Immunological Consequences Of Chemically Induced Generalized Motor Epilepsy

In Partial Fulfillment Of The Requirement's Of A Master's Degree In Psychology

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By

Roland Chretien ©

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ABSTRACT

The purpose of this study was to determine if the stress created by a generalized motor seizure was significant enough to alter the immune response. Criterion measures of antigen binding capacity (ABC) to human serum albumin (HSA) were obtained from 39 female and 36 male Satinder's Heterogenous Strain (SHS) albino rats. Within 30 seconds after a second HSA injection (booster), rats were either injected with METRAZOL to produce generalized (motor) convulsions, or with CYCLOPHOSPHAMIDE, an immunosuppressive drug (comparator treatment). A third group served as a saline-injection control and a fourth as an undisturbed control group. Multivariate analysis of variance (Manova) demonstrated a highly significant immunosuppression 4 days later in rats that had displayed brief motor convulsions and, had been administered cyclophosphamide. Injection and undisturbed controls did not differ significantly from each other. By the 10^{th} day after the booster and treatments, ABC measures for the drug treated rats (METRAZOL,CYCLOPHOSPHAMIDE) were not significantly different from controls. However on the 10^{th} day females showed significantly higher ABC measures than did males. The degree of immunosuppression seen on day four was moderately correlated to the severity of the seizure observed. These results suggest that the seizuring of an animal is a stressful event capable of suppressing humoral immunity, and that the degree of suppression is positively correlated to the severity of the seizure.

Major neuro-chemical systems in the brain, specifically gabanergic and noradrenergic systems, exert powerful control over the hypothalamus (DeFeudis, 1984). Since the hypothalamus has been shown to profoundly affect the immune system (IS), it was suspected that impairment in gamma-aminobutyric-acid (GABA) functioning (Leviel & Naquet, 1977), and depletion of norepinephrine (NE) (Engel & Sharpless, 1977) associated with seizure activity could also influence the IS. Such an effect would likely be mediated by accessing the glucocorticoid-associated-circuit (Besedovsky, del Rey & Sorkin, 1985).

The possibility that the GABA system might participate in the modulation of immunity has important clinical implications. The nature of GABA's role within the central nervous system (CNS) suggests that its' integrity is required for the maintenance of a normal healthy body. Not surprisingly, the GABA system has been implicated in the etiology of epilepsy, depression, schizophrenia, anorexia nervosa and anxiety. Moreover, aberrations of IS functioning reported in some of these various psychiatric disorders (Solomon, 1981) lend further credibility to the idea that GABA may be acting, as a primary effector in the external modulation of immunity.

If the hypothalamus can modulate immunity, then alterations in its' activity caused by seizure activity might induce changes in immunoreactivity. To test this hypothesis the following experiment was designed. Humoral immunity was monitored after brief generalized motor seizures were induced by metrazol. This type of seizure in animal models is analogous to Grand mal activity in humans. To determine the absolute level of immunosuppression, cyclophosphamide (a well known immunosuppressive drug) was used as a comparator effect (Ader & Cohen, 1975).

It is important to note that this study provided data on two endpoint measurements. Namely; 1) the behavioral observation of 'seizure activity', and 2) the monitoring of 'humoral immunity'. As such, the main assumption made was that," the seizuring of an animal was a stressful event capable of influencing immunity ". A secondary assumption made was that, " the gabanergic and noradrenergic neural systems mediated this influence by accessing the glucocorticoid-associated immunoregulatory circuit ". The secondary assumption was only speculative,

and was supported by information obtained from the literature, and not from direct experimental evidence from this study.

The suspicions that these neural systems participated in this process were based on the following information about the underlying mechanisms of this Grand mal epilepsy model (i.e. 1,7) and their potential immunomodulating effects (i.e. 2-6):

1) An impairment in GABA functioning was present during seizure activity (Babington & Wedeking, 1973; Leviel & Naquet, 1977; Tanaka, Lange & Naquet, 1975).

2) The GABA system functioned as the major inhibitory neurotransmitter in the CNS, inhibiting noradrenergic, serotonergic, and dopaminergic neural systems (Guyton, 1982).

3) All of the above neural systems were implicated in the modulation of immunity with the exception of the GABA system (Hall & Goldstein, 1985).

4) The GABA system influenced the regulation of hormonal release at the level of the hypothalamic pituitary axis (HPA) (DeFeudis, 1984).

5) Hormones released from the HPA acted as intermediaries in setting up a line of communication between the CNS and the IS (Maclean & Reichlin, 1981).

6) The GABA system influenced the IS indirectly by accessing the glucocorticoid-associated immunoregulatory circuit. This was likely achieved through GABA's control over the stimulating action of both serotonin (5HT) and acetycholine (Ach) on corticotropin releasing factor (CRF) producing neurons.

7) The disinhibition of central NE associated with seizure activity could result in further elevations in CRF which could ultimately affect the IS (Callaghan & Schwark, 1976; Engel & Sharpless, 1977).

The recent viewpoint that the IS was subject to external influences has made it exceedingly difficult to study the IS in its' entirety. To address the topic in a logical sequence, knowledge of IS functioning will first be presented, followed by a discussion of the evidence in support of external modulation of immunity.

The Immune System

The major function of the IS is to defend the body against foreign substances such as bacteria, viruses, and neoplasms. The two major divisions of the IS comprise of the humoral and cell mediated systems. Both types of immunity rely on similar yet different classes of lymphocytes. The T-and B- cells are derived from bone marrow stem cells. The T-cells mature in the thymus gland while B-cells mature in the equivalent of the Bursa of Fabricus found in birds. T-cells are the primary lymphocyte involved in cell mediated immunity while the B-cells are those involved in humoral immunity. Other accessory cells involved in immune processes include: monocytes, neutrophils, macrophages and mast cells. Several subsets of T- cells including: helper T, suppressor T, and killer T cells have also been identified.

Three basic features of an immune response include: specificity, memory, and recognition of self versus non-self substances. Substances perceived as foreign bodies by the IS are called antigens and these are presented to lymphocytes by accessory cells. Each lymphocyte has been genetically programmed to respond specifically to a given antigen. The binding of the antigen to the lymphocyte initiates lymphocyte proliferation and a host of other effector responses. This is referred to as a primary response. Upon reexposure to the same antigen, a very rapid and much stronger response occurs. This magnified response is due to memory cells which were formed during the primary response. The second is appropriately called a secondary immune response.

The complement system is another major component of the IS, remaining inactive until stimulated. Once stimulated it mediates inflammatory and host defense reactions. Various functions of the complement system include: 1) chemotactic factor, acting as an alarm to direct migrating leukocytes into the infected or inflamed areas; 2) anaphylatoxin activity which increases local capillary permeability; 3) opsonin factor, which facilitates phagocytosis; 4) enhancing of virus neutralization and bacterial lysis; 5) immune surveillance against malignancy and the lysis of tumour cells (Kimball, 1986).

Behaviour To Immunity And Back Again

The observation that "mental well being" was somehow related to "physical well being" has been made throughout the centuries. However it has not been until recently that behaviour has been linked to immunity (Ader, 1981; Solomon & Amkraut, 1981). A host of factors including personality, sex, age, nutrition, sleep, drugs, neurotransmitters, neuropeptides and hormones have also been found to influence this process. The apparent interdependence of all these factors has made it exceedingly difficult to explain external regulatory mechanisms of immunity.

Much of the research has focused on excessive stress and susceptibility to disease. The relationship between stress, behaviour, adaptation and immunity has been reviewed in animals by Borysenko and Borysenko (1982) and in humans by Locke (1982). The common theme derived from these reviews suggest adaptation to stress is attained through adequate coping mechanisms and perceived control. The feeling of lost control is generally accompanied by various biological changes capable of influencing immunity.

Thus far, the likely routes by which psychoneuroimmunological influences are mediated have been adequately demonstrated. It is suggested that thoughts and emotions accompanying behaviour are linked to underlying neural processes in the brain. These neural processes affect the IS directly through the activation of the autonomic nervous system (ANS) and/or indirectly by releasing hormones via the HPA.

Direct activation of the IS via the ANS

The IS can be activated both directly and indirectly by the sympathetic branch of the ANS. The direct activation is made possible by the presence of sympathetic fibers extending to lymphoid tissue in the lymphatic organs, and by the corresponding receptors located on lymphoid cells. The lymphatic organs innervated by sympathetic fibers include the spleen, bone marrow, bursa of Fabricus, thymus gland, tonsils and lymph nodes (Bulloch, 1985). The receptors found on the IS cells can accept the catecholamines used as neurotransmitters by the sympathetic nervous system (Singh, Millson, Smith & Owen, 1979).

The indirect activation of the IS by the ANS occurs under conditions of sympathoadrenal stimulation. Hypothalamic efferent neurons stimulate the secretion of catecholamines from the adrenal medulla by innervating the splanchic nerves. Catecholamines can also be released from neuronal sources. Once released into the bloodstream, the catecholamines are free to interact at the cellular level with the IS. Again this interaction is made possible because lymphocytes have receptors capable of accepting these neurotransmitters. Thus lymphocytes can act as moving targets in setting up a line of communication between the NS and the IS.

Indirect activation of the IS via the HPA

Central neuro-chemical systems including noradrenergic, serotonergic, cholinergic, and gabanergic systems exert powerful control over hormonal releasing factors at the level of the hypothalamus. Upon stimulation of these releasing factors, corresponding hormones are released from the pituitary gland into the bloodstream . Once into the bloodstream these hormones are free to interact at the cellular level with the immunological cells. Hormones may affect various parameters of the IS through either enhancement or suppression. For instance, elevated levels of prolactin (PRL) have been shown to decrease natural killer cell (NK) activity in hyperprolactinemic females (Gerli, 1986). Conversely, an enhanced proliferative response of human peripheral blood T-lymphocytes is seen with the administration of growth hormone (GH) in vitro. Since lymphocytes carry receptors for β-adrenergic agents, GH, PRL, acetycholine (Ach), substance P, endorphins and enkephalins, a complex picture emerges for potential interaction between the CNS and the IS.

The most popular integrative model of interaction encompassing nervous, endocrine and immune systems has been set forth by Besedovsky and his colleagues. In the following section an overview of this model will be presented, and the relevance of the seizure model used in this study will be discussed.

Immunological-Neuroendocrine Feedback Circuits

Until recently the IS was thought to be autoregulatory. It is now known however that, hormones and neurotransmitters present in the microenvironment of immunological cells can influence this autoregulation. Besedovsky and his colleagues believe the activity of hormones and neurotransmitters are integrated with intrinsic IS regulatory processes, thus linking them to a common feedback circuit between neuroendocrine structures and the IS.

The glucocorticoid-associated immunoregulatory circuit, immunological cells, the sympathetic nervous system, and the brain are all key elements in the functioning of this regulatory feedback loop. I shall discuss the role of each of these elements, and describe their interdependence in completing this immunological-neuroendocrine feedback circuit. I will also discuss the role neurotransmitters play in completing these circuits. Finally, the disturbances of the GABA and catecholamine neural systems associated with seizure activity will be discussed as potential mediators in these immunological-neuroendocrine circuits.

Glucocorticoid-Associated Immunoregulatory Circuit

Glucocorticoid hormones can exert a number of effects on various parameters of the IS. Whether all of these effects are immunoregulatory is uncertain. Nevertheless there is evidence to suggest that some of the general effects of glucocorticoids are immunoregulatory. Besedovsky et al. (1975) have shown that after stimulation with sheep red blood cells (SRBC), horse red blood cells (HRBC), and trinitrophenylhemocyanin (TNP-hae), in two species (rat and mice), an increase in glucocorticoid blood levels occurs at about the same time as the peak of the immune response. The levels reached are known to be immunosuppressive (Gisler & Schenkel-Hulliger, 1971). Thus the elevations of glucocorticoids may be interpreted as regulating the peak of the immune response.

The significance of this increase was clear, yet the question as to how this elevation came about was not. To address this question, these researchers looked at immunological cells.

Immunological Cells

The basic question asked was, " Do immunological signals to endocrine structures exist that control blood glucocorticoid levels ? " (Besedovsky, del Rey, & Sorkin 1985, p.167). The answer to this question was yes. These researchers found that supernatants obtained from **in vitro** stimulation of immunologically activated cells could elevate blood corticosterone levels when injected into normal recipients (Besedovsky, del Rey, & Sorkin, 1981). They named this factor "glucocorticoid-increasing factor (GIF)". The existence of GIF has since been confirmed by other researchers (Pulley et al. 1982; Bindon et al. 1983).

Experimental evidence suggests that GIF acts at the level of the hypothalamus through CRF and does not act directly on the adrenal cortex (Besedovsky, del Rey, & Sorkin, 1983; Vahouny et al. 1983). Removal of hypothalamic function through surgical means (hypophysectomy) or chemical means (dexamethasone) prior to injection with GIF results in complete abolition of the glucocorticoid output. Furthermore it was demonstrated that intracerebroventricular (i.c.v.) injection of GIF produced an increase in glucocorticoid blood levels.

In summary this evidence supports an immunoregulatory circuit linking neuroendocrine structures to the IS. Stimulation of immunological cells induces the release of GIF, which results in an increase in blood level of glucocorticoids via the HPA. The exact nature of GIF has yet to be characterized, but it is known that thymosins and lymphokines produced by immunologically activated cells play a role in GIF.

Sympathetic Control Of The Immune Response

As previously mentioned, the sympathetic branch of the ANS innervates lymphoid organs. It is believed that peripherally, sympathetic activity has an inhibitory influence on immunological cells. Sympathetic nerve activity as reflected by NE levels were monitored during the immune response. After antigenic stimulation with SRBC, a marked decrease in NE content in rat spleen was seen on days 3 and 4, the days of the peak immune response (Besedovsky, del Rey, Sorkin, Da Prada, & Keller, 1979). The severity and persistence of this decrease was inversely related to

the magnitude of the response. No changes in the NE content of a non lymphoid organ such as the heart were noted.

These results suggest that the sympathetic branch of the ANS participates in immunoregulation. It is suggested that the phasic decrease of NE in the spleen but not in lymphoid organs, can be interpreted as " The expression of a sympathetic reflex mechanism that frees immunological cells from sympathetic influences " (Besedovsky, del Rey, & Sorkin, 1985, p.169). The mechanism underlying the NE decrease and the nature of the afferent signals has yet to be demonstrated.

The Brain And Immunoregulation

To determine if the immune response could evoke a brain response, Besedovsky, Sorkin, Felix, and Haas (1977) measured changes in electrical activity at the level of the hypothatlamus. The rate of firing of individual hypothalamic neurons were monitored after injection of SRBC or TNP-hae. In both instances a noteworthy increase in the firing of neurons in the hypothalamus occurred at a time close to the peak of the immune response. Furthermore no increase in the firing rates occurred in immunological non-responders.

It is well known that central neuro-chemical systems including noradrenergic, serotonergic, cholinergic, and gabanergic systems modulate the activity of the hypothalamus. To determine whether NE pathways in the brain were affected by the immune response, Besedovsky et al. (1983) looked at NE levels in the hypothalamus. They found a marked decrease in hypothalamic NE turnover rate 4 days after antigenic challenge, in comparison to saline-injected controls.

To determine whether the soluble products derived from the activated immunological cells mediated this effect, the supernatants from these cells were administered to rats. The results showed a reduction of NE concentration 2 hours after injection when compared with the effects of supernatants taken from non-stimulated lymphoid cells. The reduction of NE corresponded to about 50% of the reduction seen when a powerful inhibitor of NE synthesis (alpha-methyl-p-tyrosine) was administered. The researchers concluded that the activation of immunological cells

resulted in a decrease of NE synthesis in the hypothalamus. An effect which was probably mediated by a factor found in the supernatants of the immunological cells.

The finding of inhibited NE synthesis in the hypothalamus allowed an integrative model to be postulated. Firstly, NE has a known inhibitory influence on hypothalamic neurons (Nishino, 1976). Thus the decrease in NE can explain the increased firing rates seen in the hypothalamus during the immune response. It has also been shown previously that an increase in NE in the hypothalamus results in decreased activity of CRF producing neurons (Ganong, 1974). Thus the decrease of NE in the hypothalamus during the immune response possibly mediates the increase in activity in CRF producing neurons, hence providing a link to the glucocorticoid-associated circuit.

The evidence presented shows the relatedness of endocrine, sympathetic, and neuronal changes during an immune response. It is important to note that changes in neuroendocrine circuits can be initiated by immune products such as GIF. This finding suggests that psychosocial influences on the IS are not simply unidirectional brain effects mediated by neurotransmitters, hormones and sympathetic innervation. More likely these external influences probably affect the "set points" of constantly operating homeostatic immunoregulatory neuroendocrine circuits. Besedovsky and his colleagues believe that by disturbing these " set points " the results may tip the balance towards immunopotentiation or immunosuppression. They also believe that other operating neuroendocrine circuits have yet to be discovered.

The role of the GABA system within neuroendocrine circuits has been established, yet its' function as an immunomodulating agent has not. Most likely, the GABA system has an immunopotentiating effect, since reduction of GABA functioning as a result of seizure activity appears to result in temporary suppression of the immune response. This point will be discussed further, however I shall first discuss the role of neurotransmitters in neuroendocrine circuits and immunoregulation.

Neurotransmitters, Immunoregulation, And Neuroendocrine Circuits

The proposed mechanism by which brain 5HT and NE modulate immunity is depicted in Fig. 1. Both 5HT and NE regulate the release of CRF and therefore have the potential to alter the immune response. In the case of 5HT, it stimulates the release of CRF, while NE inhibits it (Hillhouse, Burden & Jones, 1975; Jones, Hilhouse & Burden, 1976).

Elevations of 5HT would therefore result in increased CRF release, which in turn would elevate adrenocorticotropin hormone (ACTH). The subsequent elevation of glucocorticoids would suppress the activity of lymphocytes. This inhibitory effect of 5HT on the immune response has been demonstrated (Devoino & Ilyuchenok, 1968; Eremina & Devoino, 1973).

In contrast, central NE levels have a stimulating effect on immunity. Brain NE exerts an inhibitory influence on CRF (Hillhouse et al. 1975). Therefore an increase in central NE would result in a reduction of blood glucocorticoid levels which in turn would be immunofacilitative. The opposite effect would be expected with a reduction of central NE. The evidence previously discussed on the role of NE in immunoregulatory neuroendocrine circuits strongly supports this role for central NE.

To view 5HT and NE as sole mediators of CNS-IS interactions would be erroneous. These mediators influence other neuroendocrine circuits, and are also influenced by other regulatory systems. One such system is the GABA system which exerts powerful inhibitory control over central Ach, 5HT and NE (De Feudis, 1984). Thus the nature of GABA's role as the major inhibitory neurotransmitter in the CNS implies that it participates in regulating immunoregulatory neuroendocrine circuits.

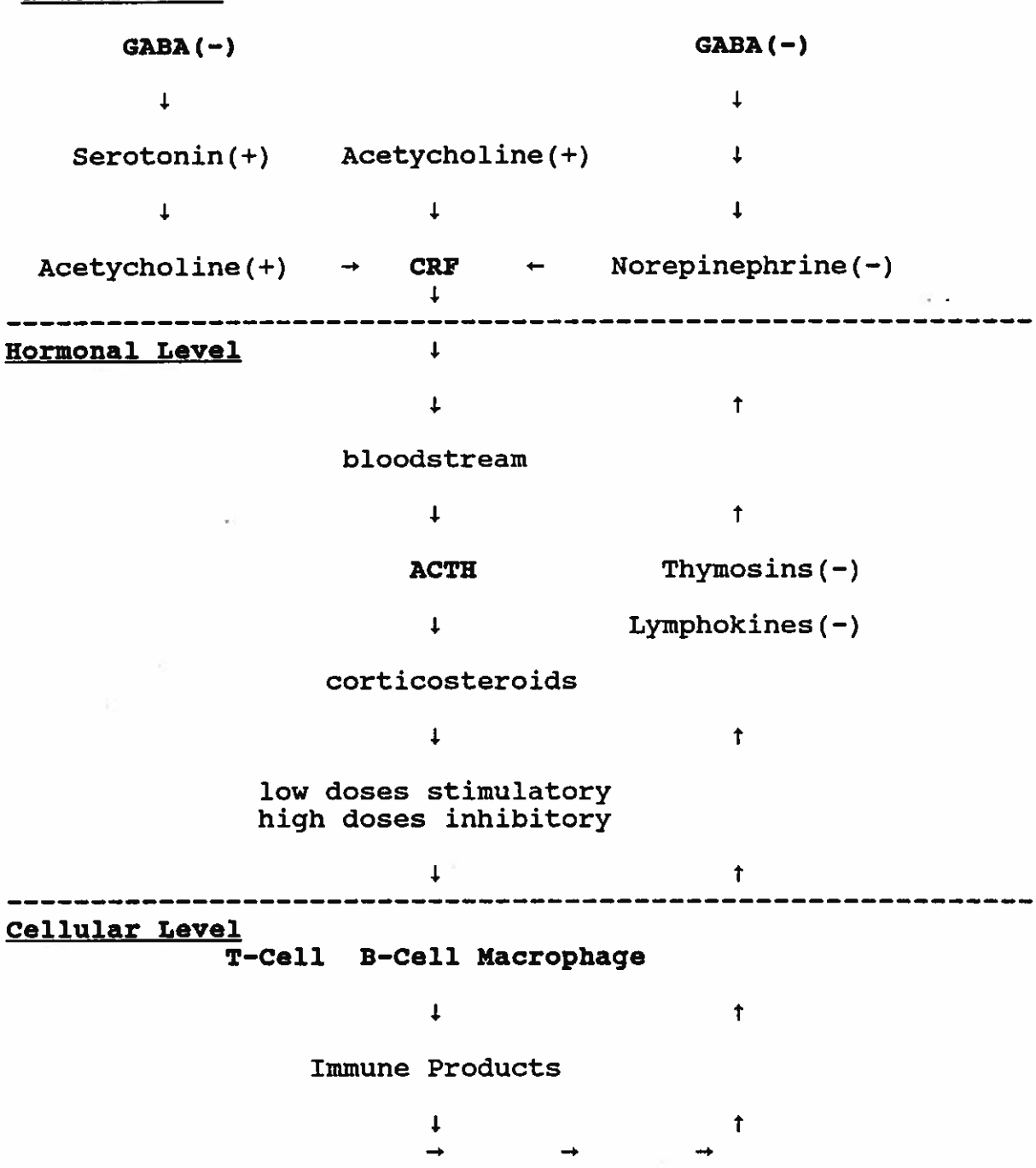

Figure 1. The GABA systems'superimposition on the integrative model of the neuroendocrine feedback circuit.

* Modified from Hall & Goldstein (1985).

The Role Of GABA In Seizure Activity, And Neuroendocrine Immune System Interactions

Disruption of the GABA system in response to seizure activity would predict a shift in balance of currently operating neuroendocrine circuits in favour of immunosuppression. This shift is probably initiated by a reduction in both GABA (Leviel & Naquet, 1977; Tanaka et al. 1975), and catecholamine functioning (Callaghan & Schwark, 1976; Engel & Sharpless, 1977). The reduction in GABA functioning would predict elevations of 5HT, Ach and NE. The elevations of 5HT and Ach would in turn predict elevations of CRF, ACTH, and corticosteroids.

In contrast, reduced inhibition of NE by GABA would predict the opposite effect. However, central catecholamines systems are believed to have an inhibitory effect on kindling activity (Bloom, Costa & Salmoiraghi 1965; McLennan and York, 1967; Stein, 1969). This raises the possibility that a disinhibition mechanism of the catecholamines underlies the kindling activity leading up to a seizure. Such a reduction of catecholamine activity would result in further elevation of CRF and ACTH.

Taken together, the reduction of GABA's inhibition on 5HT and Ach, in combination with the reduction of the catecholaminergic inhibitory effects on CRF, clearly suggest elevated CRF, ACTH, and corticosteroid levels would be present in response to seizuring. Ultimately the elevation of corticosteroids could suppress the immune response.

Sex Differences In Antibody Production

An intriguing phenomena of antibody production is seen between the sexes. Female rats will sometimes fail to produce antibodies when injected with a given antigen. This also happens with male rats but at a much lower frequency. Females also tend to produce larger amounts of antibodies than do the males. This is a common observation (Falter, personal communication, 1987).

Thus far the most accepted explanation for the occurrence of non-responders is the "clonal theory of selection". As described in Kimball's (1986) book of Introductory Immunology, the theory suggests the following scenario.

Prior to antigenic stimulation, the outcome of antibody production is dependant on the hosts' equipment to deal with the antigen. There are approximately 10 million different molecular configurations which can be recognized by a corresponding number of lymphocytes. The B-lymphocytes are alerted to the presence of antigens by accessory cells such as macrophages. If the lymphocyte possesses the corresponding molecular configuration (receptor), it will undergo transformation to an immunoblast and eventually an antibody producing plasma cell.

The key element for observing antibody production is the presence of the corresponding receptor to the given antigen. Without this initial binding the lymphocyte cannot receive the coded message to manufacture more receptors (antibodies). Despite being widely accepted as a theory explaining the existence of non-responders, an alternative explanation may be offered. For instance, Satinder (personal communication, fall, 1988), suggests that non-responders may simply be low responders. Such low responders may go undetected because the instrument of measurement is lacking in sensitivity. Both of these explanations seem plausible, nevertheless they offer no explanation as to the different representation of responders and non-responders among the sexes.

The enhanced immunological response seen in female animals has also been seen in humans. For instance, women given immunotherapy have a better prognosis than men in chronic myelocytic leukemia, in adult lymphatic and myeloid leukemia, in multiple myeloma, and malignant

myeloma (Reizenstein, 1982). These sex differences are also not well understood. Perhaps the differential response seen among the sexes is related to the differences in hormonal milieux.

Rationale Of The Study

To the best of this author's knowledge, experiments examining the effects of seizures on the IS are novel to the field of psychoneuroimmunology. The first was a pilot study conducted by this author in 1987 at Laurentian University. The initial results showed that 120 day old female rats seizured with metrazol demonstrated immunosuppression (in comparison to controls) 5 days after receiving the HSA booster shot and metrazol injection.

These results were confirmed in a subsequent study (Chretien, Persinger, & Falter, 1987). In this study two types of seizures were examined. They were generalized motor convulsions and limbic seizures respectively. The limbic seizures produced more violent seizures and resulted in more severe immunosuppression than that of generalized motor convulsions. In all experiments, immunosuppression and a rebound effect on the 10th day after the booster shot and experimental treatments (cylophosphamide & drug induced seizures) were noted.

The third experiment comprises this thesis. This study was conducted by this author at Lakehead University (1988), and is reported in detail in this document. A series of other experiments have been conducted since that time to address methodological difficulties encountered in earlier experiments (Falter, Persinger, & Chretien, 1990). These problems will be addressed in the discussion section.

No other reporting of this nature was found in the literature. Similarly there was a paucity of literature concerning GABA's role as an immunomodulating agent.

The consistency of the experimental results obtained in these studies support the rationale for studying seizure activity and the humoral immune response.

Seizure Activity And Epilepsy

The term seizure is often used to describe a disturbance of consciousness associated with epileptic episodes. These episodes have also been called convulsions, fits and attacks. However, none of these terms is completely satisfactory since seizures vary greatly in nature. Seizures are very common, with one person in 20 experiencing at least one seizure in their lifetime. A single seizure however does not qualify one as an epileptic. The prevalence of more frequent seizures is much lower, about one person in 200.

Epilepsy may be classified as partial, generalized or unclassified seizures. Partial seizures begin locally and spread. For instance in a simple partial Jacksonian seizure, the attack may begin with the jerk of a finger, a toe, or the mouth and spread throughout that region.

A complex partial seizure most commonly occurs in the temporal lobe and is characterized by the following affective and behavioral symptoms: 1) subjective feelings including repetitive thoughts, mood alterations, deja-vu or hallucinations 2) automatism, repetitive stereotyped movements such as lip smacking, chewing or acts such as undoing buttons. 3) postural changes such as in catatonia.

Generalized seizures are symmetrically bilateral without local onset: a) The Grand mal attack is marked by three stages: 1) a tonic stage in which the body stiffens and the persons' breathing stops 2) a clonic stage of rhythmic shaking 3) a post ictal stage of depression in which the patient is confused. b) The Petit mal attack is marked by a loss of awareness with a little motor activity such as blinking and/or turning the head, rolling the eyes etc... . These attacks seldom exceed 10 seconds. c) Myoclonic spasms are massive seizures consisting of a sudden flexion or extension of the body preceded by a cry. d) Akinetic seizures are usually seen in children. The child usually collapses without warning. This is very short in duration, and the child may sit up in a few seconds without any postictal depression (Kolb & Whishaw, 1985).

A host of factors may precipitate seizures in susceptible individuals. These range from hyperventilation, sleep, sleep deprivation, sensory stimuli (i.e. flashing lights, sounds, reading),

trauma, fever, emotional stress, drugs, and hormonal changes including menses, puberty, adrenal steroids and ACTH (Pincus & Tucker, 1974).

The term kindling refers to an epilepsy model which has been widely used to study this phenonenon. Kindling is marked by a progressive increase in the strength of epileptiform activity evoked by spaced (in time) and repeated electrical or chemical (i.e. metrazol) stimulation of certain brain structures. Initially weak epileptiform after discharges occur, followed by an eventual increase in the strength of both the electroencephalographic and behavioral response. In the final stages of treatment the animals respond with fully generalized electroencephalographic and motor seizures. The final outcome is a dramatic and permanent change in response, seemingly without the production of tissue damage.

A search for underlying mechanisms suggests that some of the effects are due to changes at the synapse and that these changes are widespread within the brain. The changes in synaptic functioning may be due to an increasing efficacy of excitatory synapses or a decreased efficacy at inhibitory synapses (Racine, 1978). Findings of depletions of both GABA and the catecholamines support both of these suggestions. The role of these neural systems in the kindling phenomena suggest they are further involved with on going immunoregulatory neuroendocrine feedback circuits.

The preliminary results of this research project suggests that a tentative relationship has been established between seizure activity and the IS. Since both epilepsy and the IS are intimately involved with the CNS, simultaneous study of both these phenomena may be useful in unravelling the complexities of CNS-IS interactions . To date, there has been no research conducted in search of common mechanisms of action involving epileptic behaviour and immunoregulatory functioning.

CHAPTER II

Methods

It is important to remember that the monitoring of HSA antibody production in this study was conducted in two segments. In the first segment, the animals produced antibodies (**in vivo**) in response to primary and secondary administrations of HSA suspended in normal saline. In the second segment, blood samples drawn from the animals were assayed (**in vitro**) to determine the antigen binding capacity (ABC) of HSA antibodies present in the rat serum of the immunized animals.

Radioimmunoassay (RIA) Procedure

The RIA used in the second segment was a modified version of the Farr (1958) assay. In this modified version, bovine serum albumin (BSA) was substituted by HSA. The mechanistic organization of the assay is outlined in appendix A. The objective of the assay was to determine the ABC of the antibodies contained in the rat serum, and not their actual quantity or weight.

The assay was based on an antigen-antibody reaction. The antigen in this assay was the radioactively labelled human serum albumin (*HSA). The antibodies were those contained in the serum. The rat serum was diluted to 10 different concentrations with phosphate buffer saline (PBS). Each dilution was combined with a known quantity of *HSA in a reaction mixture (refer to Table 1). The reaction mixture was then gently vortexed, and incubated at 37° c in a water bath to allow the antigen-antibody reaction to proceed.

The formed antigen-antibody complexes were then precipitated by chemical means (salting out). To salt out, 380 ml of PBS was added to each tube, followed by the addition of 500 ml of saturated ammonium sulfate (SAS). This was allowed to stand for 15 minutes. The tubes were then centrifuged at 3000 g for 30 minutes. The supernatants were then poured off except for control tubes 1, 2, & 3. The precipitates were then washed with 500 ml of 50% saturated ammonium sulfate (SAS/2) for 10 minutes at 3000 g, excluding tubes 1, 2, & 3. The supernatants were discarded except for control tubes 1, 2, & 3. The γ^{125}I rays were then counted with an ANRS gamma counter.

notes: *HSA was diluted to the appropriate concentration with radioiodination buffer containing .1% gelatine. A further dilution for radioactivity was also made by mixing the appropriate volume of 1.1 µg/ml cold HSA in radioiodination buffer solution.

Table I: Protocol content (vol. unit in µl)

tube #	phosphate buffer	control serum	*HSA	antiserum	
1	80	-	20	-	
2	80	-	20	-	
3	80	-	20	-	
4	60	20	20	-	
5	60	20	20	-	
6	60	20	20	-	
7	40	20	20	20	
8	50	20	20	10	)neat
9	55	20	20	5	
10	40	20	20	20	
11	50	20	20	10	)1/10
12	55	20	20	5	
13	40	20	20	20	
14	50	20	20	10	)1/100
15	55	20	20	5	
16	40	20	20	20	)1/1000

Once the gamma rays were measured in counts per minute (CPM), the ABC values for the various dilutions were calculated and plotted on a graph (Fig. 2). The percentages of precipitation of *HSA were viewed on the Y axis, and the dilution factors were viewed on the X axis. The calculation of the ABC values were obtained using the following formula:

ABC = (amount of *HSA)x(33 /100)x(dilution factor)x 1000

= μg *HSA / ml of serum.

An arbitrary point (33%) was chosen as a figure to calculate the ABC of the rat serum. The first two parts of the equation determines how much *HSA was bound at 33% ABC. The third part determines the dilution factor at which 33% binding occurred. The greater the dilution factor, the greater the ABC of rat serum would be. For instance, if 33% ABC occurred at a dilution factor of 17, this value should be 17 times greater than a 33% ABC occurring at a dilution factor of one. This estimate was determined through extrapolation (refer to the dotted line seen in figure 2). The final part of the equation simply converted the expression of the ABC in μg/ml of serum rather than μg/μl of serum.

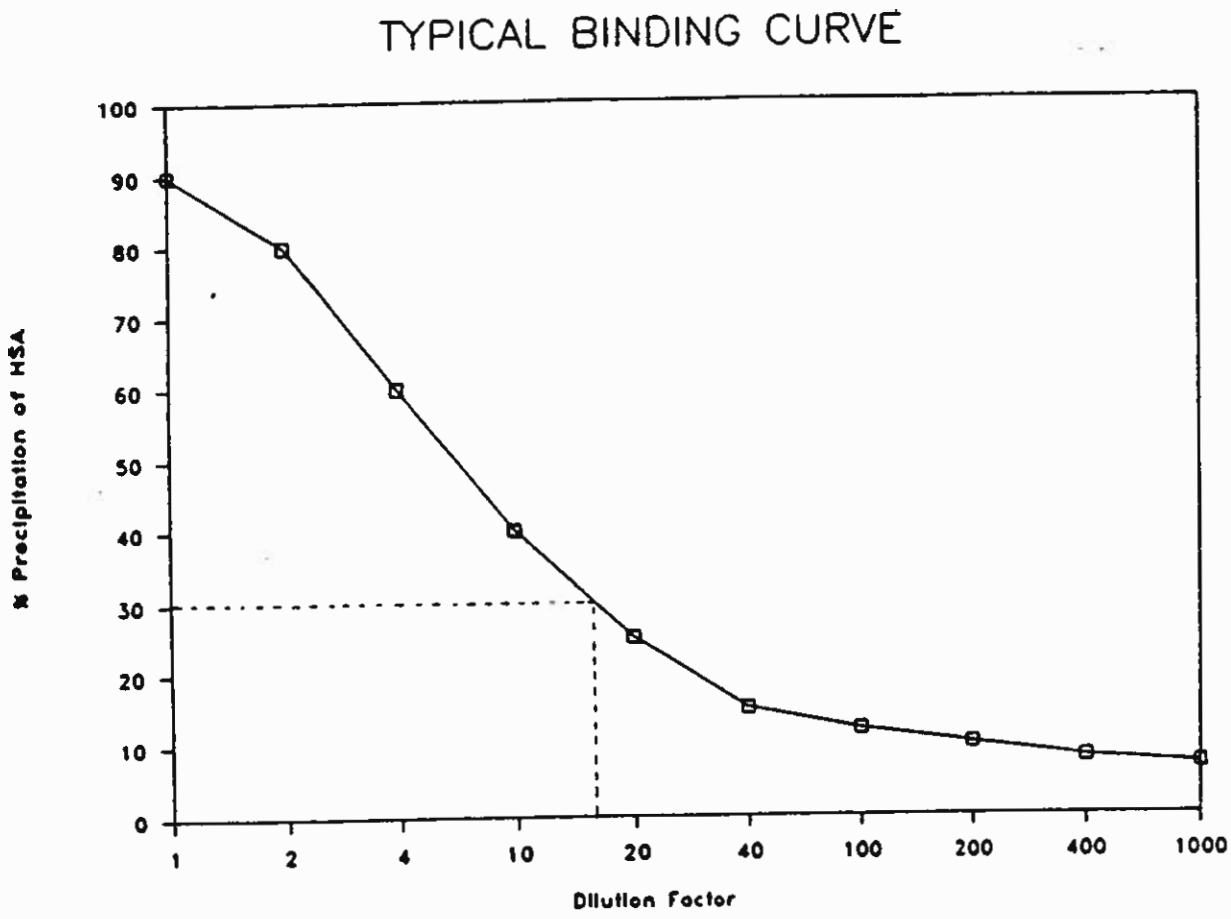

Figure 2. Typical Binding Curve

There are a few control factors that were run as part of the assay procedure. They were the maximum (tubes 1-3) and minimum (tubes 4-6) controls. The maximum measured the amount of *HSA that went into every test tube for the various dilutions. The minimum assessed how much non-specific binding of the *HSA occurred during the reaction (i.e. how much *HSA bounded to the test tubes). For a more detailed description of the calculations refer to Farr (1958).

Radioiodination Procedure

The major preparation and most crucial for the RIA was the preparation of the *HSA. This was done through the iodination of HSA with radioactively labelled ^{125}I. The iodination procedure was provided by Bio-Rad Laboratories (refer to appendix E).

The commercial preparation of lactoperoxidase and glucose oxidase combined in proportion provided enzymatic activity upon the addition of glucose. The addition of glucose activated glucose oxidase such that a continuous but small amount of hydrogen peroxide was generated. This in turn minimized the possibility of oxidizing the HSA protein. The lactoperoxidase catalyzed the peroxide oxidation of ^{125}I to I_2. The I_2 then reacted with the tyrosine residues of the HSA to produce *HSA. Refer to appendix B for schematic representation.

Once the iodination had taken place, the reaction mixture was quenched. This was achieved by running the reaction mixture through a column. By running the mixture through the column, the reacted *HSA was separated from the unreacted ^{125}I. These two radioactive compounds were then collected in a fraction collector every 5 minutes and counted for their gamma Rays. Two distinct peaks were seen when the values were plotted. The first peak showed the reacted *HSA, and the second peak showed the unreacted ^{125}I (Fig. 3).

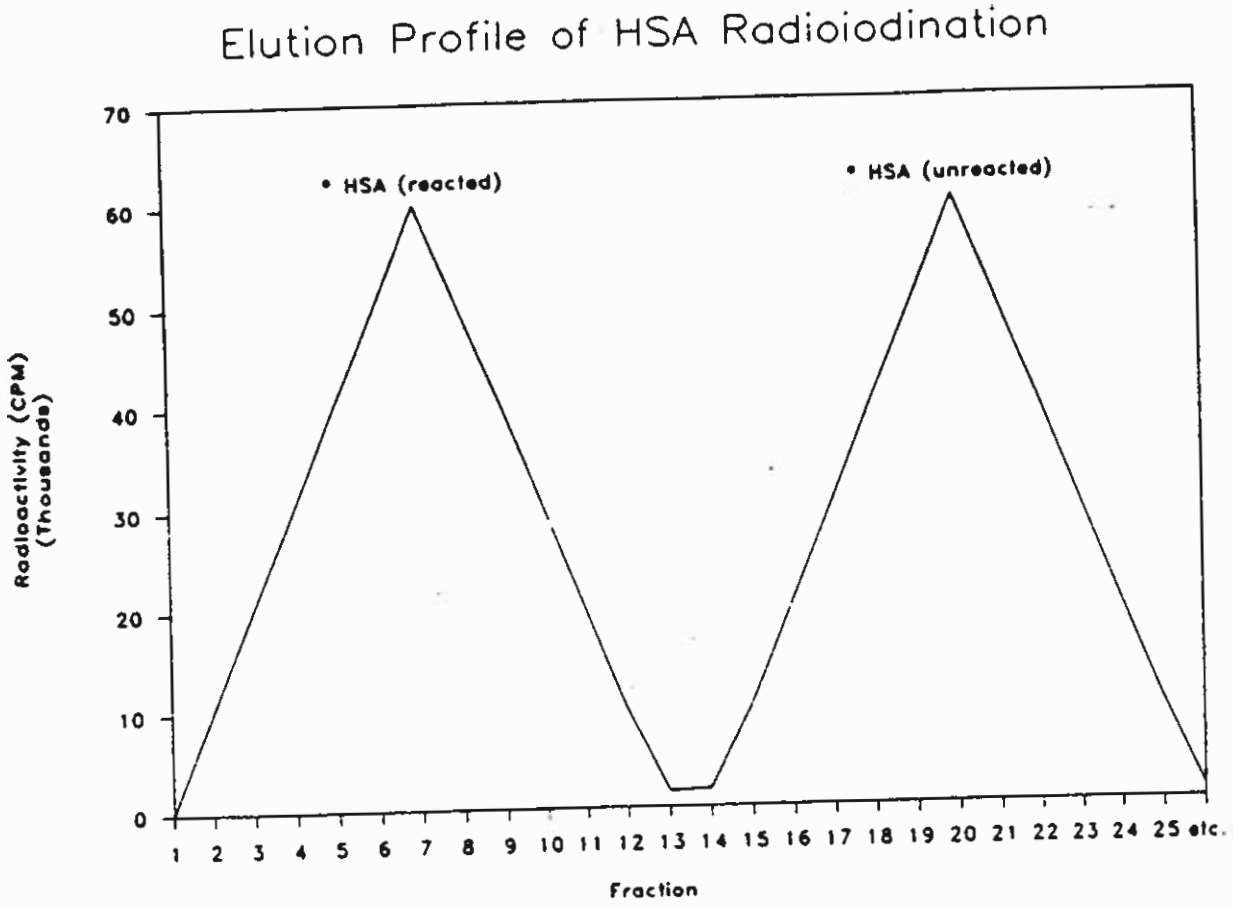

Fig. 3. Elution Profile of HSA Radioiodination.

Materials and Procedures

Refer to appendix E for a list of suppliers and their source.

Reagent preparation:

All containers used for radioimmunoassay were washed with double distilled water. Double distilled water was also used in preparation of all reagents. This precaution was taken to ensure the absence of chloride ions and bacterial contamination which may have interfered with the iodination of the HSA.

Buffer preparation:

Phosphate buffer (assay buffer) (.15M, pH 7.2)

This buffer was prepared by dissolving 20.7 grams (g) of $NaH_2PO_4.H_2O$ into approximately one litre of double distilled water, adjusting the pH to 7.2 with NaOH (aq).

Phosphate buffer (.2M, pH 7.2)

This buffer was prepared in the same fashion as described above, except 27.6 g of $NaH_2PO_4.H_2O$ was used instead.

Phosphate buffer saline (PBS) (.15M, pH 7.2)

This buffer was prepared by mixing 9 g of NaCl (.9%), 8.09 g of Na_2HPO_4 (dibasic), together with 2.45 g of KH_2PO_4 (monobasic) in one litre of doubled distilled water. The final pH was adjusted with HCL (aq).

Radioiodination buffer

Sodium phosphate buffer (NaH_2PO_4) 0.2M, was prepared by dissolving 2.75 g of NaH_2PO_4 in a 100 ml of double distilled water. A few sodium hydroxide (high purity grade) crystals ($NaOH.H_2O$) were added to the mixture. Upon mixing, 1.5 ml of the aliquot were removed for monitoring of the pH. The desired pH was achieved through further titration, using the same method. This procedure ensures that no chloride ions (CL^-) from the electrode would leak into the buffer which might contaminate the iodination process.

Other Reagents:

Saturated ammonium sulfate (SAS)

SAS was prepared by dissolving 150 g of $(NH_4)_2SO_4$ in one litre of distilled water, stirring it overnight at room temperature. The pH was adjusted to 7.2 with sodium hydroxide, and was then filtered.

Saturated ammonium sulfate 50% (SAS/2)

SAS/2 was prepared by diluting SAS by two fold with an equal amount of PBS.

Human serum albumin solution

HSA was dissolved into double distilled water and dialyzed exhaustively for four days (three changes/day). It was then centrifuged at 20000 g for 30 minutes. The pellet was discarded and the supernatant was freeze dried. A protein concentration of 6 mg HSA/ml iodination buffer was prepared for the iodination process.

Substrate solution

An amount of 0.02 g of α-D-glucose was dissolved in 1.0 ml of double distilled water and allowed to stand overnight for muta-rotation. Therefore after 17 hours the concentration of ß-D-glucose would be about 1%.

Enzymobead reagent

One vial of the reagent was rehydrated with 0.50 ml of double distilled water for 24 hours and was stored at 4° C.

Normal saline solution

This was prepared by dissolving 0.90 g of NaCl into 100 ml of double distilled water (.9% NaCl).

Preparation procedure:

Column preparation:

The G-25 Sephadex superfine resin (10 g) was suspended in 75 ml of 0.2M pH 7.2 phosphate buffer and was allowed to swell for at least 2 hours. The suspension was then gradually filled into a 25 ml burette or column (25x1.0). It was allowed to equilibrate with the running buffer at a flowrate of ≈ 0.25ml/min.. After the column was settled and packed, it was preloaded with an adequate amount (150 µl 1mg/ml) of cold HSA. This step was to ensure that the non-specific, if any, for the resins of HSA would be bounded.

Experimental Manipulations

Animals

Seventy five 100 day old Satinder's Heterogenous Strain (SHS) experimentally naive rats (39 females; 36 males) were included in this experiment. Refer to (Satinder, 1980) for a discussion of the breeding of the SHS line of rats. The animals were housed individually in standard wire

cages at a temperature of 22° C ± 1, and maintained on a 12:12 light/dark cycle (8 A.M.-8 P.M.). Food and water were available ad libitum.

Procedures

The animals were tested at 100 days of age. Prior to this they were given two weeks to habituate to the individual housing condition before being injected subcutaneously (s.c.) in the scruff of the neck with 0.1 ml (1 μg/ml) of HSA suspended in 0.9% saline solution. Eighteen days later each animal was placed in a restraint cage and .1 ml to .2 ml of tail blood was quickly taken from the tail vein and collected into heparinized microtubes. Blood was obtained by cutting the skin of the tip of the tail and gently milking the tail. Prior to this the tail was placed in a water glass for 20-30 seconds at a temperature of approximately 50° C (to promote vasodilation). Subsequent tail bleeding consisted of removing the formed scab from the previous cut. Sterilized razors were used for all tail bleeding procedures. The collected blood was marked for identification, allowed to stand for 30 min., centrifuged for 3 min. at 3000 RPM's. The serum was removed with a pasteur pipette and stored in a 10 ml vial at 4° C until assayed. The first measure obtained from the radio-immunoassay was used as a baseline measure for the primary antibody response.

Two days later the animals were injected with a second dose of HSA at the same concentration and volume as the first. After receiving the booster shot, the animals were immediately allocated to one of eight groups: 1) The undisturbed control group, 2) the saline-injected control group, 3) the metrazol (pentylenetetrazol) group and the 4) cyclophosphamide group. The above groups were further subdivided by sex (female, male) to yield eight groups. Litter mates were assigned to each of the eight groups whenever possible. The undisturbed control groups received no injections, the saline-injection control groups were injected intraperitoneally (i.p.) with 1 ml/kg of 0.9% NaCl, the metrazol groups were injected i.p. with 30mg/ml/kg of this substance, and the cyclophosphamide groups were injected i.p. with 50mg/ml/kg of cyclophosphamide. All injections occurred within 30 sec. of receiving the HSA booster.

Following the second antigenic stimulation (booster), and experimental manipulations (i.p injections), the secondary antibody response was monitored by determining antigen binding capacity (ABC) measures from rat serum taken two days, four days, and ten days later.

Our operational definition of a responder to HSA was the occurrence of a minimum of 0.02 μg/ml of ABC of the rat serum on the baseline sample. This minimum was determined as the lower end of the sensitivity level for this assay (Falter, personal communication, 1987). Only rats displaying this criterion were used in the analyses. As such the following number of rats per group (n originally in group/ n criterion, in parentheses) were included: female control (8/4), male control (6/5), female saline control (8/5), male saline control (8/5), female metrazol (11/9), male metrazol (12/10), female cyclophosphamide (12/9), and male cyclophosphamide (12/9).

Open-Field Testing

Rats receiving the metrazol treatment were immediately placed in an open field apparatus and videotaped for 30 min. following injection. Individually each rat was placed in the center of the open-field and was observed without disruption, and with the generation of white noise. Following the observation period, the animals were returned to their individual housing condition. All testing occurred between 1500 hrs. and 1700 hrs. The videotapes were viewed later by two independant raters. The rats were rated for the severity of their generalized motor seizure according to the scale of Racine (1972):

stage 1: behavioral arrest

stage 2: whisker twitching to facial clonus

stage 3: unilateral forepaw clonus

stage 4: bilateral forepaw clonus

stage 5: rearing and falling

The experimenter was strictly concerned with the monitoring of seizure activity, and not defecation or ambulation. These latter behaviours are the most commonly observed behaviours in an open-field apparatus.

The apparatus was an arena 90 cm on each side and divided into 16 equal square sections marked on the floor. The arena was made of plywood and white melamine plastic, and the walls were 45 cm high. The front wall was a sliding door of transparent plexiglas, which served as an observation screen and as a door for cleaning the arena. The arena was lighted by four 90-cm-long fluorescent lights 90 cm above the floor centre of the arena. A white noise generator produced 65dB (re 0.0002 μbar) of masking noise. (Sound intensity was measured at the level of floor centre of the arena with a General Radio sound-level meter, type 1551-C) (Satinder, 1980).

Decapitation

The serum taken from animals that were not immunized with HSA was required as control serum for the radioimmunoassay. The control serum served as carrier molecules in the antigen-antibody reaction. Only animals that were already destined to be sacrificed as part of routine colony maintenance were used.

To obtain the control serum, decapitation was performed with a guillotine, and trunk blood was collected in 25 ml centrifugal tubes. The tubes were allowed to stand for 30 min. and were then centrifuged for 20 min. at 3000 RPM's. Serum was then removed with a pasteur pipet, transferred to 10 ml test tubes and stored at 4° C.

Chapter 3

Results

Statistical Analyses

Multi analysis of variance (Manova), analysis of variance (Anova), covariance, a posteriori tests (Scheffe's), non-parametric tests (Kruskal-Wallis, crosstabs), Pearson and Spearman-Rho correlations, and data transformations were carried out on a Vax 780 system computer using the SPSS^x software. Where extreme values resulting in skewness values greater $\pm$ 1, or where homogeneity of variance test was $p \leq 0.01$, the data was transformed to square root values or log to the base 10 values to satisfy the criteria for using parametric tests. If those data transformations did not reduce these values to acceptable levels, non-parametric tests were used.

The following analysis was carried out with a multi analysis of variance. The saline-injected versus the undisturbed control groups were compared to determine if the i.p. injection received by the animals had any significant effects on the findings. The injection-control and undisturbed control groups did not differ significantly from each other. Therefore these groups were collapsed to form one group and referred to from here on as 'control group'. The subsequent Manova of experimental versus control groups was carried out to determine the effects of seizuring in comparison to both cyclophosphamide injected and control animals. The Anova's were then used to determine the major sources of significance for groups and sex factors across baseline, day 2, day 4, and day 10 ABC measures. Following these analyses, a Post hoc test (Scheffe procedure) was used to determine the specific source of variance among groups on day 4 (the day of the observed immunosuppression). Covariates for litter mate and bodyweight factors were also used with the Anova's to unmask any possible effects these factors might have on other measures. A Manova comparing bodyweight of all animals on baseline, day 2, day 4, and day 10 of the experiment was also used to determine if any significant bodyweight fluctuations occurred during the course of the experiment. All ABC measures displayed extreme heterogeneity in variance, therefore non-parametric analyses (Kruskal-Wallis) for groups and sex factors were used.

To determine the relationship between the severity of the seizure observed and the degree of immunosuppression, various correlations were carried out. The first was a Pearson correlation evaluating the interrater reliability of the scores judged by the "seizure observing" raters. Prior to this a one-way analysis of variance was carried out to determine if females differed from males in the degree of the severity of their seizure. Following this, both a Pearson and Spearman-Rho correlation of seizure ratings and ABC measures on day 4 were carried out.

The final analysis was a chi-square to determine if females and males differed in their representation of responders and non-responders.

Means and standard deviations of the absolute value of the ABC of rat serum for both saline-injected and undisturbed control groups are shown in appendix C. The Manova showed no statistically significant difference between saline-injected and undisturbed control groups ($F(1,17) = .54$, $p > .05$) or males and females ($F(1,17) = 1.79$, $p > .05$) but a significant ($F(3,51) = 58.82$, $p < .001$) difference among days (baseline, day 2, day 4, and day 10) of ABC measurement.

Means and standard deviations of the absolute values of the ABC of rat serum for experimental groups and control group are shown in appendix D. The Manova demonstrated no significant difference among control, metrazol, and cylophosphamide groups ($F(2,52) = 2.68$, $p > .05$) but a small significant difference between males and females ($F(1,52) = 4.76$, $p < .05$), the females showing higher scores than males, and a highly significant ($F(3,156) = 35.56$, $p < .001$) difference among days (baseline < day 2 < day 4 < day 10), and a highly significant day by group interaction ($F(6,156) = 18.67$, $p < .001$).

The Anova procedure revealed no significant difference among control, metrazol, or cyclophosphamide groups for baseline ($F(2,55) = .87$, $p > .05$), day 2 ($F(2,55) = 1.97$, $p > .05$, and day 10 ($F(2,55) = .32$, $p > .05$) for ABC measures, but a highly significant ($F(2,55) = 11.66$, $p < .001$) difference on day 4.

A Post hoc analysis (Scheffe's set at $p < .01$) indicated that the specific source of variance was due to the significant ($F(2,55) = 11.66$, $p < .001$) suppression of antibody formation in the cyclophosphamide (CY) and metrazol (MTZ) groups on the 4th day after the booster shot and experimental treatments.

The Anova procedure revealed that the specific source of significance for the sex difference was due to a significant ($F(1,56)=8.49$, $p < .01$) difference between female and male antibody formation levels on the 10th day. Females had significantly higher ABC measures than did males. No sex difference was detected on baseline ($F(1,56)=2.94$, $p > .05$), day 2 ($F(1,56)=3.29$, $p > .05$), or day 4 ABC measures ($F(1,56)=1.75$, $p > .05$).

Because of a problem with heterogeneity of variance for all ABC measures, non-parametric tests were carried out for group and sex factors. Kruskal-Wallis one-way analysis of variance indicated no significant difference among groups on baseline (chi-square = .59, $p > .05$), day 2 (chi-square = 4.03, $p > .05$) or day 10 (chi-square = 1.27, $p > .05$). However a highly significant difference among groups was observed on day 4 (chi-square = 4.03, $p < .001$). Further non-parametric analyses of variance comparing all possible combinations of groups on day 4 measures revealed that the specific source of variance was due to the significant (chi-square = 7.85, $p < .01$) suppression of antibody formation by the MTZ group and the CY group (chi-square = 19.93, $p < .001$) in comparison to the combined control (CTL) groups (i.e. undisturbed and saline-injected). The MTZ and CY group did not significantly differ from one another (chi-square = 3.00, $p > .05$) on day 4.

Kruskal-Wallis one-way analysis of variance indicated no significant difference between sexes on baseline (chi-square = 2.66, $p > .05$), day 2 (chi-square = 2.06, $p > .05$), or day 4 (chi-square = 3.06, $p > .05$). However a significant sex difference was found on day 10 (chi-square = 7.19, $p < .01$).

Both parametric and non-parametric procedures confirmed the findings of immunosuppression on day 4 by CY and MTZ groups compared to the CTL group, and the higher ABC levels by females than males on the 10th day. Refer to Figures 4 and 5 for a graphic representation of these results.

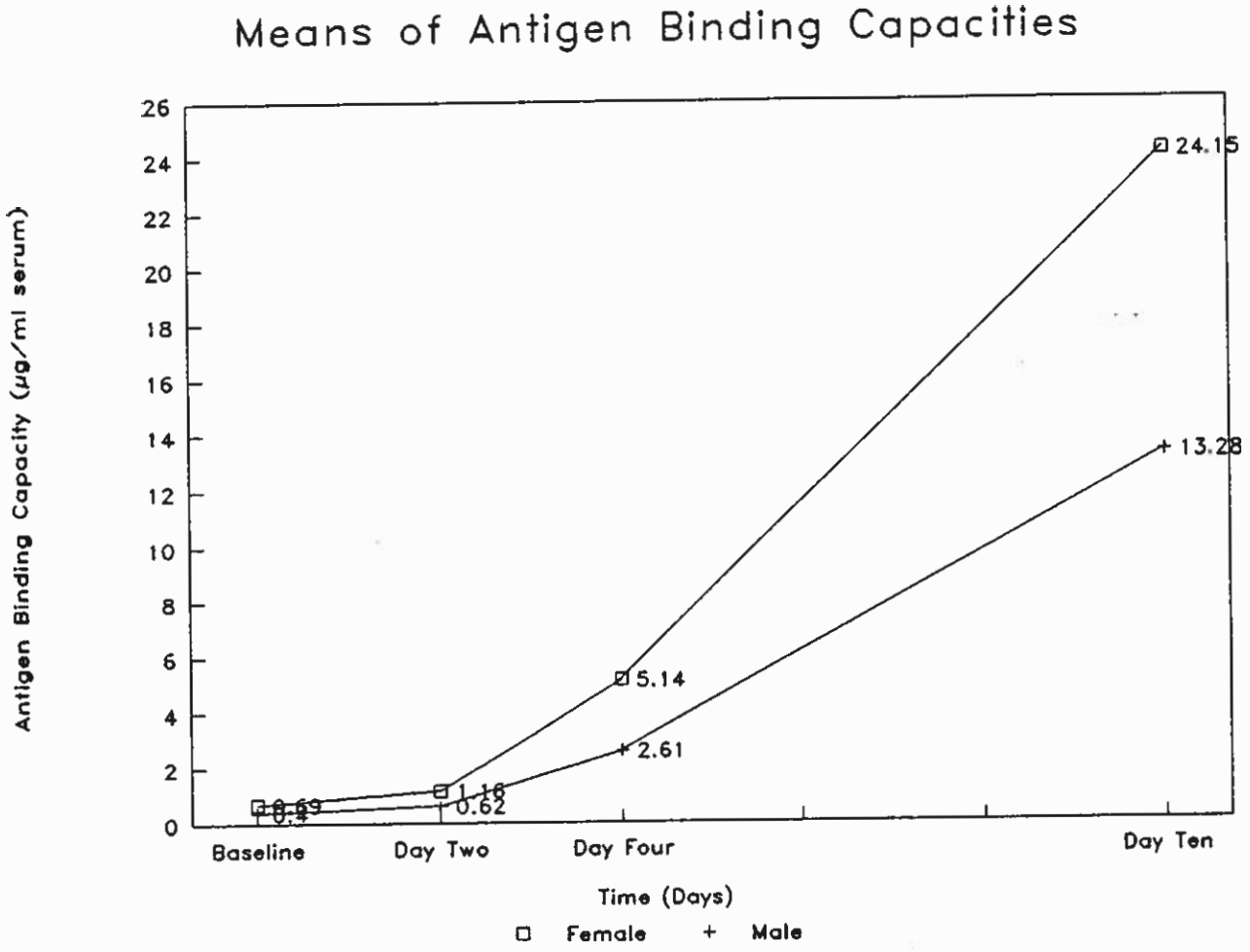

Figure 4. Means of Absolute values of antigen binding capacity for females and males from baseline to day ten measures

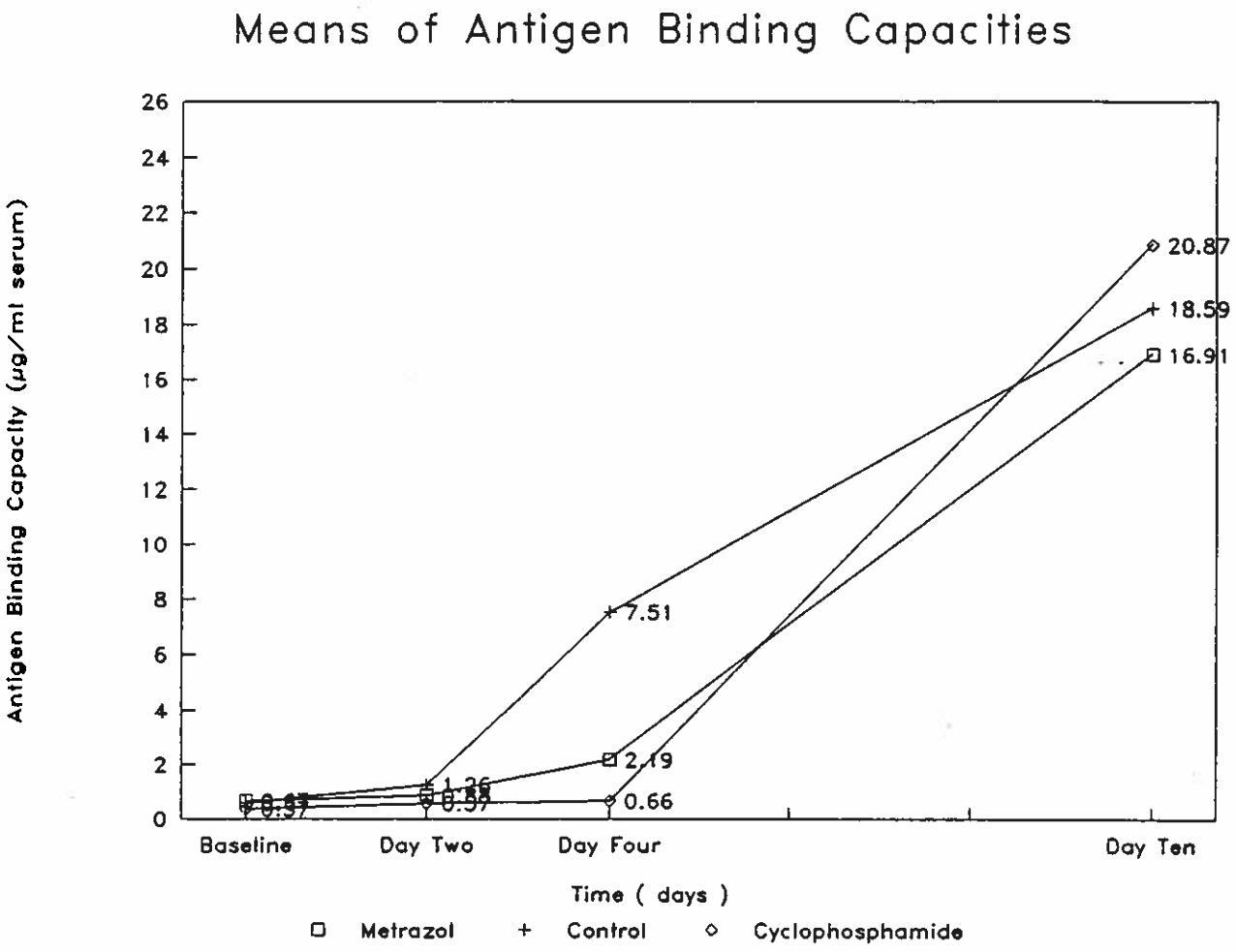

Figure 5. Means of absolute values of antigen binding capacity for control, metrazol and cyclophosphamide groups for baseline to day ten measures.

Results of the covariate analysis with the Anova's on baseline, day 2, day 4, and day 10 ABC measures, indicated that bodyweight was not significant on any of the days of ABC measurement. The litter from which the animal came from was a significant covariate on baseline ($F(1,51)=8.60$, $p<.01$), day 2 ($F(1,51=7.60$, $p<.01$), and day 10 ($F(1,51)=4.15$, $p<.05$). However litter mate number was insignificant as a covariate on day 4 ($F(1,51=1.28$,$p>.05$), the day of the observed immunosuppression. Refer to Table 2 for a presentation of the Anova analyses carried out with these covariates. Results of the Manova procedure comparing bodyweight indicated no significant bodyweight difference existed among control, metrazol, and cyclophosphamide groups ($F(2,52)=2.80$, $p>.05$). The bodyweight measures used for comparison were taken on baseline, day 2, day 4, and day 10, the same days that ABC measures were taken. The Manova procedure revealed a significant bodyweight difference between the sexes ($F(1,52)=394.53$, $p<.001$), with female animals being lighter than males.

Table 2

Analysis Of Variance Of Rat Serum Antigen Binding Capacity (ABC) For Baseline, Day 2,Day 4, And Day 10 ABC Measures: A) By Group And Sex B) By Group And Sex With Litter mate Number (LN) As A Covariate C) By Group And Sex With Bodyweight (BW)As A Covariate At The Time Of ABC Measurement

)	Baseline			Day 2			Day 4			Day 10		
ource	df	MS	F	df	MS	F	df	MS	F	df	MS	F
roup(G)	2	.07	.52	2	.56	2.1	2	14	15***	2	1.9	.58
ex(S)	1	.43	3.1	1	.89	3.2	1	2.9	3.2	1	25.2	7.6**
x S	2	.01	.09	2	.01	.02	2	.11	.12	2	.21	.06
esidual	52	.14	-	52	.27	-	52	.91	-	52	3.31	-

B)	Baseline			Day 2			Day 4			Day 10		
	df	MS	F	df	MS	F	df	MS	F	df	MS	F
Covariate (LN)	1	1.1	8.6*	1	1.9	7.6**	1	1.8	1.3	1	13.4	4.15*
Group(G)	2	.11	.52	2	.51	2.01	2	14.3	15.4***	2	1.96	.60
Sex(S)	1	.18	1.4	1	.42	1.65	1	2.44	2.63	1	18.3	5.66*
G x S	2	.03	.20	2	.01	.04	2	.10	.11	2	.45	.14
Residual	51	.12	-	51	.25	-	51	.92	-	51	3.24	-

C)	Baseline			Day 2			Day 4			Day 10		
	df	MS	F	df	MS	F	df	MS	F	df	MS	F
Covariate (BW)	1	.29	.73	1	.26	.20	1	17.1	.80	1	6.04	.03
Group(G)	2	.31	.78	2	1.7	1.3	2	253	11.6***	2	29.8	.13
Sex(S)	1	2.4	6.0	1	6.9	5.3	1	29.3	1.3	1	1073	4.9*
G x S	2	.12	.29	2	.14	.10	2	9.90	.45	2	.25	.001
Residual	51	.40	-	51	1.31	-	51	21.9	-	51	217.9	-

*p < .05 **p < .01 ***p <.001

A one-way analysis of variance indicated that females did not differ from males in the degree of the severity of their seizures ($F(1,20)=2.75$, $p > .05$).

A Pearson correlation showed a very good level of interrater reliability of the "seizure rating observations" as seen by a positive ($r = .96$, $p < .01$) significant correlation.

A Pearson correlation assessing the degree of relationship between the severity of the seizure and the immunosuppression observed on day 4 showed a negative ($r = -.37$ $p > .05$) insignificant correlation.

A non-parametric Spearman-Rho correlation revealed a significant (-.42, $p < .05$) negative correlation between the severity of the seizure and the immunosuppression observed on day 4 (Figure 6).

The final chi-square analysis showed that female and male animals did not differ significantly ($df = 1$, 0.52, $p > .05$) in their number of responders versus non-responders.

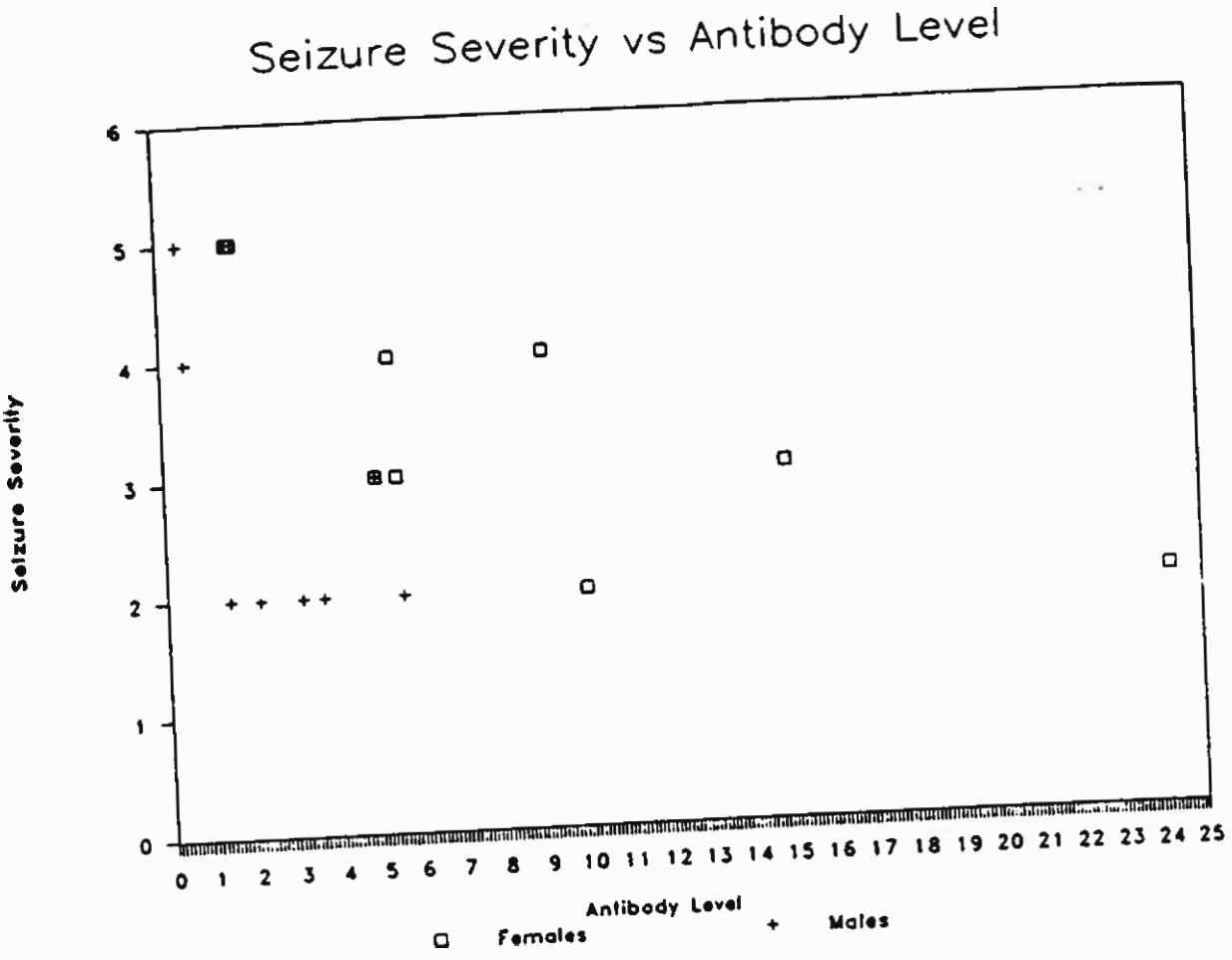

Figure 6. Scatterplot of ABC Measure Vs. Severity of Seizure

CHAPTER IV

Discussion

The results indicate that generalized motor convulsions elicited by metrazol produce significant immunosuppression that is comparable to the immunosuppression induced by cyclophosphamide, a well known immunosuppressive agent. The immunosuppression was defined by the reduced antibody levels on the 4th day following the second HSA antigenic challenge compared to rats that served as controls.

The severity of the seizure was moderately correlated to the degree of immunosuppression. This finding is consistent with earlier findings comparing limbic seizures with generalized motor convulsions (Chretien et al. 1987). In that study, the results indicated that limbic seizures evoked immunosuppression that was comparable to cyclophosphamide. The immunosuppression was also significantly more than that evoked by metrazol. The limbic seizures also caused brain damage to amygdaloid, entorhinal, and ultimately hippocampal structures (Persinger, Makarec, & Bradley, 1987).

Despite the absence of statistically significant difference between the cyclophosphamide and metrazol group on day 4 in this study (this problem will be addressed later in the discussion), the pattern of results were consistent in the Chretien et al. (1987) study, and this thesis. The values of the cyclophosphamide groups were lower in comparison to the metrazol groups on the day of the measured immunosuppression, yet the rebound effect on day 10 was greater for the cyclophosphamide group. An explanation for this effect is not clear, but it does suggest that mild suppression might be more prolonged than severe immunosuppression (such as that produced by limbic seizures and cyclophosphamide). Alternatively, a greater rebound effect may be expected when a greater disturbance (i.e. immunosuppression) occurs. This effect is likely due to a greater adjustment on the homeostatic mechanism within this immunological neuroendocrine circuit.

The rebound effect was seen on the 10th day when all animals showed similar HSA antibody levels by this time, despite displaying reduced antibody levels on day 4. This suggested that the IS may possess a certain resiliency. The final outcome (10th day) suggests suppression of the

immune response but no disruption was present. Otherwise values obtained on the 10th day would have remained at the 4th day levels. The occurrence of a rebound effect by the suppressed groups supports the view of Besedovsky et al. (1985) that intrinsic "set points" are maintained by operating immunological-neuroendocrine circuits.

The expected sex difference in the amount of antibody formation produced was evident on the 10th day. The differential response is a common observation (Falter, personal communication, 1987) with females producing a greater amount of antibodies than males. This difference was not observed on baseline, day 2 or day 4 measures. This lack of significant difference on the baseline measure may be due to a smaller range of values seen in the primary immune response in comparison to the secondary immune response. The greater magnitude of the secondary response would facilitate the detection of statistically significant differences.

The lack of statistically significant differences on day 2 and day 4 between the sexes may be due to immunosuppression. The values of the ABC measures on these days do not follow the normal increment expected if suppression were not present. Furthermore, the day 2 measure was taken fairly early after the HSA booster. This implies that a marginal increase in antibody production would be present because lymphocyte proliferation is at its' earliest stage. Despite lacking statistical significance on baseline, day 2, and day 4 measures, all values obtained for females were higher than those of the males, and this difference was strongly expressed (statistically significant) by the 10th day.

There was another sex difference that was also expected but was not clearly expressed. Usually females have a greater number of non-responders than do the males. In this study, the numbers supported that idea with the females having 12 non-responders and the males having only 9. Nevertheless the difference was not statistically significant. This lack of significant difference may simply be an artifact. This point will be addressed later on in the discussion.

Covariates for bodyweight, and the litter differences were examined to determine if they had any non-specific effects on the results. Bodyweight fluctuations were considered because severe bodyweight loss can influence the general health of the animals and subsequently affect the immune response. Litter mate effects were considered because the genetic differences of one

litter of animals may differ significantly from another, therefore causing differential responses to experimental treatments (Borysenko & Borysenko, 1982).

Statistical analysis showed no significant changes in bodyweight fluctuations over the course of the experiment for all animals. This ruled out bodyweight as a contributing factor. The litter from which the animals were drawn did show up as significant covariate on baseline, day 2, and day 10, but not on day 4 ABC measures. Despite being a significant covariate, this factor did not influence the final results of the Anova procedure for groups, since differences were neither increased or reduced on any of the four ABC measures. It did however reduce the differences slightly for the sex factor (refer to Table 2). This finding suggests a differential response of the sexes may be related to litter differences.

Alternatively, the inconsistency of sex differences may be due to the injection procedure used in administering the HSA. The lack of the use of an adjuvant in this case could have resulted in a wide variation in both the magnitude and frequency of response. Persinger & Falter (personal communication, Spring, 1988) report that the use of an adjuvant in conjunction with HSA results in a greater magnitude of response (antibody formation) and an increase in the frequency of responders in comparison to animals (rats) injected with HSA suspended in normal saline (such as in this study).

The use of an adjuvant results in a more uniform pattern of response amongst the animals and a subsequent reduction in the heterogeneity of variance. The adjuvant presumably primes the immune system by stimulating macrophages and possibly preventing the HSA from dissipating into the microenvironments. An adjuvant was not used in this study because this author was not aware of this effect. Such an oversight may have accounted for the extreme heterogeneity of variance observed, and could possibly explain the peculiarities seen in the sex differences. The differences being a lack of expression in the magnitude of the primary response, and the lack of significant difference in the number of non-responders and responders amongst male and female subjects.

Despite being a common observation, the exact mechanism accounting for the differential response of males and females to immunization has not been demonstrated. Nevertheless sex differences related to hormonal milieux may contribute to this phenomenon.

For instance, sex differences in the metabolism of corticosterone C^{14} have been demonstrated to be double the rate in female plasma in comparison to males (Fischer rats). The liver is primarily responsible for the disappearance of corticosterone from the plasma due to steroid hydrogenase (Glenister, and Yates, 1961). Differences in the rate of metabolism between the sexes has been shown to result from differences in the activity of this enzyme system and not from differences in blood flow. Since high levels of corticosteroids are known to be immunosuppressive, the quicker clearance rate seen by females might account for their greater magnitude of response. Further studies elucidating the source of male-female differences in antibody levels are required.

Because of the multitude of actions and interactions occurring at the level of the CNS after a metrazol-induced seizure, a plausible mechanism of action for the observed immunosuppression is not easily postulated. Nevertheless, speculations can be made based on one's assumptions and supported by information in the literature.

In the present study the main assumption made was that the induced seizure is a stressful event capable of suppressing antibody formation. Secondly, this suppression may have been the result of reduced GABA functioning and a depletion of central catecholamines associated with seizure activity. The reduced inhibitory influences of these neural systems on the production and release of CRF could ultimately account for the observed immunosuppression.

The evidence in support of the first assumption comes from the measurement of two endpoints. These being, seizure activity at the behavioral end, and the measurement of ABC's of rat serum at the biological end. Support for the second assumption is speculative, and is the result of synthesized information about the role GABA plays in epilepsy, neuroendocrine control and, the IS (Racine, 1972; DeFeudis, 1984).

It is possible that two other mechanisms of action may have accounted for the metrazol induced immunosuppression. On the one hand, it is reasonable to suggest that metrazol mediated

its effect via the same mechanism as that of cyclophosphamide. This suggestion was considered because of the similar time course of immunosuppression followed by both experimental groups. Furthermore the degree of immunosuppression was not significantly different for both groups. Alternatively, it is possible that the effects of the metrazol were simply non-specific side effects that were occurring outside of the CNS.

Both of these possibilities can be controverted quite strongly on the basis of their pharmacodynamics. In the first instance, it is known that cyclophosphamide mediates its' pharmacological actions at the cellular level of the IS. It is also known that metrazol exerts its' action primarily at the level of the CNS (Lewin & Esplin, 1961). This suggests that the time element is irrelevant of the mechanism of action, since both of these drugs are acting at different sites.

The second possibility is also easily defeated. It is known that metrazol is rapidly and equally distributed throughout the tissues, and that it is also rapidly inactivated in the body (Epslin & Woodburg, 1956). By virtue of this rapid metabolism, it highly unlikely that any non-specific side effects of this drug could be influencing the week long metabolic processes required to form antibodies.

More likely, the disturbance of the IS was likely due to the subversion of CNS functioning caused by metrazol. The acute stress caused by seizuring is in itself likely to elicit an IS reaction via the HPA and/or the ANS.

The results from the present study suggest a tentative relationship between generalized motor convulsions and immunosuppression exists. The results also suggest that the degree of immunosuppression is related to the severity of the seizure experienced by the animal. This point was supported by the correlation of seizure severity and ABC measures in this study, and by comparing ABC measures between limbic seizures and generalized motor seizures in a previous study (Chretien et al. 1987).

Despite these findings, the results have raised several questions which should be answered in subsequent studies. One of these questions is whether or not the use of an adjuvant would significantly alter the immune response of the SHS line of rats? If so, could an improved

uniformity in response account for the reduced expression of sex differences seen in this study? Finally, do genetic differences affect the results significantly?

A comparison of both studies suggest they do. As stated earlier (Chretien et al. 1987), showed that the immunosuppression experienced by the metrazol group was significantly less than both cyclophosphamide and limbic seizure groups which did not differ from each other. However in this study the immunosuppression experienced by the metrazol group was comparable to that of the cyclophosphamide group. Despite using heterogeneous strains in both studies, it appears that the reaction from the Wistar rats obtained from a breeder in Quebec differed from the SHS line bred at Lakehead University.

Further studies of this phenomenon should focus on answering these questions. Subsequent studies should explore the mechanism of action underlying these findings. In the meantime it would be premature to generalize these results to humans. Rats are a steroid sensitive species (Claman, 1975), and would therefore react strongly to any changes of corticosteroid activity induced by generalized motor seizures. Conversely, humans are a steroid resistant species, therefore they would likely be minimally affected by elevations of corticosteroids.

Nonetheless, the suggestion that epilepsy and the GABA system are involved in the modulation of immunity is a new finding, and it provides a model of study for those interested in studying both phenomena simultaneously.

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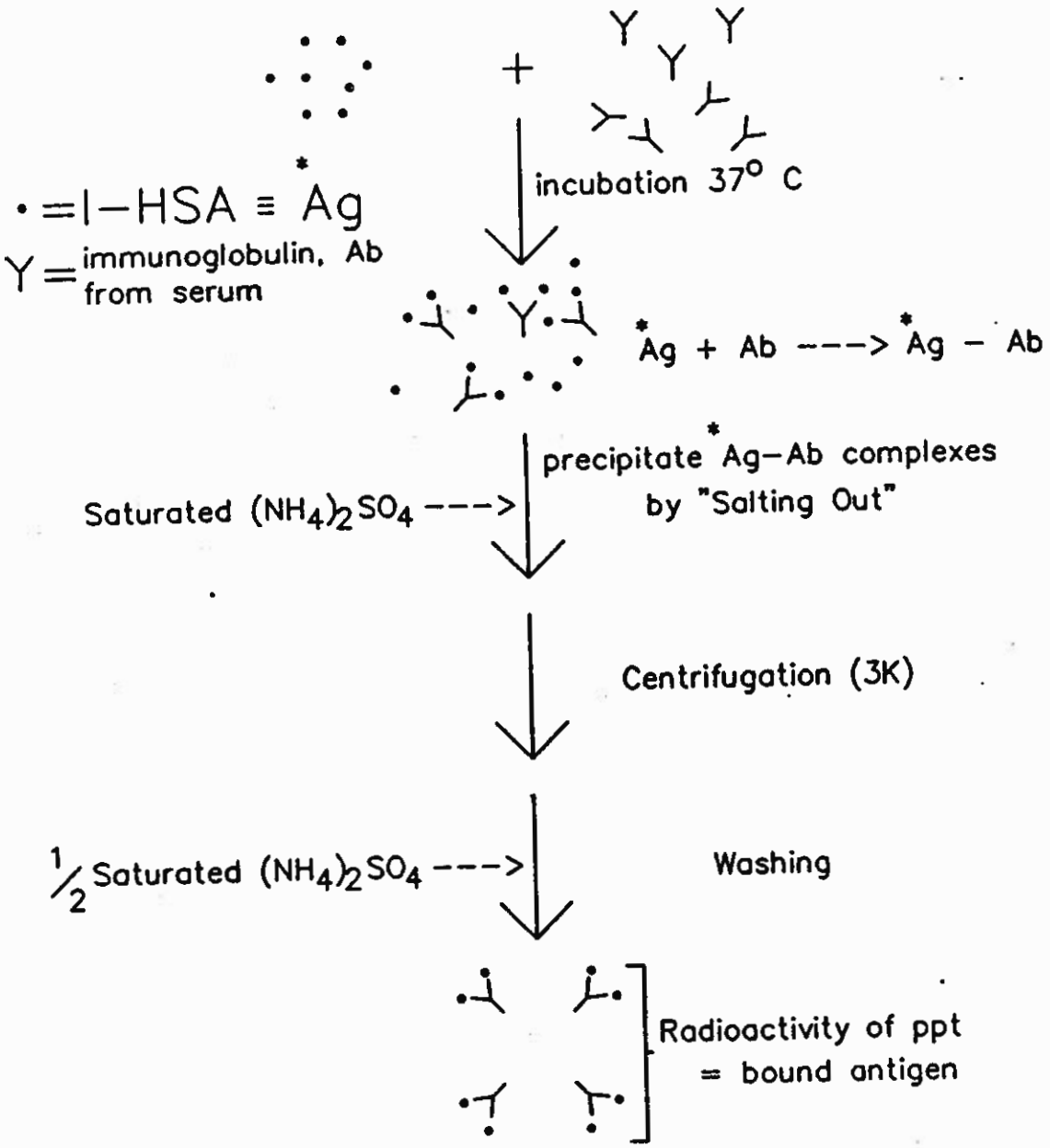
Abbreviations

ABC	Antigen Binding Capacity
Ach	Acetycholine
ACTH	Adrenocorticotropin Hormone
Anova	Analysis Of Variance
ANS	Autonomic Nervous System
BSA	Bovine Serum Albumin
CNS	Central Nervous System
CTL	Control
CRF	Corticotropin Releasing Factor
GABA	Gamma-aminobutyric acid
GH	Growth Hormone
GIF	Glucocorticoid-increasing factor
HPA	Hypothalamic-pituitary-axis
HRBC	Horse Red Blood Cells
5-HT	Serotonin
HSA	Human Serum Albumin
*HSA	(Iodine I^{125}) labelled HSA
IS	Immune System
Manova	Multiple Analysis Of Variance
NE	Norepinephrine
NK	Natural Killer Cells
NS	Nervous System
6-OHDA	6-Hydroxydopamine
PBS	Phosphate Buffer Saline
SAS	Saturated Ammonium Sulfate
SAS/2	50% Saturated Ammonium Sulfate

SHS	Satinder's Heterogenous Strain
SIC	Saline Injection Control
SRBC	Sheep Red Blood Cells
TNP-hae	Trinitrophenylhemocyanin
UC	Undisturbed Control

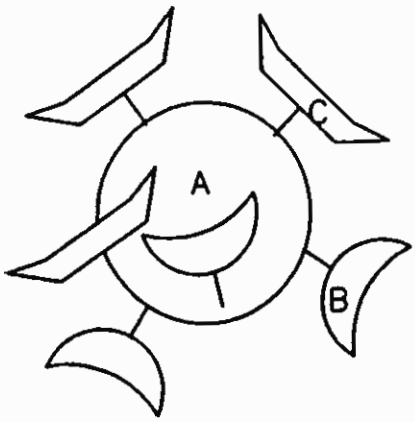
Appendix A

RIA MECHANISM



Appendix B

SCHEMATIC RADIOIODINATION



A) ENZYMOBEAD

B) LACTOPEROXIDASE

C) GLUCOSE OXIDASE

GLUCOSE

H_2O, O_2

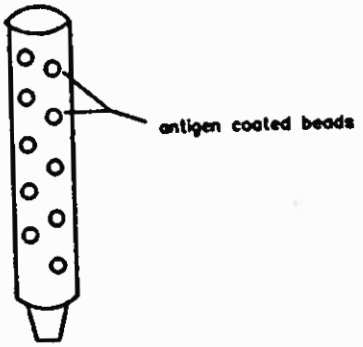
HYDROGEN PEROXIDE

$^{125}I^-$

$^{125}I_2$

PROTEIN

RADIOIODINATED PROTEIN



↓

fraction collector

↓

γ– count

Appendix C

Means (M) and Standard Deviations (SD) Of The Antigen Binding Capacity Of Rat Serum On Baseline (BL), Day Two (D2), Day Four (D4), And Day Ten (D10) Measurements For Saline-injection Control (SIC) Vs. Undisturbed Control (UC)

Sex	Treatment	Measure	BL	D2	D4	D10
F	UC	N	5	5	5	5
		M	0.40	1.02	7.60	22.42
		SD	0.25	0.96	5.53	14.20
F	SIC	N	6	6	6	6
		M	0.88	1.89	10.56	23.33
		SD	0.60	1.50	9.85	22.00
M	UC	N	5	5	5	5
		M	0.50	1.06	5.70	12.43
		SD	0.74	1.60	8.20	15.96
M	SIC	N	5	5	5	5
		M	0.56	0.96	5.88	13.98
		SD	0.64	1.13	5.70	11.95
Entire Sample		N	21	21	21	21
		M	0.60	1.27	7.60	18.30
		SD	0.58	1.30	7.40	16.55

N = Number

Treatment: Undisturbed Control (UC), Saline-injected Control (SIC)

Sex: Female (F), Male (M)

Appendix D

Means (M) And Standard Deviations (SD) Of The Antigen Binding Capacity Of The Rat Serum On Baseline (BL), Day Two (D2), Day Four (D4), And Day Ten (D10) Of Measures for Control, Metrazol, And Cyclophosphamide Groups

Sex	Treatment	Measure	BL	D2	D4	D10
F	CTL	N	11	11	11	11
		M	0.66	1.50	9.09	22.92
		SD	0.51	1.33	8.08	18.41
M	CTL	N	10	10	10	10
		M	0.53	1.01	5.76	13.21
		SD	0.65	1.29	6.67	13.32
F	MTZ	N	9	9	9	9
		M	0.92	1.29	2.68	22.93
		SD	1.23	1.87	2.53	19.64
M	MTZ	N	10	10	10	10
		M	0.40	0.51	1.75	11.48
		SD	0.45	0.74	2.01	11.09
F	CY	N	9	9	9	9
		M	0.50	0.70	1.00	26.58
		SD	0.38	0.17	0.84	11.55
M	CY	N	9	9	9	9
		M	0.26	0.33	0.32	15.15
		SD	0.20	0.35	0.23	10.29
Entire Sample		N	58	58	58	58
		M	0.55	0.90	3.64	18.64
		SD	0.65	1.18	5.50	15.07

N = Number

Treatment: Control (CTL), Metrazol (MTZ), Cyclophosphamide (CY)

Sex: Female (F), Male (M)

Appendix E

List of Suppliers

Materials Purchased:

Sodium Iodide (^{125}I) :

Carrier free NaI* with a specific activity of 17 Ci/mg (at a 100% isotopic enrichment), having a half-life of 60.2 days, was purchased from ICN Radiochemicals, Division of ICN Biochemicals, P.O. Box 19536.

Enzymobead Radioiodination Reagent:

It was purchased from Bio-Rad Laboratories, 32nd and Griffin Avenue, Richmond, California, 98408, U.S.A..

Sephadex G-25 Superfine Resin:

This was purchased from Pharmacia, 2044 St. Regis Boulevard, Dorval, Quebec.

Human Serum Albumin:

This was purchased from Sigma Company. P.O. Box 14508, St. Louis, Missouri, 63178, U.S.A..

Monobasic Phosphate, Phenol (C_6H_5CH), α-D-Glucose, Sodium Chloride (NaCl), Ammonium Sulphate ($(NH_4)_2SO_4$), Sodium Hydroxide Monohydrate Superpure ($NaOH.H_2O$):

These were all purchased from BHD Chemicals, 350 Evans Ave., Toronto, Ont. M8Z 1K5.

Hypodermic 26G needles, Serum Seperators and Heparanized Hematocrit Capillaries:

These were purchased from Fischre Scientific, 111 Scotia Court, Whitby, Ontario.

Polypropylene RIA Tubes:

These were purchased from Terochem Laboratories Ltd., Box 8118, Edmonton Alberta, T6H 4PI.

Metrazol and Cyclophosphamide:

These were purchased from Sigma Company. P.O. Box 14508, St. Louis, Missouri, 63178, U.S.A..