oliverio, & Puglisi-Allegras, 1988; Okimura & Nigo, 1986)), variations in neurochemical utilization and synthesis (Anisman et al., 1991; Anisman & Zacharko, 1992; Krehbiel et al., 1986), and differential lymphocyte generation and activation over the course of the immune response, or after exposure to a particular stressor paradigm (Esterling & Rabin, 1987; Lysle et al., 1987; Rinner et al., 1992; Teshima et al., 1991). It is also important to recognize the potential for bidirectional communication between the immune and central nervous systems. Given this interaction, many diverse immunological outcomes engendered by stressors are possible, and in fact may account for the inconsistent data of the effects of various stressor severities on the functioning of the immune system.

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APPENDIX A

Table 1a (Experiment 1)

**Analysis of Variance of the PFC Responses to SRBC of BALB/cByJ
and DBA/2J Mice as a Function of Day of Injection**

Anova table for a 2-factor Analysis of Variance on Y_1 : pfc

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
strain (A)	1	45.564	45.564	4.145E-5	.9949
day (B)	5	276372888.347	55274577.669	50.281	.0001
AB	5	26284400.329	5256880.066	4.782	.0006
Error	107	117627337.879	1099320.915		

Table 1b (Experiment 1)

**Analysis of Variance of the Serum Antibody Titer
Response to SRBC in BALB/cByJ and DBA/2J Mice
as a Function of Day of Injection**

Anova table for a 2-factor Analysis of Variance on Y_1 : titer

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
strain (A)	1	5.514	5.514	4.355	.0393
day (B)	5	216.492	43.298	34.201	.0001
AB	5	19.764	3.953	3.122	.0113
Error	108	136.727	1.266		

Table 2a (Experiment 2)

Analysis of Variance of the PFC Response to SRBC in BALB/cByJ and DBA/2J Mice as a Function of Stressor Severity and Timing of Stressor Application Relative to the Development of the Peak Immune Response

Anova table for a 3-factor Analysis of Variance on Y_1 : pfc

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
strain (A)	1	165750.771	165750.771	.453	.5012
trtmnt (B)	2	814861.4	407430.7	1.114	.3291
AB	2	136730.311	68365.156	.187	.8296
day (C)	5	4579047.905	915809.581	2.503	.0296
AC	5	2866904.648	573380.93	1.567	.1675
BC	10	3121619.81	312161.981	.853	.5773
ABC	10	4837803.294	483780.329	1.322	.2149
Error	521	100589022.015	365813.862		

Table 2b (Experiment 2)

**Analysis of Variance of the Proportion of No Treatment Means
of the PFC Response to SRBC in BALB/cByJ and DBA/2J Mice
as a Function of Stressor Severity and Timing of Stressor
Application Relative to the Peak Immune Response**

Anova table for a 3-factor Analysis of Variance on Y₁: Ppn of Mean

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
strain (A)	1	.5	.5	4.658	.0314
trtmnt (B)	2	.238	.119	1.111	.3299
AB	2	.039	.02	.182	.8337
day (C)	5	1.332	.266	2.484	.0308
AC	5	.829	.166	1.546	.1739
BC	10	.933	.093	.87	.5613
ABC	10	1.438	.144	1.341	.2054
Error	521	55.883	.107		

Table 2c (Experiment 2)

Analysis of Variance of Antibody Titers of BALB/cByJ and DBA/2J Mice At Various Intervals Following Stressor Exposure

Anova table for a 3-factor Analysis of Variance on Y₁: Titer

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Strain (A)	1	22.822	22.822	14.962	.0001
Stressor Trtmnt (B)	2	12.711	6.355	4.167	.0162
AB	2	14.952	7.476	4.901	.0079
Day (C)	5	25.37	5.074	3.327	.0059
AC	5	16.443	3.289	2.156	.0583
BC	10	7.78	.778	.51	.883
ABC	10	17.218	1.722	1.129	.3392
Error	379	578.085	1.525		

Table 3 (Experiment 3)

**Analysis of Variance of BALB/cByJ Mice Exposed to 150 μ A Footshock
either on Day 4 or Day 5 and Receiving Either a Single Injection of
Saline or Propranolol Prior to Stressor Treatment**

Anova table for a 3-factor Analysis of Variance on Y_1 : PFCs

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
trtmnt (NS or SH) (A)	1	1008331.579	1008331.579	5.151	.0251
DAY (4or5) (B)	1	787768.384	787768.384	4.024	.0473
AB	1	5.942	5.942	3.036E-5	.9956
DRUG (sal or prop) (C)	1	588995.095	588995.095	3.009	.0856
AC	1	1701917.228	1701917.228	8.694	.0039
BC	1	542097.583	542097.583	2.769	.0989
ABC	1	49256.595	49256.595	.252	.6169
Error	112	21925173.659	195760.479		

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