CONDITIONAL IMMUNOMODULATION

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CONDITIONAL IMMUNOMODULATION

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ABSTRACT

Interest in central nervous-immune system interactions was inspired largely by the work on conditional immunomodulation (Ader and Cohen, 1975, 1985). Ader and Cohen (1975) reported that rats trained such that a taste cue (saccharin, SAC) predicted an injection of an immunosuppressant (cyclophosphamide, CY) subsequently exhibited a conditional immunosuppression in response to SAC alone.

Experiments 1-3 were designed to evaluate reports of conditional immunosuppression after training with SAC and CY. Rats received either one or three training sessions in which SAC presentation was followed by CY injection. Upon reexposure to the SAC, antibody formation to sheep red blood cells was assessed. Rats which were trained with SAC and CY had antibody titers that did not differ from saline controls, while rats which SAC and CY in a nonpredictive manner had suppressed antibody titers.

Experiments 4 and 5 assessed the effectiveness of environmental and drug state cues in signalling CY injections. In Experiments 4 and 5, the effect of training was to attenuate the unconditional immunosuppression produced by the CY.

Experiments 6 and 7 further assessed conditioning with pentobarbital predicting CY. After conditioning, some

animals were rested until the residual effects of the CY had dissipated. When reexposed to the pentobarbital after this period, these animals had antibody titers that were significantly elevated above those of animals that never received the CY. Other animals received seven pentobarbital injections during the rest period. These animals demonstrated extinction of the conditioned response.

Experiment 8 assessed the effectiveness of conditioning when the unconditional stimulus was antigenic rather than pharmacologic. After sensitization, rats received antigen injections paired with an environmental cue. Upon reexposure to the cue, trained rats had increased levels of rat mast cell protease II in their serum. As this protease is found only in mucocsal mast cells, these results suggest that mucosal mast cells may be activated by a cue for antigen.

In a final experiment, physiological correlates of conditional mast cell activity were examined. Ussing chambers were employed to assess responsivity of intestinal epithelium after exposure to a cue for antigen. Results indicated that sensitivity to <u>in vitro</u> antigen was altered in rats exposed to a cue for antigen. These results, and those of the previous studies, support the hypothesis that the nervous and immune systems communicate, and that this communication may be functionally important.

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INTRODUCTION

"Il est impossible d'admettre que les phenomenes si compliques que present - l'immunite, puissent s'effectuer dans l'organisme sans l'intervention du systeme nerveux...."

Metal'nikov (1934)

Role de Systeme Nerveux et des Facteurs Biologiques et Psychiques dans L'Immunite.

"In all types of immune or allergic reactions an individual acquires specific information (learns) from contact with an antigen without the mediation of the nervous system...."

Sell, (1975)

Immunology, Immunopathology and Immunity.

Psychoneuroimmunology is an emerging discipline concerned with understanding the ways in which the central nervous system (CNS) and immune system (IS) communicate and influence each other. As the name implies,

psychoneuroimmunology combines knowledge from psychology, neuroscience, and immunology and attempts to integrate these disciplines which have previously been largely distinct. This task is often difficult, as the language and concerns of workers within each discipline may be very different. The task is important, however, as it is becoming obvious that it may be impossible to develop a comprehensive understanding of either the immune or central nervous system without regard for the other system. The knowledge gained from an integrated study of these two systems, while providing a new way to conceive of the CNS and IS, may also lead to new ways in thinking about a variety of diseases that have both CNS and IS involvement.

Some of the areas that have contributed information about the ways in which the immune and nervous systems influence each other include neuroanatomy, electrophysiology, neurochemistry, psychosomatic medicine, and animal learning. The following chapter will describe briefly the accumulated evidence obtained by researchers who have examined CNS-IS interactions from a variety of these perspectives. Work from the area of animal learning and classical conditioning will be discussed in greater detail in a subsequent chapter, as the experiments conducted for this thesis were derived from previous work in classical conditioning.

CHAPTER 1: Evidence for Central Nervous and Immune System Communication

This chapter briefly describes work from a number of areas which have contributed to the development of psychoneuroimmunology as a distinct discipline. The purpose of the chapter is primarily to provide a summary of the work which has led to the belief that nervous-immune interactions exist, and that they are functionally important.

1.1: Evidence for CNS-IS communication: Common origins

Besedovsky and Sorkin (1981) have argued that the concordance between the development of the immune and neuroendocrine systems during the development of various mammalian systems "can hardly be fortuitous". It may or may not be reasonable to assume that the central nervous and immune systems are interdependent during development, as it would hardly be surprising if precocial young with highly developed neural responses also exhibited well-developed immune responses, while altricial young which have a variety of partially developed systems also had immune and nervous systems that were not yet complete. The finding that immune-associated tissues, such as the thymus,

may contain neurally derived tissue (Le Douarin, 1974) also provides little support for more than an anatomical association between these tissues.

Reports that the CNS and IS depend on each other for normal development provide a somewhat more convincing argument that the immune and nervous system have a functional interaction. Besedovsky and Sorkin described several developmental disturbances that they believe support the notion of CNS-IS interaction during ontogeny. They described the hypopituitary dwarf mouse which shows defective cell-mediated immunity along with a deficit in growth hormone and thyrotropin. Not surprisingly, congenitally thymusless mice show deficiencies in T-cells and also altered neuroendocrine responses, while bursectomized chicken embryos also show immune and neuroendocrine alterations. These results do little more than illustrate that alterations in immune function sometimes correspond with altered neuroendocrine function. Besedovsky and Sorkin (1977) have shown development under germ-free conditions results not only in marked underdevelopment of the total mass of lymphoid tissue, but also in reduced noradrenaline content. Since it is less obvious why germ free conditions should act directly upon the development of the CNS, the finding that CNS parameters are altered after development in germ free environments

suggests involvement of the immune system in nervous system development. The Besedovsky and Sorkin (1977) report illustrates the sort of evidence necessary to suggest more than an anatomical association of the nervous and immune system during development. Generally, then, there is some evidence to suggest ontogenic interdependence between the CNS and IS, but this evidence must be scrutinized with the awareness that the co-occurrence of events is a long way from the inference of causality.

1.2: Evidence for CNS-IS communication: Innervation

Anatomical studies have provided evidence that the nervous and immune systems communicate. Studies have determined that all of the major lymphoid organs are innervated (for reviews see Bulloch, 1985, 1987).

From late embryonic life, bone marrow is the source of stem cells which will differentiate to become immune cells. The bone marrow is innervated by nerves arising at the level of the spinal cord where the bone is located. Nerves accompany arteries through the nutrient foramen into the center of the bone marrow, and there is substantial innervation of the sinusoidal parts and parenchymal elements where hematopoiesis occurs. Developmentally, myelination of these nerves is completed just prior to the onset of hematopoiesis, suggesting that these functional

nerves may influence the development of stem cells. It thus appears possible that nerves may influence immune cells at the earliest stages of immune cell production.

Anatomical studies of the mouse have shown that the thymus, the critical organ for T-cell differentiation, is innervated by vagal nerves, superior cervical and stellate ganglia of the cervical and thoracic sympathetic chain, and the recurrent laryngeal nerves (Bulloch, 1985). Further studies in rats and chicks have shown similar patterns of innervation (Bulloch, 1987). Thus the potential exists for nerves to influence T-cells during critical periods of T-cell education.

A secondary lymphoid organ, the spleen, is also innervated. The majority of nerves travel into the parenchyma and terminate in the white pulp, the region in which T and B cells are distributed. The lymph nodes, appendix, gut-associated lymphoid tissue, and Peyer's patches show similar patterns of innervation, with lymphocytes having close and unrestricted access to the terminals and synaptic vesicles of the nerves. Felten, Felten, Carlson, Olschowka and Livnat (1985) have shown the presence of noradrenergic and possible presence of peptidergic nerve fibres in all of these tissues. Thus a variety of sites may serve as junctions for nerve-immune communication.

In fact, Ottaway (1984) has suggested that a primary means of neurophysiological influence of the immune system might be by regulation of the migratory patterns of lymphocytes through lymphoid tissues. Vasoactive intestinal peptide (VIP) is found in abundance in much of the enteric nervous system (from which many of the nerves that innervate lymphoid tissues arise). Ottaway has reported that changing the expression of VIP receptors on T-cells can alter the migratory patterns of the T-cells through the Peyer's patches and mesenteric lymph nodes. He has proposed that the presence of VIP receptors contributes to the way in which T-cells interact with the specialized high endothelium of the postcapillary venules of these tissues. More recently he reported the presence of VIP containing nerves in Peyer's patches, providing an anatomical basis for the notion that VIP may influence the migration of lymphocytes from blood into Peyer's patches (Ottaway, Lewis & Asa, in press). The studies by Ottaway are some of the first attempts to go beyond a simple anatomical description of the presence of nerves in lymphoid tissues in search of functional roles for these nerves.

1.3: Evidence for CNS-IS communication: Electrophysiology

Stein, Schiavi and Camerino (1976; see also Korneva, 1987) have reviewed early electrophysiological studies that found evidence for neuroimmune interactions. Following the typical pattern of early electrophysiologists, many lesion studies were performed, in which alterations in immune functions were examined after ablations of various parts of the brain. Freedman and Fenichel (1958) reported that midbrain lesions in the guinea pig retarded death by anaphylaxis. More specifically, Szentivanyi and Filipp (1958) demonstrated that bilateral focal lesions in the tuberal regions of the hypothalamus prevented lethal anaphylactic shock in the guinea pig and rabbit. Szentivanyi and Szeleky (1958) reported that lesions in the same region also protected the animals against lethal histamine shock (suggesting that changes in antibody production could not fully account for the phenomenon). Lupareilo, Stein and Park (1964) found that anterior, but not posterior lesions of the hypothalamus could inhibit anaphylaxis in the rat. From similar studies, Stein et al. (1976) reported that guinea pigs with anterior hypothalamic lesions did not respond to low dose ovalbumin administration after previous sensitization. They did respond to injections of 1.5 mg of ovalbumin, but the mortality rate in lesioned animals was thirty six percent,

compared with seventy three percent in sham operated controls. The unfortunate problem with many of these studies is that they did not control for the possibility that the primary effect of the lesions was not on immune function but on the ability of target tissues to exhibit typical responses to immune mediators. That is, if the lesions had a direct effect of smooth muscle responsiveness, for example, then the intense bronchospasm that appears to be the immediate cause of death in many animals might well be reduced. In fact, more recent studies provided support for the possibility that lesions may produce direct or indirect effects on target tissues that are distinct from immune-mediated events. Van Oosterhout and Nijkamp (1987) lesioned the anterior hypothalamus and sensitized guinea pigs to egg albumin. They then challenged the animal and studied the reactivity of the trachea. They concluded that the protection from anaphylaxis observed after lesioning was due in part to reduced reactivity of tracheal smooth muscle, although they do not postulate a mechanism by which this reactivity is reduced. Thus while early studies were suggestive, they must be interpreted with the realization that hypothalamic lesions may result in a variety of systemic changes which may or may not involve the immune system. This is not to say that studying the involvement of brain structures in

anaphylaxis is not useful, but that other factors must also be considered when examining this hypothalamus-anaphylaxis relationship.

Other investigators have considered the effects of lesions on levels of circulating antibodies. Korneva and Khai (1964) reported that lesions of the posterior ventral hypothalamus in rabbits suppressed the production of complement-fixing antibodies, while lesions in other parts of the hypothalamus, the thalamic structures, the caudate nucleus and the posterior commissure did not alter the immune process. More recently, studies have looked in the opposite direction for relations between the brain and antibody production. Besedovsky, Del Rey, Sorkin, da Prada, Burri and Honegger (1983) have shown a decreased noradrenaline turnover in the hypothalami of rats at the time of peak antibody response to sheep red blood cells. Besedovsky et al. (1983) postulated that the effect was due to soluble mediators released from activated immune cells because the administration of supernatants from stimulated cells mimicked the events that occurred after <u>in vivo</u> immunization. They also argued that the decreased turnover was a result of increased firing rates in the ventromedial hypothalamus. This argument is based on previous studies (Besedovsky, Sorkin, Felix and Haas, 1977, see also Besedovsky and Sorkin, 1981) which showed alterations in

the activity of ventromedial hypothalamic nuclei which corresponded to increases in antibody titer after immunization with sheep red blood cells. Besedovsky and Sorkin (1981) proposed that lymphokines were responsible for mediating communication between the immune cells and the brain, but they have not yet isolated specific mediators involved in this process.

Saphier, Abramsky, Mor and Ovadia (1987) have recently shown increases of up to 300 % in multiunit electrical activity in the preoptic / anterior hypothalamus on the fifth day after immunization of SRBCs to rats. Saphier et al. also suggested that the increases are modulated by a humoral factor; their candidate of choice was adrenocorticotropic hormone, although they acknowledged that any of a variety of lymphokines might provide information to the neurons of the target area. Sharma, Thakur, Maurya, Garg and Singh (1987) also recently showed increases in hypothalamic firing rates after sensitization with Prosopis juliflora antigen. Sharma et al. showed corresponding alterations in acetylcholinesterase activity in the hypothalamus, and they suggested that a cholinergic transmitter might be involved in the neuroimmune response.

Abramsky, Wertman, Reches, Brenner and Ovadia (1987) studied the effects of anterior hypothalamic lesions on the development of experimental allergic encephalitis (EAE) in

Lewis rats. They found that the development of EAE was prevented after electrolytic destruction of the anterior hypothalamus, and inhibited after depletion of dopamine and norepinephrine by injections of 6-hydroxydopamine and reserpine. They suggested that these neurotransmitters might be involved in mediating the neuroimmune response.

Based on the studies reviewed above, it appears that electrophysiological studies, while not without complications, may contribute to an understanding of the factors involved in CNS-IS communication pathways. As has become obvious, there is good evidence that areas of the hypothalamus are sensitive to, and perhaps important in immune responses, but the neurochemical messengers that allow for hypothalamus - immune system integration remain speculative. As the next section will discuss, the pool of potential nerve-immune communicator molecules is increasing, but the precise functional role of any of the potential messengers remains to be determined.

1.4: Evidence for CNS-IS communication: Chemical messengers

Roth and colleagues (Roth, LeRoith, Shiloach & Rubinovitz, 1983) have suggested that intercellular communicators began evolutionarily and embryonically as local tissue factors acting on surrounding cells. The change from local tissue factors to "hormones",

"neurotransmitters" and "lymphokines" may represent a change in the evolution of increasingly complex series of target cells rather than in changes in communicatory molecules. That is, in advanced organisms it is the anatomical relationship between the secretory cell and its target which has changed through evolution.

Viewed from this perspective, the notion that the nervous and immune system may share common messengers is not surprising. As research progresses, it becomes increasingly apparent that the majority of chemicals involved in neural communication can exert either a direct or indirect effect on the immune system. These include the endorphins, the enkephalins, adrenocorticotropin, cortisone and other steroids, substance P, vasoactive intestinal peptide, somatostatin and others (see Table 1.1). Similarly, lymphocytes and other immune cells are known to synthesize a variety of factors with neuroendocrine effects (see Table 1.2), including the thymosins, serotonin, adrenocorticotropin, gamma-endorphin, thyroid-stimulating hormone, and human chorionic gonadotropin-like peptide.

In addition to direct effects of chemical messengers on the nervous and immune systems, the chemical messengers from these two systems may act indirectly to influence each other. For example, nervous system events which influence hormones such as estrogens, progestins and androgens may

Table 1.1: Immunotransmitters with Effects on the Central Nervous System

Immunotransmitter	CNS Activity (Reference)
Interleukin-1	increased ACTH and cortisol (Woloski et al., 1985) increased thermostatic setpoint (Sztein et al., 1981) Binds opiod receptors (Morley et al., 1987) Induces slow wave sleep (Krueger et al., 1983)
Interleukin-2	increased ACTH and cortisol (Westley et al., 1987)
Alpha-interferon	Analgesia (Blalock & Stanton, 1980) Catatonia (Blalock & Stanton, 1987) Lethargy (Abrams et al., 1985) Depression (Abrams et al., 1985) Anorexia (Abrams et al., 1985) increased slow wave sleep (Adams et al., 1984) decreased REM latency (Adams et al., 1984) altered cognition (Adams et al., 1984) altered affect & personality (Adams et al., 1984) decreased opiate withdrawal (Reyes-Vasques et al. 1984)
Endorphins	corticotropin-like activity (Smith et. al, 1987)
Endogenous pyrogen	increased thermostatic setpoint (Murphy et al., 1980))
C3A	Anorexia (Morley et al., 1987)

Table 1.1: Continued

Immunotransmitter	CNS Activity (Reference)
Lymphokines	decreased hypothalamic norepinephrine & Stimulated glial cells (Fontana et al., 1980)
Thymosin F3	Binds opiod receptors (Spangelo et al., 1987)
Thymosin Beta 4	increased LH and testosterone (Spangelo et al., 1987)
Muramyl peptides	increased high amplitude slow wave sleep (Krueger & Karnovsky, 1987)
Cyclosporin	decreased opiate withdrawal syndrome (Dafny et al., 1985) Analgesia (Dafny et al., 1985)
Tuftsin	Activiates hypothalamic-pituitary-thyroid axis (Morley et al., 1987)
Neuroleukin	Promotes survival of motor and sensory neurons (Gurney et al., 1986)
Lymphocyte-derived factor	Maintains neuronal viability (Gozes et al., 1983)
Nerve growth factor	Maintains neuronal viability (Thorpe et al., 1987)
Glucocorticoid releasing factor	Cortisol production by adrenal cortex (Besedovsky et al., 1981)

Table 1.2: Neurotransmitters with Effects on Immune Cells

Neurotransmitter	(Reference)
Vasoactive Intestinal Peptide	(Ottaway & Greenberg, 1984)
Somatostatin	(Stanisz et al., 1986)
Substance P	(Payan et al., 1984) (Stanisz et al., 1986)
Adrenocorticotropin	(Johnson et al., 1982) (Westley et al., 1987)
Alpha-endorphin	(Johnson et al., 1982)
Beta-endorphin	(Van Epps & Saland, 1984)
Enkephalins	(Faith et al, 1984) (Plotnikoff et al., 1985)
Cortisone	(Selye, 1950)
Prostaglandins	(Ahlqvist, 1981)
Serotonin	(Devoino et al., 1975) (Boranic et al., 1987)

influence immunological activity by altering the efficacy of the thymus (see Ahlqvist, 1981). Understanding the role of various messengers is also complicated by the fact that many chemical communicators can act locally as neuro/immuno transmitters or humorally as hormones depending on sites of release and action.

Studies have shown the presence of receptors for various chemicals on both immune and nerve cells. Studies have also demonstrated that viability, proliferation and function of both immune and nerve cells can be affected by the actions of chemical communicators from both systems. However, much research remains to be done before the functional significance of physiological levels of the messengers of interest are described. As indicated above, chemical messengers may act through so many feedforward and/or feedback circuits before reaching the cell of interest that the potential for interactions between various cells and substances is daunting to contemplate. Perhaps the only simple fact in a discussion of nervous-immune chemical messengers is that the once widespread belief in the autonomy of the immune system is now untenable.

1.5: Evidence for CNS-IS communication: Stress

Studies of the influence of "psychological" stressors have been cited in support of a CNS-IS association. Many studies have been performed, and a variety of species, stressors and immunological measures have been employed (see Table 1.3). It should be noted that the stressors employed in several studies might exert physiological effects distinct from the desired psychological stress effect. That is, stressors such as cold swim (Aarstad, Gaudernack & Seljelid, 1983) or wheel running (Hara & Ogawa, 1983) might exert immunologic effects that are as much the result of hypothermia or exercise as they are of the "psychological" effects of the stress. These studies are included because they involve stress and immunity, but interpretations of such studies must be taken with caution until the possibility of confounds from the properties of the stressor can be controlled. Other stressors, such as conditioned fear (Lysle, Cunnick, Fowler & Rabin, 1988) and sound (Monjan & Collector, 1977) appear to be more purely "psychological", and as such, better suited for analyses of the effects of psychological stressors on immunological functioning.

Without examining studies in detail, the general pattern of results suggests that acute or short-term exposure to a stressor results in reduced immunological

Table 1.3: Stress and Immunologic Function: Animal Studies

Reference	Species	Stressor	Immune response & effect
Hill et al (1967)	monkey	noise & light	Ab to BSA reduced
Solomon et al (1968)	mouse	preweanling handling	Ab to flagellar antigen reduced
Solomon (1969)	rat	overcrowding electric shock	Ab reduced (to flagellar Ab increased antigen)
Gisler et al (1971) Gisler (1974)	mouse	rotation restraint overcrowding	PFC to SRBC reduced
Joasco & McKenzie (1976)	mouse	overcrowding	Lymphocyte proliferation reduced
Edwards & Dean (1977)	mouse	overcrowding	Ab to typhoid antigen reduced titres
Monjan & Collector (1976)	mouse	auditory	T & B cell proliferation suppressed at 15 days: enhanced at 25 days
Monjan & Collector (1977)	mouse	high intensity sound	Lymphocyte proliferation & cytotoxicity reduced in short term, increased in long term
Schultz et al (1979)	mouse	restraint	Macrophage tumoricidal activity reduced
Pavlidis & Chirigos (1980)	mouse	restraint	Macrophage tumoricidal activity reduced
Dadhich et al (1980)	rat	restraint	Ab to typhoid antigen reduced
Reite et al (1981)	monkey	separation	Lymphocyte proliferation reduced

Table 1.3: Continued

Reference	Species	Stressor	Immune response & effect
Blecha et al (1982)	mouse	restraint	DTH to SRBC reduced Contact sensitivity increased
Laudenslager et al (1982)	monkey	separation	Lymphocyte proliferation reduced
Aarstad et al (1983)	mouse	cold swim	NK cell activity reduced
Boranic et al (1983)	rat	restraint	Phagocytosis & Ab dependant cytotoxicity reduced post 2 days stress; enhanced after 4
Hara & Ogawa (1983)	mouse	wheel running	Ab to SRBC reduced
Laudenslager et al (1983)	rat	escapable shock inescapable shock	Lymphocyte proliferate not reduced Lymphocyte proliferation reduced
Sato et al (1984)	mouse	conditioned fear	PFC to SRBC reduced
Shavit et al (1985)	rat	footshock	NK activity & survival after tumor decreased
Shavit et al (1986)	rat	footshock	NK specific cytotoxic activity reduced
Croiset et al (1987)	rat	passive avoidance	PFC to SRBC & lymphocyte proliferation reduced
Esterling & Rabin (1987)	mouse	rotation	PFC & Ab to SRBC reduced
Ghoneum et al (1987)	rat	isolation	NK cell activity reduced reduced only in aged rats
Irwin & Livnat (1987)	mouse (C57BL/6J) (DBA/2J)	footshock	NK specific cytotoxic activity reduced not reduced
Laudenslager et al (1987)	monkey	mother/infant separation	Primary Ab response reduced

Table 1.3: Continued

Reference	Species	Stressor	Immune response & effect
Kandil & Borysenko (1987)	mouse	rotation	NK cell activity reduced
Lysle et al (1987)	rat	signalled shock	Lymphocyte reactivity reduced
Pollock et al (1987)	mouse	surgery	NK cell cytotoxicity reduced
Lysle et al (1988)	rat	conditioned fear	Lymphocyte proliferation reduced

activity, while chronic or repeated exposure to stressors results in either an attenuation of the suppressive effects of stress or an actual enhancement of immunological response. The best support for this suggestion comes from studies in which the effects of a stressor at several time points was examined (Boranic, Gabrilovac, Manev, Pericic, Poljak-Blazi & Sverko, 1983; Monjan & Collector, 1977; see also Gorczynski, Macrae & Kennedy, 1984). Each of these studies report an initial suppression and a subsequent enhancement of immunological activity in response to the stressors. It is interesting to speculate that the initial suppression observed after exposure to a stressor represents a component of the "fight or flight" metabolic adjustment. That is, an animal preparing to deal with a potentially harmful stressor might channel resources towards immediate needs, leaving the immune system temporarily depleted of resources (energy/nutrients). Such an interpretation is limited by a lack of knowledge concerning the extent to which the immune system is subject to homeostatic adjustments of the organism as a whole.

The resultant enhancement after long term stress may represent a simple rebound effect or a more complex response. Interpreting the effects of long-term or repeated stressors is difficult because it is probably unreasonable to ignore the effects of learned responses at

this time. That is, acute exposure to a stressor implies minimal numbers of exposures and little time for the animal to learn much about factors associated with the stressor. Thus it might not be unreasonable to expect that the immunological alterations are, in fact, the result of a stressor and not what is learned about the stressor. On the other hand, long-term studies of the effects of stress often give repeated exposure to the stressor, with predictive contextual cues (being placed in a restraint apparatus, for example) reliably predicting the stressful stimulus itself. Thus it is difficult to determine whether the immunological alterations observed after repeated stressors are the result of repeated stressors alone or, more likely, confounded with the responses the animal has learned to make in anticipation of the stressor. If stressors have unconditional immunological effects, then presumably cues associated with the stressors may elicit conditional immunological effects. The enhancement observed after repeated or long-term stress may represent a conditional compensation for the suppressive effects of the stressor. Many "stress" studies, then, might also be interpreted as studies of conditional immunomodulation. Regardless of whether the effect of a psychological stressor is ultimately exerted via unconditional or

conditional responses, these studies do support the hypothesis that CNS events can result in IS alterations.

Recently, several studies of the effects of stress on immune function in humans have been reported (See Table 1.4). While these studies are correlational, and must take into account the variety of factors (nutrition, sleep, drug consumption) that might also correlate with both stress and immune function, they do suggest that acute stress and life changes leave people more susceptible to the onset of disease. Studies of the effect of psychosocial factors and immune function in humans will be considered in the next section.

1.6: Evidence for CNS-IS communication: Personality, emotion and disease

Psychosomatic medicine has been devoted to an elucidation of the various psychosocial components involved in, or correlated with, a variety of diseases (for reviews see Miller 1983; Rogers, Dubey & Reich, 1979; and Weiner, 1982). Of particular interest here are diseases with known or proposed immunological involvement. Included in these are infectious diseases (Levenson, Hamer, Hart, & Kaplowitz, 1987; Plaut & Friedman, 1981), neoplasias or cancers (Fox, 1981; Riley, Fitzmaurice & Spackman, 1981) and autoimmune and hypersensitivity diseases (Pearson,

Table 1.4: Stress and Immunologic Function: Human Studies

Reference	Stressor	Immune parameter
Bartrop, et. al. 1977	bereavement	Lymphocyte proliferation, T & B cell numbers
Dorian, et. al., 1982	examinations	Lymphocyte proliferation, T & B cell numbers
Schleifer et. al. 1983	bereavement	Lymphocyte proliferation T & B cell numbers
Arnetz, et. al., 1987	unemployment	Lymphocyte proliferation, Reactivity to tuberculin
Dorian, et. al, 1987	occupational (tax season for accountants)	NK cell activity, Helper to supressor T cell ratio
Halvorsen & Vassend 1987	examinations	Lymphocyte proliferation T & B cell numbers
Kiecolt-Glaser, et. al., 1987	caregivers of Alzheimer's patients	Helper to suppressor T cell ratio, Antibody levels to Epstein-Barr virus
Kiecolt-Glaser, et. al., 1987	marital quality and disruption	Lymphocyte proliferation, Antibody levels to Epstein-Barr virus

1988; Solomon, 1981). The diseases of most relevance to subsequent discussions (see Chapter 5) are hypersensity and autoimmune diseases. Asthma has long been thought to involve psychosocial factors (Falliers, 1969; Khan, 1973, 1974; MacKenzie, 1886, Moore, 1965), and treatment strategies for controlling asthma based on relaxation and biofeedback techniques have been developed (Creer, 1974; Harding & Maher, 1982). Although the accuracy and interpretation of some of these reports has been questioned (Pastorello, Codecasa, Pravettoni, Zara, Incorvaia, Froldi & Zanussi, 1987; Pearson, 1988) evidence to support the involvement of psychosocial factors in at least components of the onset and severity of asthma continues to accumulate (see Chapter 5).

Other researchers have examined the influence of psychologic factors on atopic diseases such as urticaria, psoriasis and alopecia (Hansen, 1981; Lyketsos, Psaras, & Lyketsos, 1985), chemical sensitivities (Schottenfeld, 1987) and food and drug allergies (Selner, Staudenmayer, Koepke, Harvey & Cristpoher, 1987). Schottenfeld (1987) has argued that cognitive processes typically play a role in magnifying the severity of such conditions regardless of the original aetiology of the disease, and that behavioral techniques are useful in alleviating symptoms associated with allergies.

Patients with autoimmune diseases have also been extensively studied for the presence of psychsocial correlates. Rheumatoid arthritics have been, and continue to be extensively studied (Koehler, 1987; Pow, 1987; Solomon & Moos, 1965) for the presence of distinguishing personality factors that correlate with presentation and progression of the disease. Patients with intestinal diseases, which may involve immune components, have also been favored for assessment (Salim, 1987a, 1987b) and factors such as personality type and ability to cope with stress, have been correlationally linked to peptic ulceration and other forms of gastrointestinal distress.

Many of these studies provide support for correlations between psychosocial factors and immunological over- and under-responsiveness; the number of personality factors that have in some way been associated with one disease or another is staggering. Solomon and Moos (1965) give a particularly effective account of early attempts to define the factors correlated with rheumatoid arthritis, which highlights the dangers of correlational, small-sample studies. More recent studies have improved their sampling and matching techniques, but they remain largely suggestive rather than conclusive.

The role of individual differences in studies of this sort must be great; based more on opinion than

evidence, I would suggest that on a case-by-case basis, determining the psychological stressors and factors that are associated with an individual's disease might be useful in controlling the disease and reducing the individual's distress. On a population basis, however, it is doubtful that a search for the psychosocial factor(s) resulting in or even strongly correlating with disease X, will be especially useful. Describing such a factor can only increase the probability of identifying potential sufferers of a specific disease; that is, it seems to be a largely descriptive rather than interventionist endeavor, and one that is unlikely to result in decreasing the numbers of sufferers of a specific disease. The best that is likely to be done is to decrease the suffering of the individual should the disease develop. This is not a trivial endeavor, but it requires different approaches than do population studies.

1.7: Summary

It has become obvious that psychoneuroimmunology finds support from a variety of previously distinct disciplines. Another discipline, animal learning, has also contributed to knowledge about influences of the CNS on the IS. Our work which will be described in later chapters relied on paradigms developed by researchers in classical

conditioning, and on studies of conditional immunomodulation; therefore, studies of conditional immunomodulation will be described in more detail than the studies mentioned above. The next chapter will examine historical and recent accounts of conditional immunomodulation.

CHAPTER 2: Studies of Conditional Immunomodulation

2.1: Early studies of conditional immunomodulation

Metal'nikov and Chorine, working at the Pasteur Institute in France, were the first researchers to formally examine the influence of classical conditioning on the immune system. Metal'nikov and Chorine conceived of the immune system as a series of highly complicated internal reflexes (1926), which might be influenced in ways similar to those described at that time by Pavlov in the Soviet Union (1923). They reasoned that immunological reflexes might also be subject to the influence of classical conditioning, and to test this hypothesis they performed a number of experiments. A typical experiment involved administering daily injections of an antigen (tapioca, B-anthracoids, staphylococcus filtrate, the unconditional stimulus or US) to a guinea pig. After administration of such antigens, increases in the numbers of immune cells were apparent in the peritoneal exudate (unconditional response, or UR). The antigen injections were paired with an immunologically neutral stimulus (tactile stimulation; conditional stimulus or CS). After 15 or 20 daily pairings, the guinea pigs were left undisturbed until the

peritoneal exudate returned to normal (12-15 days). Metal'nikov and Chorine then challenged the animals with only the tactile stimulus. They found that animals which received only the CS showed increased numbers of polynuclear leukocytes (conditional response, CR). Peritoneal exudate was obtained before reexposure to the CS and at various times after presentation of the CS, with each animal thereby acting as its own control. Clearly other controls were lacking, and the numbers of guinea pigs were small; nonetheless, these experiments provided the first demonstration of the involvement of conditioned cues in immune responses.

Metal'nikov and Chorine then conducted a slightly different experiment. They trained animals as before, but shortly after reexposure to the CS they gave the animals an injection of Cholera vibrios. A naive animal was given a similar Cholera injection. While the naive animal died several hours later, the conditioned animals survived. Again, many alternative explanations are possible in the absence of appropriate controls, but the finding is suggestive. Metal'nikov and Chorine suggested that conditional responses were defense reactions that provided the animal protection against potential homeostatic disruption. Their analysis of conditioning bears remarkable similarity to modern theories of learning as

adaptive processes (e.g. Hollis, 1982). Furthermore, Metal'nikov and Chorine suggested that conditioning might play an important role in diseases, and that conditioning factors might account for a large part of the symptomatology observed in chronic illnesses such as asthma. They argued that changes in living conditions, to remove factors associated with illness, could benefit the patient. As will become apparent in later discussions, we are only now re-discovering and re-affirming what was so apparent to Metal'nikov and Chorine over half a century ago.

Dolin, Krylov, Lukianenko and Flerov (1960), in one of the last reviews of studies of conditional immunomodulation in the Soviet Union, pointed out a conflict that is still not fully resolved. They argued that the evidence from various laboratories, from various species and large numbers of animals, left no doubt about the possibility that conditional factors can influence the immune system. But they also pointed out that some "microbiologists still, however, deny the possibility of any cortical regulation of immunological reactions from the higher divisions of the central nervous system" (p. 889). They then described a series of studies which examined the effects of conditional responses on anaphylaxis, antibody formation, and mortality from tetanus toxin. Dolin et al.

(1960) emphasized the importance of conditioning as a defensive mechanism, and stated that the ways in which the immune system responds to a challenge is determined in large part by "readjustment" of the nervous system. Unfortunately, however, the days of psychoneuroimmunology in the Soviet Union were numbered. One reason for this was a number of failures to replicate across laboratories. As well, there was a lack of results describing mechanisms or ways in which the immune and nervous systems might communicate. The widely held view in the autonomy of the immune system became the dominant one, while conditioned immune responses largely faded into obscurity for the next twenty years (see Ader, 1981 for a review).

While explicit reports of conditioned immune responses are absent in the literature from about 1960 to 1975, a certain number of studies appeared before and during that time which examined the interaction of the nervous and immune systems from other perspectives. A number of these studies have been combined into a book (Locke, Ader, Besedovsky, Hall, Solomon, Strom & Carpenter, 1985). These studies used electrophysiological, neuroendocrine, and behavioral data to support the hypothesis that there is an interaction between the immune and nervous system (mentioned in Chapter 1). Thus work to support CNS-IS associations progressed slowly until Ader

and Cohen (1975) rediscovered the phenomenon of conditional immunomodulation; that existing body of work, however, provided support for Ader and Cohen's assertions concerning the potential significance of CNS-IS communication.

2.2: Ader and Cohen: Rediscovering the phenomenon

In their 1985 review, Ader and Cohen described the "serendipitous" discovery which led them to perform studies of conditional immunomodulation. Ader and Cohen reported that they were conducting taste aversion studies using a taste (saccharin, SAC) CS and a pharmacologic (cyclophosphamide, CY) US. They noticed a certain mortality rate among their conditioned animals, and upon examination realized that the likelihood of an animal dying appeared to correlate with the amount of SAC consumed during the training session. Knowing that CY is a potent immunosuppressant, it occurred to them that perhaps they were conditioning the immunosuppressive properties of CY to the SAC.

Ader and Cohen (1975) reported a formal experiment in which they tested the hypothesis that SAC could become associated with the immunosuppressive properties of CY in such a way that subsequent reexposure to the SAC could elicit a conditional immunologic alteration. Their animals were exposed to SAC followed shortly by a CY injection (on

Day 1) and were reexposed to the SAC CS (on Days 4 and 7). To measure immunoreactivity, Ader and Cohen employed a hemagglutination in which animals were immunized on Day 4 (shortly before reexposure to the CS) and blood was drawn on Day 10. Conditioned animals had antibody titers that were suppressed relative to animals which received CY in a noncontingent manner, thus the CR mimicked the unconditional response, and resulted in an augmented immunosuppression. It appeared that this might explain why animals from the original study showed increased mortality after conditioning; the augmented immunosuppression induced by a CR to SAC might render the animals more susceptible to infection. Decreased immunoreactivity might well lead to increased mortality rates.

This initial report of Ader and Cohen led to a renewed interest in conditional immunomodulation. The next sections will discuss subsequent studies of conditional immunomodulation, and the variety of manipulations that have been performed. Questions that arise from the Ader and Cohen study will also be addressed in detail.

2.3: Use of pharmacologic unconditional stimuli

Shortly after the initial reports by Ader and Cohen (1975), two replications appeared. Rogers, Reich, Strom and Carpenter (1976) and Wayner, Flannery & Singer (1978)

used experimental paradigms similar to those employed by Ader and Cohen (1975) and reported augmented immunosuppression in animals reexposed to the SAC CS. Interestingly, Wayner et al. reported that one reexposure to the SAC CS was not sufficient to produce a conditioned suppression, while three reexposures to the SAC CS also did not produce a conditioned suppression. None of the studies have been able to determine why two reexposures to the SAC appears to produce optimal conditional effects.

Wayner et al (1978) also reported that they were unable to produce a conditional response using Brucella abortus, a T-cell independent antigen. In another study, however, Cohen, Ader, Green and Bovbjerg (1979) did report successful conditioning using the hapten 2,4,6-trinitrophenyl coupled with a relatively thymus-independent carrier, lipopolysaccharide. Thus early attempts to determine whether the phenomenon required thymus or T-cell participation did not produce conclusive results. More recently Schulze, Benson, Paule & Roberts (1988) reported that they were able to condition a T-cell dependent, but not a T-cell independent antibody response to SRBCs. Clearly the issue of whether T-cell dependence is necessary for a conditioned antibody response is as yet unresolved.

Many studies employing a SAC-CY paradigm have shown that a variety of responses can be successfully conditioned

(see Table 2.1). Among these was an intriguing study conducted by Ader and Cohen (1982). They used NZ/B mice, which are genetically disposed to develop a disease that is considered to be a model of systemic lupus erythmatosis. Since these mice have autoimmune disease, CY is beneficial inasmuch as it retards onset of nephritis and subsequent morbidity. Paired group mice which received CY paired with SAC interspersed with SAC alone exposures, produced a delay in the onset of nephritis that was statistically not different from CY injections that delivered twice the quantity of CY in a noncontingent manner. Mice which received similar quantities of SAC and CY as the paired animals, but in a noncontingent manner, developed nephritis significantly sooner than mice in the paired and high dose-CY groups. This study remains as one of the best demonstrations of the potential significance of conditional immunomodulation.

Other immune parameters studied include graft-versus-host disease (Bovbjerg, Ader and Cohen, 1982), adjuvant-induced arthritis (Klosterhalfen and Klosterhalfen, 1983), plaque forming cell responses (Gorczynski, Macrae & Kennedy, 1984; McCoy, Roszman, Miller, Kelly & Titus, 1986), reduced mitogen induced lymphoproliferation (Neveu, Dantzer & Le Moal, 1986), reduced NK cell activity (O'Reilly & Exon, 1986) and

Table 2.1: Conditional immunomodulation with pharmacologic USs

Reference (species)	CS	US	Immunologic response
Ader & Cohen (1975) Rogers et al (1976) Wayner et al (1978) Ader et al (1979) Ader et al (1982) (rat)	Sac	CY	reduced serum antibody to SRBC
Cohen et al (1979) (mouse)	Sac	CY	reduced serum antibody to TNP-LPS
Ader & Cohen (1982) (NZB mice)	Sac	CY	reduced mortality rate from SLE
Bovbjerg et al (1982) (mouse)	Sac	CY	reduced graft-versus-host response
Gorczynski et al (1983) (mouse)	Sac	CY	either suppression or enhancement of antibody to SRBC
Jenkins et al (1983) (rat)	Sac-LiCl	SRBC	increased antibody to SRBC
Klosternalfen & Klosterhalfen (1983) (rat)	Sac	CY	reduced adjuvant-induced arthritis
Bovbjerg et al (1984) (rat)	Sac	CY	reduced graft-vs-host response
Gorczynski et al (1984) (mouse)	Sac	CY	reduced PFC response to SRBC
Gorczynski et al (1985) (mouse)	Sac	CY	increased tumor-induced mortality increased T-suppressor function
Ghanta et al (1985) (mouse)	Camphour	Poly I:C	increased NK cell activity
Dyck et al (1986) (mouse)	Env	Poly I:C	attenuated NK cell tolerance
McCoy et al (1986) (rat/mouse)	Sac	CY	suppressed PFC response

Table 2.1: Continued

Reference (species)	CS	US	Immunologic response
Neveu et al (1986) (mouse)	Sac	CY	reduced lymphocyte proliferation
O'Reilly & Exon (1986) (rat)	Sac	CY	reduced NK cell activity
Bovbjerg et al (1987) (mouse)	Sac	CY	increased DTH to SRBC
Ghanta et al (1987) (mouse)	Camphour	Poly I:C	decreased tumor growth increased survival
Hiramoto et al (1987) (mouse)	Sac-LiCl	CY	reduced NK cell activity
Husband et al (1987) (rat)	Sac	Levamizole	increased helper:suppressor ratio
Schulze et al (1988) (Balb/c mice)	Sac	CY	decreased T-cell dependent response no effect on T-cell independent response
Solvason et al (1988)	Sac-LiCl	IFN-beta	augmented NK cell activity

increased delayed type hypersensitivity to sheep red blood cells (Bovbjerg, Cohen & Ader, 1987). All of these studies reported that conditional responses influenced the magnitude of the immune parameter in question.

The results of several studies, however, suggested that conditional immunologic responses might be more complicated than they had appeared at first. In an attempt to systematically explore factors influencing conditional immunological response, Gorczynski, Macrae and Kennedy (1984) conducted a series of eight studies. They observed conditional immunosuppression five times, conditional immunoenhancement twice, and no effect once, using the plaque-forming cell response to SRBC as the immunologic measure. They reported that diurnal effects appeared to be important determinants of whether conditional immunoenhancement or immunosuppression was observed. As well, age, stress, and adrenal function were important considerations. Aged, chronically stressed and adrenalectomized mice did not exhibit conditional immunosuppression. As a behavioral index of ability-to-condition, Gorczynski et al. reported that mice which exhibited low open field activity were particularly likely to exhibit conditional immunosuppression. Thus Gorczynski et al. demonstrated that a variety of physiological and

behavioral factors may be important in determining whether a conditional immunosuppression can be observed.

More recently, other researchers (Schulze, Benson, PAul & Roberta, 1988), also reported variability in the conditioned response to SAC after SAC-CY training. Schulze et al. trained animals with a single SAC-CY trial; they then rested the animals for two weeks. Some animals were then reexposed to the CS and immunized four days later. Other animals were reexposed to the CS after two days and immunized two days later. Another group of animals was both reexposed and immunized four days after the end of the rest period. Finally, two further groups of animals were immunized on the fourth day after the rest period, but not reexposed to the CS until two or four days after that (six or eight days after the end of the rest period). Thus reexposure to the SAC CS occurred either two or four days before immunization, on the same day as immunization, or two or four days after immunization. Schulze et al. failed to observe conditional immunosuppression when animals were reexposed to SAC on the same day they were immunized (design which most closely mimics other studies). Schulze et al. did find conditional immunosuppression if animals were reexposed to the SAC four days before, or two days after SRBC immunization, but not two days before, or four days after. They provide no explanation of why certain

reexposure days are effective while others are not; clearly, however, these results support the notion that a variety of subtle factors may infuence the likelihood of observing a conditional immunosuppression.

Krank and MacQueen (1988) reported that mice trained with either an environmental cue or a simultaneous presentation of environmental and taste (SAC) cues, did not show conditional immunosuppression. In fact, they reported that animals which received a signal for CY had antibody titers that were significantly increased above those obtained from mice which received unsignalled CY injections. One explanation for the discrepancy between this result and the reports of Ader and Cohen is that the effectiveness of the SAC taste cue is altered in the presence of other, nongustatory cues. Krank and MacQueen had no group of mice trained only with SAC and CY, however, and this group would be necessary to insure that in the absence of the environmental cue mice demonstrated a conditional immunosuppression to SAC. Thus, while it has become clear that a SAC-CY conditioning paradigm can produce conditioned responses, several studies suggest that a variety of factors may influence the likelihood of observing conditional immune responses.

Several studies have also examined the possibility of observing conditional responses to immunoenhancing

pharmacologic agents. Ghanta, Hiramoto, Solvason and Spector (1985; see also Ghanta, Hiramoto, Solvason & Spector, in press; Solvason, Ghanta & Hiramoto, 1988) have performed a series of experiments using polyinosinic-polycytidlic acid (Poly I:C), a synthetic polyribonucleotide which raises natural killer (NK) cell and interferon activity. Ghanta and his colleagues have paired Poly I:C administration with both SAC-lithium chloride complex CSs and camphour odours. They reported conditional increases in natural killer cell activity, with conditioned animals showing NK activity three times that of animals which were conditioned but not reexposed to the CS (camphour). Recently Ghanta and his colleagues have demonstrated enhanced survival in tumor bearing mice conditioned with camphour and poly I:C. Twenty percent of the conditioned mice lived 120 days (end of study) beyond training with no evidence of clinical disease; all mice in nonconditioned groups were dead by day 61. In addition, the median survival rate was longest in the conditioned group.

Other researchers working with Poly I:C (Dyck, Greenberg & Osachuck, 1986) have shown tolerance development to repeated injections of Poly I:C. Dyck et al. proposed that these results suggest a correspondence with Pavlovian influences on tolerance development that

have been demonstrated for other drugs, primarily drugs of abuse (see Siegel, 1988 and Macrae, Scoles and Siegel, 1987, for reviews). Thus immunoenhancing agents may also act as efficient unconditional stimuli in conditional immunomodulation studies.

2.4: Use of antigenic unconditional stimuli

A number of researchers have used antigens as the unconditional stimulus (see Table 2.2), generally egg albumin (EA) or bovine serum albumin (BSA). One of the first of these studies was performed by Ottenberg, Stein, Lewis and Hamilton (1958), who examined conditional asthma in the guinea pig. They sensitized animals with EA injections, and afterwards paired a distinctive chamber with aerosolized EA. Using an index of physiologic distress, they determined that animals developed conditioned respiratory attacks in response to the chamber alone. They monitored such signs as rapid breathing, gasping, piloerection, dilation of alae nasi, and cyanosis and convulsion. They then determined that after repeated exposure to the chamber, they were able to extinguish the conditioned response. While this study demonstrated no direct immune system involvement, it is possible that the symptomatology observed was the result of activation of the immune cells involved in producing anaphylactic responses

Table 2.2: Conditioning with Antigenic USs

Reference (species)	CS	US	Conditional Response
Ottenberg et al (1958) (guinea pig)	Env	EA	increased respiratory distress
Justesen et al (1970) (guinea pig)	Env	EA	increase in peak-to-peak amplitude of plethysmogram
Russell et al (1984) (guinea pig)	Odor	BSA	histamine release
Dark et al (1987) (guinea pig)	Odor	BSA	histamine release
Djuric et al (1987) (rat)	Sac	EA	avoidance of Sac
Peeke et al (1987) (guinea pig)	Odor	BSA	histamine release
Djuric et al (1988) (rat)	Sac	EA	avoidance of Sac
Markovic et al (1988) (rat)	Sac	EA	avoidance of Sac

(a further discussion of this possibility is presented in Chapter 5).

A study performed by Justesen, Braun, Garrison & Pendleton (1970) supported the earlier report of conditional anaphylaxis by Ottenberg et al. (1958). Justesen and his colleagues developed a whole body plethysmographic technique for use in guinea pigs. During an asthmatic response, the pressure differential between the inspiratory peak and expiratory peak of each respiratory cycle is greater than during basal respiratory levels. The plethysmographic apparatus was designed to measure pressure differentials; thus a physiologic measure, rather than gross visual inspection, was employed to assess responsiveness of the animals to both unconditional (bronchoconstrictor) or conditional (environmental) cues. Justesen et al. (1970) reported that they were able to measure conditional increases in peak-to-peak amplitude of the plethysmogram in response to a sham challenge with distilled water. In addition, Justesen et. al. report both extinction and spontaneous recovery of the CR.

Justesen et al. then attempted to determine the pathways involved in producing the conditional response; they reported that they were able to abolish CRs using central (atropine) and peripheral (methscopolamine) cholinergic blockers. Although Justesen et al. do not

directly implicate immune cells in the conditioned response, they do argue for the probable importance of histamine and/or serotonin, both of which are contained in the mast cells of the immune system. As will be discussed more completely in Chapter 5, these mast cells are thought to release mediators such as histamine and serotonin during an anaphylactic response.

Further attempts to understand conditional asthmatic responses have been undertaken (Russell, Dark, Cummins, Ellman, Callaway & Peeke, 1984; Dark, Peeke, Ellman & Salfi, 1987; Peeke, Dark, Ellman, McCurry, & Salfi, 1987). Russell, Dark, Peeke and colleagues have shown conditional increases in histamine in serum after training with bovine serum albumin as the US and an odor CS. In their initial paper (Russell et. al., 1984) they suggested that conditioned histamine release implicates immune system involvement in production of the anaphylactic CR. While the results are certainly suggestive, as Russell et al. point out, histamine is found in many tissues. Thus, as in the studies performed by Ottenberg et al. and Justesen et al., the immune system has been implicated, but not determined to be influenced by CNS activation.

Further studies by Dark et. al. (1987) confirmed that histamine can be released in response to an odor CS for antigens; they also found that they were able to

extinguish the CR after reexposing the animals to only the odor CS at five weekly intervals. In another study (Dark et. al., 1987) they reported that animals which were stressed prior to conditioning (by handling) showed significant CRs to a CS. Animals which had not been stressed prior to conditioning did not exhibit the CR. Inasmuch as their initial report of conditioned histamine release described no explicit prior stressor, but significant CRs, this study appears to represent at least a partial failure to replicate. Dark et. al. have no good explanation for this discrepancy. They do make an argument that in the first experiment the animals were subject to the stress of an ether anesthetic; in the subsequent experiment, however, the animals were anesthetized with ketamine. They do not discuss the reason that ether anesthetic, but not ketamine anesthetic, should be an effective stressor.

Furthermore, in their initial experiment they argued that they handled their animals intentionally to *reduce* stress, whereas in the more recent experiment, they presumed with little justification, that handling acted as an effective stressor in their paradigm. They presented cortisol levels for test days only. Stressed animals had higher cortisol levels than non-stressed animals, but the cause of this increase may or may not be a result of

stress. As well, stressed animals' cortisol levels differed within groups only when a second CS+ exposure was compared with a CS- trial. Cortisol levels on the first test day (first CS+ exposure) were not significantly different from those obtained on a control trial (CS- exposure). No reason for this variation is presented. Thus although they provide evidence for a conditional histamine alteration, a role for either stress or the immune system is speculative.

Using another approach, a group of Yugoslavian researchers (Djuric, Markovic, Lazarevic & Jankovic, 1988; Markovic, Djuric, Lazarevic & Jankovic, 1988; Djuric, Markovic, Lazarevic & Jankovic, 1987) have recently shown that an anaphylactic response can produce a taste aversion if presentation of the taste CS (SAC) is associated with administration of the allergen (EA, the US). Djuric et al. (1988) reported a positive correlation between severity of anaphylaxis (measured by behavioral analysis, hematocrit and rectal temperature) and severity of the subsequent conditioned aversion. Djuric and colleagues take the observed anaphylaxis to represent an index of immune system activity, and the demonstrated conditioned aversion as evidence of an interaction between central nervous and immune system events. While the data are appealing, what is lacking is a clear measure of immune system involvement.

A generalized conclusion from the above studies is that, inasmuch as anaphylaxis represents immune system involvement, there is good evidence that antigen can act as an effective US in studies of conditional immunomodulation. Because none of the above studies directly monitor immune system activity, however, it is difficult to evaluate how much of the CRs observed are a result of CNS-IS communication.

2.5: Summary

While the evidence clearly supports conditional involvement in a variety of immune response systems, what is lacking in the literature are successful attempts to elucidate specific CNS and IS components involved in producing conditioned immune responses. As well, attempts to understand these studies within the framework of modern learning theories have been largely absent. The following chapters describe studies performed using both pharmacologic (Chapters 3 and 4) and antigenic (Chapters 5 and 6) stimuli. In Chapters 3 and 4 we describe experiments designed to evaluate the effect of a variety of conditioning manipulations on cues for CY. Given the apparently capricous nature of conditioned responses observed after training with SAC-CY, our first experiments attempted to elucidate some of the factors involved in

producing a CS-CY association. This is an important issue to address, as many studies have used, and continue to use, SAC-CY associations to observe conditional immunomodulation. CS-CY associations have not, however, been previously studied with explicit regard for modern theories of animal learning. We hoped to obtain a better understanding of a CS-CY association by manipulating several parameters of this association and evaluating results in terms of prevalent theories of conditioning.

CHAPTER 3: Conditional Immunomodulation Following Training with Cyclophosphamide

3.1: Introduction to Experiments 1-5

The many reports of conditional immunological responses (discussed above), have been inspired primarily by the work of Ader and Cohen (1975, 1985). They found that pairing saccharin (SAC) with the immunosuppressive drug, cyclophosphamide (CY), resulted in an association developing between the flavor and the drug. Rats demonstrated this association not only by displaying an aversion to SAC, but also by displaying CY-like immunosuppression in response to SAC. That is, when animals with a history of SAC-CY pairings were reexposed to SAC in conjunction with an antigenic challenge, they displayed compromised immunological functioning. Although these results were subsequently replicated in other studies by Ader and Cohen and colleagues (see Ader & Cohen, 1985), as well as by others (Rogers, Reich, Strom, & Carpenter, 1976; Wayner, Flannery, & Singer, 1978), there have been failures to replicate the results of Ader and Cohen (Gorzczynski, et al., 1984; Krank & MacQueen, 1988).

Some investigators have suggested that such conditional impairment of immunological activity is enigmatic, in view of data and theory concerning the adaptive nature of conditional responses (CRs). That is, it has been hypothesized that CRs provide a mechanism by which organisms are better able to deal with environmental demands (e.g., Pavlov, 1927, p. 14; Hollis, 1982) or adapt to repeated stimulation (e.g., Siegel, Krank, & Hinson, 1987; Wagner, 1976). Some investigators have suggested that it is difficult to suggest an adaptive process conferred by CS-elicited immunosuppression in an organism confronted with an antigenic challenge (e.g., Greenberg, Dyck, & Sandler, 1984; Hinson, 1985; Krank, 1985; Revusky, 1985b; Siegel et al., 1987; Weiss, 1985).

The issue of the adaptiveness of CRs in general, or immunological CRs in particular, is complex. It may well be the case that organisms respond with actions that are generally adaptive, rather than with actions that are appropriate to artificial and unusual situations (created, for example, by injection of immunosuppressive drugs). Thus, the conditional immunosuppression (and conditional increase in mortality) reported by Ader and Cohen (1975) was not "adaptive," but may have been an unfortunate by-product of processes that are generally beneficial (see MacQueen, MacRae, & Siegel, 1989). Indeed, there are

circumstances in which conditional immunosuppression prolongs survival, as in the Ader and Cohen (1982) report discussed above, in which mice with autoimmune disease lived longer after training with SAC and CY.

There are also demonstrations of SAC-elicited immunoenhancement (rather than immunosuppression) following SAC-CY pairings. Krank and MacQueen (1988) recently reported such conditional immunoenhancement in mice. Similarly, Gorczynski et al. (1984) reported that, in some experiments, mice trained with a procedure similar to that of Ader and Cohen (1985) evidenced conditional immunoenhancement. In other experiments, Gorczynski et al. (1984) also demonstrated SAC-elicited immunosuppression, and concluded that circadian and exogenous stress factors contribute to the expression of the various immunological CRs.

Inasmuch as SAC-elicited immunosuppression is difficult to evaluate within theories concerning the functional significance of CRs, the purpose of the present experiments was to further assess the conditioning of immunomodulation following training with CY. In addition to evaluating the effects of SAC-CY pairings on SAC-elicited immunomodulation, we evaluated other CS modalities in some experiments.

3.2: Experiment 1

Experiment 1 was similar to that of Ader and Cohen (1975), although some control groups were based on those used by those investigators in subsequent experiments (see Ader & Cohen, 1985). One group of rats (paired group) received a single SAC-CY pairing. A second group also received SAC and CY, but there was a 24-hr interval between the flavor and drug (unpaired group). A third group, although exposed to SAC, never received CY, and served as a baseline group from which to measure immunological fluctuations (saline group). Finally, the design of the experiment included a group that, like the paired group, learned a saccharin aversion, but was trained with lithium chloride (LiCl) rather than CY. Any SAC-induced immunological alterations seen in rats in this LiCl group would be attributable to the acquisition of a SAC aversion (and resulting restricted SAC intake), rather than to an association between SAC and an immunosuppressive drug.

The LiCl group permitted evaluation of an alternative explanation of conditional immunomodulation seen in paired group subjects. Some investigators have suggested that demonstrations of conditional immunomodulation are artifactual to stress effects inherent in taste aversion conditioning. That is, SAC would acquire immunosuppressive properties following association with any

aversive agent, not only an immunosuppressive drug (Kelley, Dantzer, Mormede, Salmon, & Aynaud, 1984, 1985; Klosterhalfen & Klosterhalfen, 1985).

Method

Subjects and adaptation. Forty-eight male, Charles River (CD) rats (purchased from Charles River Breeding Farms, Quebec, Canada), weighing 250-300 gm, were housed in individual cages with free access to food. They were adapted, over a five-day period, to a limited fluid access schedule: each day water was available for a 15-min period (scheduled sometime between 10:00-10:30 AM). Rats were maintained on this drinking schedule throughout the experiment. Rats were assigned randomly to each of four groups (n/group = 12).

Procedure. On Day 1 (which immediately followed the last day of adaptation to the drinking schedule), rats in the unpaired group received a 0.1% sodium saccharin solution during their usual 15-min drinking period. All other groups received 15 min access to water on this day.

The next day (Day 2) was the conditioning day. Unpaired-group rats received 15-min access to water and all other rats received 15-min access to SAC. One-half hour after this fluid access period, all rats received an intraperitoneal (ip) injection. Rats assigned to the unpaired and paired groups were injected with CY (50 mg/kg

in a volume of 1.5 ml/kg). Rats assigned to the saline group were injected with an equivalent volume of physiological saline. Subjects assigned to the LiCl group were injected with LiCl (0.15M at a volume of 6 ml/kg).

Three days later (Day 5), 30-min prior to the fluid access period, all animals received ip injections of sheep red blood cells (SRBCs) (2 ml/kg of a 1% solution, approximately $3x10^8$ cells/ml). Thirty minutes after administration of this antigen, all rats received SAC solution during their normal drinking period. Thus, in accordance with the procedure of Ader and Cohen (1975), rats were again presented with SAC on the day of the antigenic challenge. This constituted the first reexposure day.

Rats received their second reexposure day on Day 8 (i.e., SAC replaced water as the fluid during the drinking period). Blood was assayed on Day 11. Rats were anesthetized (Inovarvet, 0.4 ml/kg, i.p.), and approximately 1 ml of blood was collected via cardiac punctures when subjects no longer responded to a tail pinch (approximately 5-15 min after injection of anesthetic). The blood was centrifuged, and the serum was heat-inactivated at 56°C for 30 min. Antibody titrations were performed using the hemagglutination technique described in appendix A.

Data analyses. Saccharin-aversion was assessed by computing a SAC preference proportion: For each subject, the volume of SAC consumed on each of the three SAC-consumption days (the conditioning day, and each of the two reexposure days) was expressed as a proportion of that subject's water consumption (the mean volume of water consumed on the two days preceding each SAC-consumption day). Thus, a preference proportion of less than 1.0 indicates a SAC aversion.

Results

Aversion learning. The mean saccharin preference proportions observed in all groups on the conditioning day, and each of the two reexposure days, is shown in Figure 3.1. As can be seen, the groups were similar on the conditioning day, and SAC aversions were acquired by subjects in both groups that received SAC-drug pairings on the conditioning day (paired and LiCl groups). This is revealed by the lower preference proportions displayed by these subjects on the first reexposure day than on the conditioning day. Subjects that received a SAC-saline pairing (saline group), and subjects trained with a 24-hr interval between SAC and CY (unpaired group) displayed no evidence of a SAC aversion (see Figure 3.1).

An analysis of variance of the conditioning-day SAC preference proportions indicated no significant differences

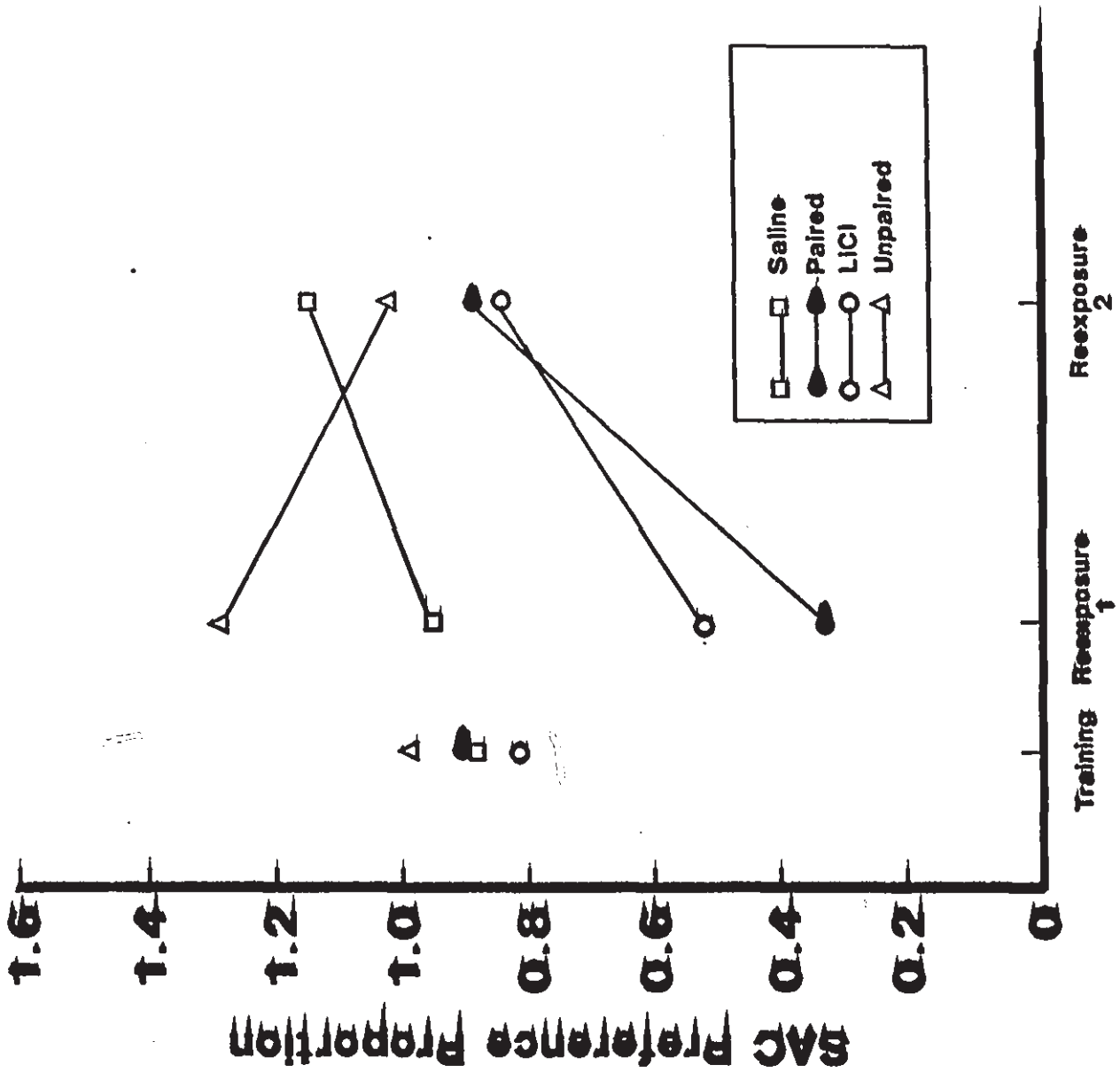

Figure 3.1: Mean Sac Preference proportions:
Experiment 1.

between groups [$F(3,44)=1.79$]. A repeated-measures analysis of variance of reexposure-days preference proportions indicated that the groups differed significantly [$F(3,44) = 17.62$, $p < 0.001$]. The interaction between group and reexposure day was also significant [$F(3,44) = 10.06$, $p < 0.001$]. The source of the interaction is apparent in Figure 3.1; subjects in paired and LiCl groups displayed extinction of their SAC aversions on the second reexposure day, whereas subjects in the unpaired and saline groups continued to display normal SAC consumption. Dependent t tests (df=11) were performed to assess the changes across reexposure. Both paired and LiCl groups evidenced significant extinction of the SAC aversion from the first to the second reexposure day (t = 5.74, $p < .001$ and t = 3.93, $p < .005$, respectively). The SAC preference proportions between the two reexposure days did not differ for the unpaired and saline groups (t = 1.83 and t = 1.90, respectively).

Hemagglutination antibody titers. The mean titer values for each group are presented in Table 3.1. As indicated in Table 3.1, unpaired-group subjects displayed the lowest titers. The titers of the other three groups were similar (see Table 3.1). An analysis of variance of the data in Table 1 indicated that the groups differed significantly in titer-values [$F(3,44) = 8.47$, $p < 0.001$]. Subsequent

Table 3.1

Mean Antibody Titers For Each Group (Experiment 1).

Group	N	Titer (± SEM) (log_2)
Paired	12	5.1 ± 0.33
LiCl	12	4.9 ± 0.31
Saline	12	4.7 ± 0.33
Unpaired	12	3.0 ± 0.30

pairwise comparisons (Newman-Keuls) revealed that the unpaired group differed significantly from each of the other three groups (all ps < .01), but differences between paired, saline, and LiCl groups were not significant.

Discussion

Rats in both groups injected with a drug after SAC (paired and LiCl) acquired comparable SAC aversions. No such aversion was seen in saline or unpaired rats, indicating that the pairing was necessary for the association between the flavor and the drug.

Titers of subjects with no exposure to CY (groups saline and LiCl) did not differ. Thus, mere acquisition of a SAC aversion (or ensuing limited ingestion of SAC) does not induce alterations in hemagglutination antibody titers. In agreement with earlier reports, subjects in these two groups displayed higher titers than subjects in the unpaired group, reflecting the residual immunosuppressive effect of CY administered on the conditioning day 10-days earlier (e.g., Ader & Cohen, 1985). However, in contrast with earlier reports, paired-group subjects (that also received CY 10 days earlier) did not display even lower antibody titers than unpaired-group subjects. In fact, paired group subjects responded as did subjects that never received CY. The results suggest that reexposure to the

CY-paired flavor elicited immunological responses that counteracted the effect of the immunosuppressive drug.

3.3: Experiment 2

Inasmuch as the results of Experiment 1 were different than those reported by others (see Ader & Cohen, 1985), Experiment 2 was conducted to assesses the reliability of the results of Experiment 1. In addition, the effects of conditioning were assessed with different control groups than those employed in the previous experiment.

In Experiment 1, the residual effects of conditioning-day CY on immunosuppression was evaluated in unpaired group rats (that were injected with CY 24-hr after SAC exposure). It is conceivable, however, that these subjects learned an inhibitory association between SAC and CY as a result of the long delay between presentation of the flavor and immunomodulator (Hinson & Siegel, 1980). In the present experiment, the residual effects of CY were evaluated in a group that received CY but no SAC during conditioning (US alone group).

In Experiment 1, the effect of SAC alone on immune functioning was assessed in the saline group. Of course, since subjects in this group did not learn a SAC aversion they drank much more SAC on reexposure days than did paired

group subjects. In the present experiment, the effect of SAC alone was assessed in rats ingesting the same amount of SAC as paired group rats. This was accomplished with a yoking procedure. Each animal in this yoked group was randomly matched to an animal in the paired group and, throughout the experiment, drank a quantity of SAC provided to them based on the amount consumed by their paired-group partner.

Finally, the design of the present experiment included a group that (like the paired group) received a SAC-CY pairing on the conditioning day, but received no subsequent reexposure to SAC. The results obtained from subjects in this nonreexposed group provided further indication of the residual effects of conditioning-day CY administration on immune functioning, but in subjects that had been trained to avoid SAC. Inasmuch as they received no reexposure to the CY-paired flavor, there can be no conditional immunological responses to modulate immune functioning in these nonreexposed subjects.

Method

The subjects were 48 rats of the same age and strain as those used in the previous experiment. They were maintained as described previously, including adaptation to a single 15-min per day drinking period. Animals were randomly assigned to one of the four groups (*n*/group = 12).

The preparations of CY, SAC, and SRBC, as well as the procedure of hemagglutination antibody assessment, were as described previously.

On Day 1, subjects in the paired and nonreexposed groups received SAC in their usual drinking period, and, one-half hr later, were injected with CY. Subjects in the US alone condition received water in their drinking period but were injected with CY in a manner similar to subjects in the paired and nonreexposed groups. Animals in the yoked group received a quantity of SAC equal to that consumed by their partner in the paired group. They had access to the amount of SAC consumed by their partner for one-half hour immediately following the SAC access period of the paired group. The extra drinking time was allotted to insure that each yoked-group subject drank all the SAC consumed by their paired-group partner. Following SAC consumption, yoked subjects received an ip saline injection.

As was the case in Experiment 1, subjects were challenged with SRBCs and received their first reexposure day three days after the conditioning day. On this reexposure/challenge day, all animals received injections of SRBCs. One-half hour later, rats in the paired, US alone, and yoked groups received SAC. For paired and US alone subjects, the flavored solution was presented in the

regular 15-min drinking period in place of water. Yoked-group subjects again received a quantity of SAC equivalent to that consumed by their partner in the paired group. The nonreexposed group was not reexposed to the SAC, and simply received water as usual on the reexposure day. Three days later subjects received a second reexposure day: paired and US-alone subjects were presented with SAC during their fluid access period, and each yoked subject drank the amount of SAC consumed its paired-group partner (nonreexposed subjects received water as usual). Three days after the second reexposure day, subjects were anesthetized, blood was sampled, and a hemagglutination test was performed.

Results

Aversion learning. Saccharin preference proportions were calculated as in Experiment 1 (for each subject that received SAC, amount of SAC consumed was expressed as a proportion of mean water consumption on the two days preceding each SAC-consumption day). Although nonreexposed subjects consumed water on each of the two reexposure days, "preference proportions" were calculated in the same manner as that employed for SAC-consuming subjects. The mean preference proportions observed in all groups on the conditioning day, and each of the two reexposure days, are shown in Figure 3.2.

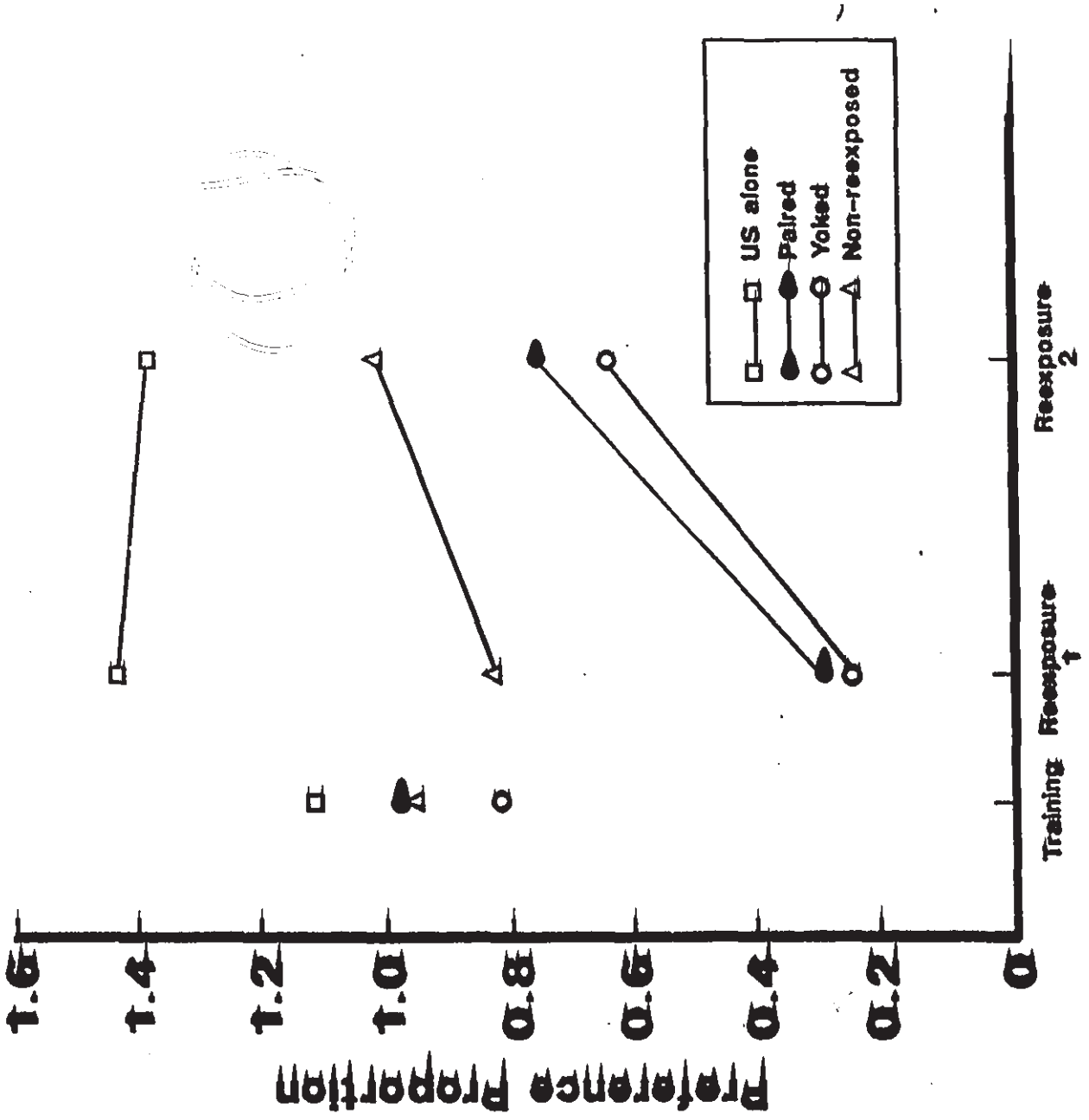

Figure 3.2: Mean Sac preference proportions:
Experiment 2.

As can be seen, the groups were similar on the conditioning day, and a SAC aversion was apparent in paired-group subjects on reexposure days. Yoked subjects displayed SAC preference proportions similar to those seen in paired subjects. Although yoked animals received an amount of SAC that was equated with that consumed by their partner from the paired group, they drank water freely. The amount of water that the yoked animals consumed on the days before SAC presentation forms the denominator of their preference proportion. Thus although the animals in the paired and yoked groups received equivalent amounts of SAC, the preference proportions vary slightly reflecting differences in the amount of water consumed by animals in each group on the days before the reexposure days (see Figure 3.2).

There was no evidence of SAC aversion in US alone subjects; thus, prior CY not paired with SAC did not decrease subsequent SAC consumption. As expected, nonreexposed subjects simply continued to consume water on days designated as reexposure days. An analysis of variance of the conditioning-day SAC preference proportions indicated no significant differences between groups [$F(3,44) = 1.58$]. A repeated-measures analysis of variance of reexposure days preference proportions indicated that

the groups differed significantly [$F(3,44) = 26.26$, $p < 0.001$].

There were two groups with free access to SAC during reexposure sessions, paired and US alone. The taste aversion in paired group subjects was evidenced by the fact that they displayed significantly lower SAC preference proportions than US-alone group subjects on both reexposure days (reexposure day 1, $t(22) = 4.07$; reexposure day 2, $t = 3.32$; both $ps < .001$).

Hemagglutination antibody titers. The mean titer values for each group are presented in Table 3.2. As expected, yoked-group subjects, that received no CY, evidenced the highest titers. Subjects in both control groups that were administered CY on the conditioning day (nonreexposed and US alone) demonstrated the lowest titer values. Paired-group subjects, also administered CY on the conditioning day, again displayed no evidence of conditional immunosuppression. Indeed, as was the case in Experiment 1, paired subjects displayed less immunosuppression than groups with equivalent exposure to CY (see Table 3.2). An analysis of variance of the data in Table 3.2 indicated that the groups differed significantly in titer-values [$F(3,44) = 5.10$, $p < .005$]. Subsequent pairwise comparisons (Newman-Keuls) indicated that the yoked group had significantly higher titer values than

Table 3.2

Mean Antibody Titers For Each Group (Experiment 2).

Group	N	Titer (± SEM) (log_2)
Yoked	12	4.5 ± 0.23
Paired	12	3.9 ± 0.24
Nonreexposed	12	3.1 ± 0.34
US alone	12	3.4 ± 0.28

either of the two CY-experienced control groups (nonreexposed and US alone) (p =.01 and p =.05, respectively). This yoked group did not differ significantly from the paired group (that also was injected with CY on the conditioning day); however, the difference between the paired and US alone group was not significant.

Discussion

As expected, paired-group subjects acquired a SAC-CY association, as revealed by a SAC aversion. No such aversion was seen in subjects with the same CY exposure but no SAC-CY pairing (US alone). Also, as expected, US-alone and nonreexposed subjects (administered CY on the conditioning day) evidenced compromised immune functioning in comparison to yoked subjects (with no exposure to CY).

The present experiment, like the prior experiment, provided no evidence of conditional immunosuppression. That is, paired-group subjects did not display greater immunosuppression (lower titer values) than did equally-CY experienced subjects in the US alone and nonreexposed groups. Indeed, the titer level of the paired group did not differ significantly from that seen in the CY-naive yoked group.

The results of Experiment 1 strongly suggested that, in paired subjects, SAC counteracted the effects of CY. In that experiment, paired subjects displayed significantly

greater antibody titers than did unpaired subjects. The relevant comparison in the present experiment (paired vs. US alone groups) is in the same direction, but not statistically significant. One interpretation of this finding is that a single SAC-CY pairing produces only a weak conditional immunomodulatory response.

3.4: Experiment 3

The design of Experiment 3 was similar to that of Experiment 2, except that the number of conditioning trials was increased from 1 to 3. In addition, the US-alone control group used in the previous experiment was replaced with an unpaired control group (which was exposed to SAC, as well as CY, during the conditioning phase of the experiment).

In the previous experiments, there was a three-day period between the single CY administration and SRBC challenge. In the present experiment, CY-injected rats were given three injections of the immunosuppressive drug. Inasmuch as the effects of CY are cumulative and long-lasting (Calabresi & Parks, 1985), it was thought appropriate to design the experiment such that there was a longer period between final CY injection and assessment of immune functioning.

Method

The subjects were 48 rats of the same age and strain as those used in the previous experiments. They were maintained as described previously, including adaptation to a single 15-min per day drinking period. The preparations of CY, SAC, and SRBC, as well as the procedure of hemagglutination antibody assessment, were as described previously.

Following adaptation to the drinking schedule, subjects received three conditioning days. There was a six-day interval between each conditioning day. On the day prior to each conditioning day, unpaired-group subjects received SAC during their drinking period (subjects in the other groups received water as usual). On each conditioning day, subjects in the paired and nonreexposed groups received SAC, while unpaired subjects received only water. One-half hour after drinking, subjects in these three groups were injected with CY. Subjects in the yoked group were matched with animals in the paired group and received a quantity of SAC equivalent to that consumed by their partner. As in the previous experiment, yoked-group rats had access to SAC for one half hour immediately following the paired group (the extra access time insuring that they consumed all allotted SAC). Yoked rats received a saline injection 30 min after SAC consumption.

A 14-day recovery period followed the third conditioning day. For the first 9 recovery days, subjects were given free access to water. For the final 5 recovery days, the 15-min-per-day water access period was reinstituted. Following the recovery period, subjects were challenged with SRBCs and received their first CS-reexposure day. One-half hour after SRBC injection, subjects in the paired and unpaired groups received SAC in their regular drinking period. Subjects in the yoked group again received a quantity of SAC equivalent to that consumed by their partner in the paired group. Subjects in the nonreexposed group were not reexposed to the SAC, but rather received water as usual.

The second reexposure day occurred three-days later. The second reexposure day was conducted as the first (except that SRBCs were not administered). Antibody levels were determined with a hemagglutination assay three days after the second reexposure day.

Results

Aversion learning. Figure 3.3 depicts the mean SAC preference proportions for all groups on each of the three conditioning days and two reexposure days. During the conditioning phase of the experiment, subjects in both paired and nonreexposed groups received SAC-CY pairings. As can be seen in Figure 3.3, both groups displayed

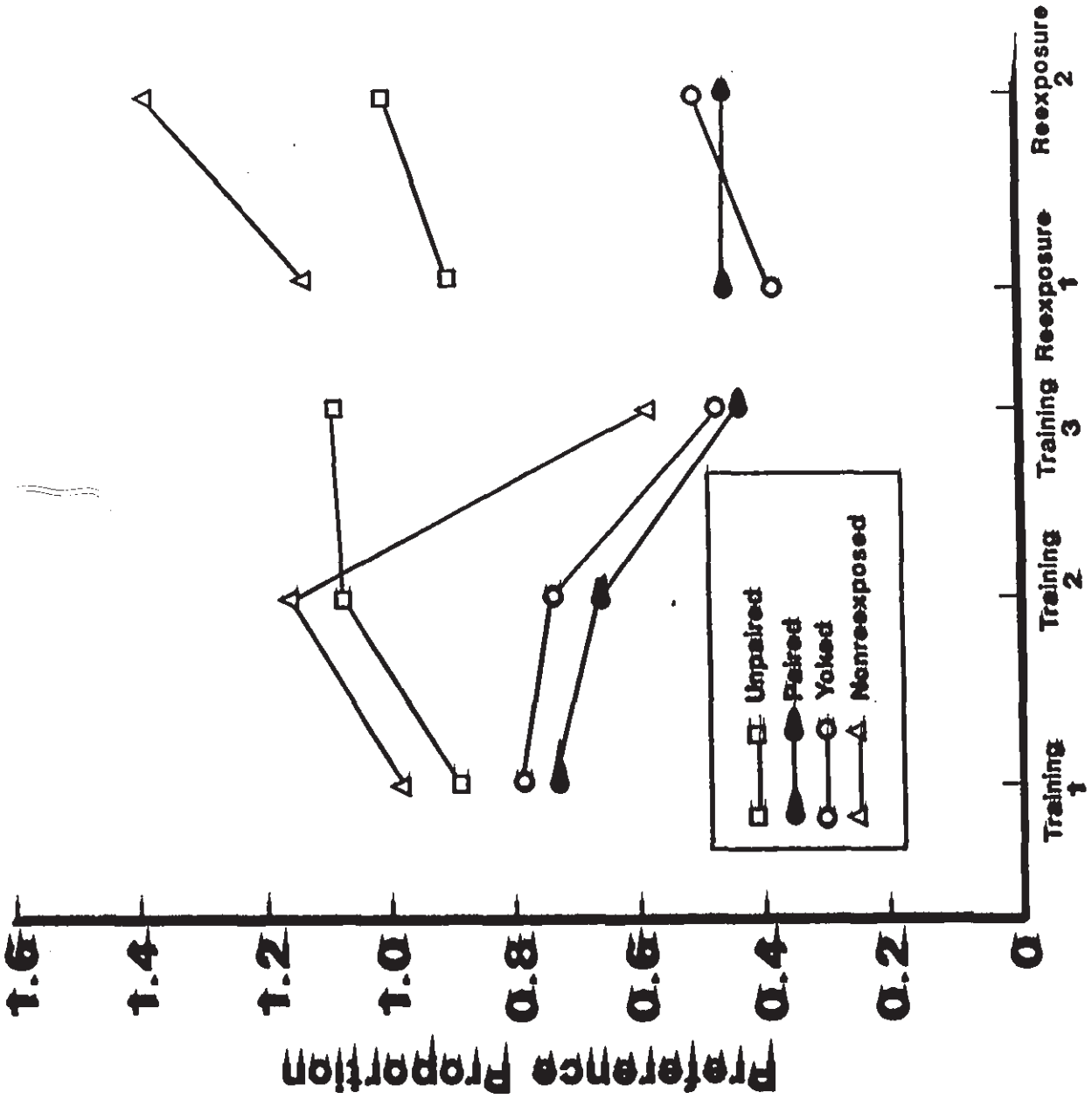

Figure 3.3: Mean Sac preference proportions:
Experiment 3.

acquisition of a SAC aversion, although (for unknown reasons) the course of acquisition differed between the two groups. Unpaired subjects did not display any aversion to SAC (ingested 24-hr prior to CY). As expected, yoked subjects displayed preference proportions comparable to those seen in paired subjects on all conditioning days (see Figure 3.3).

By the final conditioning day, the effects of the training were apparent. Three groups had unrestricted access to SAC on this day (paired, unpaired, and nonreexposed). A one-way analysis of variance of the preference proportions of these three groups was statistically significant [$F(2,33) = 14.08$, $p < .001$]. Newman-Keuls pairwise comparisons indicated that the unpaired group displayed significantly higher SAC preference proportions than any of the other groups ($p < .01$), with the differences between the nonreexposed, and paired groups not approaching significance.

The effects of the training manipulations were also apparent on the two reexposure days. Nonreexposed subjects, of course, drank only water on these days. Unpaired subjects continued to display no evidence of a SAC aversion. The low SAC preference proportions of paired group rats indicate that the SAC aversion acquired during conditioning persisted over the recovery period. The

similarly low SAC preference proportions of yoked rats indicate that they were comparable to the paired rats not only in SAC intake (which was intentionally equated), but also in terms of water intake on the days preceding each reexposure day (which formed the denominator of the saccharin preference proportion). A mixed design analysis of variance of the reexposure data indicated a significant effect of groups [$F(3,44) = 13.34$, $p < 0.001$]. On both reexposure days paired-group rats displayed significantly lower SAC

preference proportion ratios than unpaired-group rats ($t = 2.63$, $p < 0.01$ and $t = 5.29$, $p < 0.001$, respectively).

Hemagglutination antibody titers. The mean titer values for each group are presented in Table 3.3. Once again, yoked-group subjects (that received no CY) evidenced the highest titers. Subjects in both control groups that were administered CY on the conditioning day (nonreexposed and unpaired) demonstrated the lowest titer values. Exposure to the immunosuppressive drug, however, does not necessarily compromise immunological response to the SRBC challenge. Paired-group subjects did not display the low titer values seen in other CY-exposed subjects (see Table 3.3).

An analysis of variance of the assay scores summarized in Table 3.3 indicated a significant groups

Table 3.3

Mean Antibody Titers For Each Group (Experiment 3).

Group	N	Titer (± SEM) (log_2)
Yoked	12	3.9 ± 0.19
Paired	12	3.6 ± 0.21
Nonreexposed	12	1.7 ± 0.44
Unpaired	12	2.5 ± 0.39

effect [F (3,44) = 9.39, $p < .001$]. Subsequent pairwise comparisons (Newman-Keuls) indicated that although the difference between paired and yoked groups was not significant, each of these groups differed significantly from both the nonreexposed and unpaired groups ($ps < .05$).

Discussion

The results of the present experiment confirmed the results of the previous experiments. Moreover, by increasing the number of SAC-CY pairings from one to three, the immunomodulatory effect of the CY-associated flavor was more clearly evidenced.

The immunosuppressive effects of CY were apparent, even 21 days after CY administration, in the low antibody titers of subjects in the unpaired and nonreexposed groups (compared to the titer value seen in yoked subjects, that never received CY). Reexposure to the CY-paired CS had a salubrious effect in paired subjects. Despite their history of CY administration, paired subjects responded to the antigenic challenge with antibody levels comparable to those seen in rats not previously exposed to CY (i.e., yoked subjects). Indeed, the paired group displayed titer levels significantly greater than both the group that received CY at the same time but had no opportunity to acquire the SAC-CY association (unpaired rats), and the

group that had acquired the SAC-CY association but was not reexposed to the SAC (nonreexposed rats).

Results of the first three experiments confirm the results of Ader and Cohen and colleagues (see Ader & Cohen, 1985) in indicating that SAC-CY pairings result in a SAC aversion. These investigators also reported that such pairings cause SAC to acquire CY-like immunosuppressive properties. No such conditional immunosuppression was revealed in our research. In fact, when SAC had become an effective signal for CY, it functioned to ameliorate the immunosuppressive effect of the drug. Experiment 4 was designed to assess further the reliability of such conditional immunocompetence. In the next experiment, as in Experiment 3.3, paired rats received three CS-CY pairings, but the ability of a nongustatory cue to become associated with the immunosuppressive drug was evaluated.

3.5: Experiment 4

The design of Experiment 4 was similar to that of the earlier experiments, except that an audio-visual cue, rather than a flavor, was used as the CS. Inasmuch as exposure to such a cue (unlike exposure to a flavor) is not dependent on the subject maintaining contact with the stimulus, there was no need to include a group to control for CS exposure (the LiCl group in Experiment 1 and the

yoked group in Experiments 2 and 3). Thus, the design of Experiment 4 included just three groups: paired, unpaired, and CS alone.

In the previous experiments, subjects assigned to different groups drank different amounts of fluid, depending on their conditioning history. Such fluid consumption would be expected to affect food consumption, thus there were no independent analyses of group assignment and weight. In contrast, the present experiment did not involve taste-aversion learning, thus there was no need to maintain the subjects on a limited fluid access schedule. Inasmuch as CY induces gastrointestinal distress (Calabresi & Parks, 1985), it might be expected that the groups with various conditioning histories might differ in weight over the course of the experiment (see Krank, Hinson, & Siegel, 1984; Krank & MacQueen, in press). Specifically, if the CR acts to attenuate the suppressive effects of CY, it might also attenuate the effect of the drug on body weight. Thus, in the present experiment we report weight data, as well as hemagglutination titer levels.

Methods

Subjects. The subjects were 36 rats of the same sex, age, and strain as those used in the previous experiments. They were maintained as described previously, except that water, as well as food, was freely available

throughout the experiment. Twelve rats were randomly assigned to each of the three groups.

Apparatus. The CS was presented while the subjects were placed in a clear plastic cage in one drawer of a concrete-encased (soundproof) filing cabinet. An incandescent light in the drawer (28 volts D.C., .07 amp) flashed at 300 msec intervals, and background noise resulted from the operation of a ventilation fan. Conditional stimulus presentations consisted of placement in this environment for 30 min.

The preparations of CY and SRBC, as well as the procedure of hemagglutination antibody assessment, were as described previously.

Procedure. As in the previous experiment, there were three conditioning days, with a six-day interval between each conditioning day. On the day prior to each conditioning day, subjects in the unpaired group were transported from the colony room, placed in a drawer of the cabinet, and exposed to the CS for 30 min. Animals in the paired and CS alone group were not disturbed at this time. Twenty-four hours later, unpaired group animals were injected with CY in the colony room. On this day, subjects in the paired group were placed in the cabinet for 15 min, briefly removed and injected with CY, and returned to the cabinet for another 15 min. Subjects in the CS alone group

were treated as paired subjects, except the injection consisted of physiological saline rather than CY.

After the third conditioning trial, all subjects received a 14-day recovery period during which they were left undisturbed in their home cages. Reexposures to the CS were conducted in accordance with procedures used in the earlier experiments. On the first reexposure day (immediately after the recovery period) subjects were challenged with SRBCs, and, one-half hour later, placed in the CS environment for 30 min. Three days later, subjects received a second reexposure to the CS. Three days after the second reexposure, blood was sampled and antibody levels were once again assayed with a hemagglutination assay.

Data analyses. In the previous experiments, SAC aversion provided an index of the CS-CY association. In the present experiment, there was no such behavioral evaluation of the CS-CY association. However, as discussed, the fact that all subjects had free access to both water and food throughout the experiment permitted evaluation of the weight changes in the non-deprived subjects in all groups. Weight change was evaluated by determining the change in animals' weights from the first conditioning day to the assay day.

Results

The titer levels and weight gains observed in the three groups are presented in Table 3.4. As expected, unpaired-group subjects (which received CY on the three conditioning days) displayed immunosuppression in comparison with CS-alone subjects (which never received the immunosuppressive drug). In agreement with the results of the previous experiment, paired-group subjects displayed little evidence of suppressed immunological activity (see Table 3.4).

An analysis of variance of the endpoint dilution scores summarized in Table 3.4 indicated that the differences between groups was significant [$F(2,33) = 9.08$, $p < .001$]. Newman-Keuls pairwise comparisons indicated that subjects in the paired and CS alone groups did not significantly differ, and both groups displayed significantly higher antibody production than the unpaired group (*p*s < .01).

As indicated in Table 3.4, the greatest weight gain was shown by subjects that never received CY (CS alone), and the smallest weight gain was observed in subjects receiving unsignalled CY injections (unpaired), with an intermediate weight gain observed in subjects receiving signalled CY.

Table 3.4

Mean Antibody Titers and Weight Changes for Each Group (Experiment 4).

Group	N	Weight Change (± SEM) (Grams)	Titer (± SEM) (log_2)
CS alone	12	152.8 ± 9.0	4.0 ± 0.33
Paired	12	144.0 ± 6.4	3.9 ± 0.29
Unpaired	12	122.2 ± 17.0	2.3 ± 0.35

Analysis of variance of these weight gain data, however, did not attain conventional levels of statistical significance.

Discussion

Once again, a CS paired with an immunosuppressive drug eliminated the residual suppressive effects of that drug. Unlike Experiments 1-3 (and most other CY conditioning experiments), the CS used in this experiment was not a distinctive flavor. Rather, an audio-visual CS was used, but the results were very similar to those obtained in Experiments 1-3 with a SAC CS. In the present experiment, conditioning manipulations did not lead to significant group differences in weight gain.

3.6: Experiment 5

The purpose of Experiment 5 was to evaluate immunological conditioning with yet another CS for CY. There is evidence that associations readily form between sequentially presented drugs (e.g., Lett, 1983; Revusky, 1985a; Taukulis, 1982, 1986a, 1986b). For example, Siegel (1988) recently demonstrated that pentobarbital-morphine pairings are readily established. Indeed, Revusky (1985b) suggested that drug states in general, and the state induced by pentobarbital in particular, might be especially appropriate CSs in studies of CY conditioning.

Method

The subjects were 36 rats of the same sex, age, and strain as those used in the previous experiments. As was the case in Experiment 4, subjects in the present experiment had free access to food and water. The preparations of CY and SRBC, as well as the procedure of hemagglutination antibody assessment, were as described previously. All pentobarbital injections were ip, at a sub-anesthetizing dose of 20 mg/kg, in a volume of 0.615 ml/kg.

The design of the present experiment was similar to that of the previous experiment, except that the CS was pentobarbital, rather than an audio-visual stimulus. Thus, there was no need to transport animals from the colony room to the CS environment, and all rats remained in their cages in the colony throughout the experiment. Also, in the present experiment, as in the previous experiment, we evaluated the effects of the conditioning manipulations on the subjects' weights.

As in Experiments 3-4, there were three conditioning days, with a six-day interval between each conditioning day. On the day prior to each conditioning day, subjects in the unpaired group were injected with pentobarbital. Subjects in the paired and CS alone group were not disturbed at this time. Twenty-four hr later, unpaired

subjects were injected with CY. On this day, subjects in the paired group received two injections, with a 15-min interval between injections. The first injection consisted of pentobarbital, and the second of CY. Subjects in the CS alone group were treated as paired subjects, except the second injection consisted of physiological saline rather than CY.

As in the previous experiment, subjects received a 14-day recovery period after the third conditioning trial. They were then challenged with SRBCs and reexposed to pentobarbital. A second reexposure to pentobarbital occurred three days after the first. Three days later, blood was sampled and the hemagglutination test was performed.

Results

The titer levels and weight gains observed in the three groups are presented in Table 3.5. As expected, unpaired-group subjects (that received CY on the three conditioning days) displayed immunosuppression in comparison with CS-alone subjects (that never received the immunosuppressive drug). In agreement with the results of the previous experiments, paired-group subjects displayed little evidence of suppressed immunological activity. In addition, weight-gain data paralleled the immunological

Table 3.5

Mean Antibody Titers and Weight Changes For Each Group (Experiment 5).

Group	N	Weight Change (± SEM) (Grams)	Titer (± SEM) (log_2)
CS alone	12	156.4 ± 5.6	4.8 ± 0.44
Paired	12	160.0 ± 7.8	4.9 ± 0.34
Unpaired	12	89.8 ± 18.3	2.9 ± 0.40

data. Rats in the unpaired group gained less weight than rats in the other groups (see Table 3.5).

An analysis of variance was performed on the endpoint dilution values summarized in Table 3.5. There was a significant difference between the experimental groups [$F(2,33) = 8.24$, $p < .002$]. A Newman-Keuls test revealed no difference between the CS alone and paired group. The unpaired group was significantly different from both the paired and CS alone groups (ps < .01).

An analysis of variance of weight-gain data presented in Table 3.5 indicated a significant difference between groups [$F(2,33) = 10.97$, $p < .001$]. Newman-Keuls comparisons indicated no significant difference between the paired and CS alone group, but a significant difference between the unpaired group and both the paired and CS alone group (ps < 01).

Discussion

The results indicate that pentobarbital is an effective cue for CY administration. Pairing the barbiturate with the immunosuppressive drug caused the pentobarbital to enhance immunological competence, such that paired subjects did not differ from subjects that had not received CY. These results obtained with pentobarbital as a CS for CY parallel our findings with respect to an

audio-visual CS (Experiment 4) and SAC CS (Experiments 1-3).

In paired group animals, pentobarbital not only attenuated the immunosuppressive effect of CY, but also attenuated the anorexigenic effect of the CY as well. That is, unpaired rats gained less weight than CS-alone rats. However, the effect of CY on weight was not apparent in paired rats; paired group rats gained as much weight as the control group animals that never received CY.

3.7: General Discussion of Experiments 1-5

In all experiments, paired group rats received a CS paired with CY. In Experiments 1-3, the CS was SAC. Results of these experiments confirmed that the flavor can serve as an effective signal for the immunosuppressive drug. In agreement with previous reports, a taste aversion to SAC was evident after SAC-CY pairing. Contrary to most reports, however, was the nature of the observed conditional immunological response. There was no evidence of conditional immunosuppression. Rather, paired group subjects responded to the antigenic challenge as did subjects that never received the immunosuppressant. That is, the usual effect of CY in compromising immunological functioning was attenuated or eliminated by reexposure to SAC.

The findings of Experiments 1-3 were confirmed in Experiments 4-5, which used nongustatory CSs. The results of these final two experiments indicated that both audiovisual (noise and flashing light) and pharmacological (pentobarbital) cues were also effective signals for CY injection. Following pairing with CY, these cues protected animals from the immunosuppressive effects of the drug. The effect of the CS-CY association was also seen in subjects' weights. Cyclophosphamide reduces weight gain in rats, but this effect was attenuated in paired group rats (the effect was statistically significant with the pentobarbital CS, but not with the audiovisual CS).

The finding that CY-paired CSs counteract the immunosuppressive effects of CY are congenial with theoretical analyses of pharmacological CRs in general (Eikelboom & Stewart, 1982; Matysiak & Green, 1984; Obál, 1966), and immunological CRs in particular (Greenberg et al., 1984; Hinson, 1985; Krank, 1985; Revusky, 1985b; Siegel et al., 1987; Weiss, 1985). However, these results are apparently contrary to reports that such CSs elicit CY-like immunosuppressive CRs (Ader and Cohen, 1985; Rogers, et al, 1976; Wayner et al., 1978).

Krank and MacQueen (1988) and Gorczynski et al. (1984) reported conditional immunological modifications similar to those reported here. Additional research is

needed to evaluate the circumstances favoring the various outcomes. It would appear, however, that CRs following training with immunosuppressive drugs, in common with CRs seen following training with a variety of other drugs, may compensate for drug effect (see Siegel, 1988).

It is not clear why my results do not correspond with those presented by Ader and Cohen. A clue to the explanation may be found in experiments performed by Krank and MacQueen (1988). Krank and MacQueen found that in the presence of both SAC and environmental stimuli (simultaneously presented), conditional immunoenhancement is observed. It is known that different CSs may elicit different CRs (Garcia and Koelling, 1967). It may the case that SAC generally produces immunosuppressant CRs, while environmental cues result in immunoenhancing CRs. To the extent that an inadvertent environmental stimulus overshadowed our SAC CS, we may have observed a CR in response to the inadvertent environmental CS rather than to the intended SAC CS.

As we did not replicate Ader and Cohen, we have not been able to reconcile their results with theories of CRs, inasmuch as the functional significance of conditional immunosuppression remains elusive. One explanation is based on the notion that when confronted with a homeostatic disturbance, animals will generally perform a behavioral

adjustment before a physiological adjustment, presumably because the behavioral adjustment is fast, reversible, and often energetically cheap (see Greenburg, Dyck, & Sandler, 1984). Animals trained with a SAC-CY association and then confronted with SAC, may attempt to avoid the CY by avoiding the SAC (evident in the SAC aversion). Animals trained with an environmental or drug cue have no such ability to avoid either the CS or the expected US. These animals may then be forced to make a physiologic adjustment (compensate for the immunosuppressive effect of CY), which by making the appropriate behavioral adjustment the SAC animals have presumably avoided. Although there is little support for this hypothesis, it should be easily testable by removing the animals' ability to avoid SAC (forced consumption) or by increasing the animals' ability to avoid an environmental CS (escapable environment). While the potential functional significance of conditional immunomodulation is intuitively obvious, explanations of this sort of obtained data have not yet been elucidated.

The present results, particularly those of Experiment 5 (conditioning with a sedative and CY), may be relevant to the design of treatment schedules for patients receiving therapeutic administration of immunosuppressants. Patients receiving CY are sometimes pretreated with sedatives, such as pentobarbital. Concern about this

practice has been raised by demonstrations that barbiturates modify the pharmacodynamics of CY via their effects on microsomal enzymes of the liver (Hoshi, Kanzawa, & Kuretani, 1969). However, inasmuch as this effect appears minimal (Jao, Jusko, & Cohen, 1972), pentobarbital is combined with CY in clinical settings (Donelli, Colombo, & Garattini, 1973). Since a variety of cues, including pentobarbital, are readily associated with CY, it is possible that a CY administration regimen involving pentobarbital pretreatment favors the development of a pentobarbital-CY association. The effects of such inadvertent cuing of the immunosuppressant should be considered in the design of treatment schedules for patients undergoing chemotherapy for neoplastic disease.

CHAPTER 4: Acquisition and Extinction of a Pb-CY Association

4.1: Experiments 6 and 7

The following experiments are similar to those described in the previous chapter, and were designed to address some of the issues raised by that series of experiments, particularly Experiment 5. Experiment 6 is an extinction study, in which animals were initially trained with Pb and CY and subsequently were either extinguished by repeated exposures to the Pb, or were simply rested. Experiment 7 was a replication of Experiment 6; inasmuch as the results of Experiment 6 were novel and interesting, we thought it reasonable to confirm their reliability in a separate experiment.

The first reason for performing Experiment 6 was to provide a replication, in a different species, of the ability of Pb to act as an effective cue for CY. Experiment 5, in which a sedative preceded an immunomodulator, mimicked a potential therapeutic schedule. As a result of the sedative-immunomodulator pairings, the sedative was able to alter the effectiveness of the immunomodulator. If such pairings occur during clinical

administrations of immunomodulators, it would be useful to know how to enhance or degrade the effect of such associations on the therapeutic efficacy of the immunomodulator. In order to enhance or degrade the effectiveness of a Pb-CY association, it is necessary to define the sorts of manipulations to which the PB-CY association is sensitive. A second purpose of this experiment, therefore, was to attempt to understand the ways in which the Pb-CY association is modifiable by conditioning manipulations.

A final purpose of this experiment was to examine the animals' abilities to produce immunoenhancing responses. In previous experiments, we observed immunoenhancement from a baseline of suppressed immunologic activity. CY suppressed the immune response, and the CR from paired group animals acted to return the animals to a normal (no CY) level of immune functioning. We reasoned that by increasing the time from training to reexposure, we might be able to eliminate the residual effect of the CY. The issue was then whether animals would show compensatory immunoenhancement above normal levels of antibody production to SRBCs. Animals which were trained with Pb-CY, but were not reexposed to the CS during the extinction phase, should retain an active Pb-CY association. By reexposing these animals to Pb only after enough time had

elapsed to eliminate the residual effect of CY, we expected to be able to determine whether these animals would now produce antibodies at levels above those of nonsuppressed animals.

Method

Design

During the conditioning phase of the experiment, mice were injected with a subanesthetizing dose of Pb once a week for three weeks. This barbiturate was the CS. Groups differed with respect to the relationship between this Pb injection and injection of CY. Mice in the paired group were injected with CY 20 min after each Pb injection. Mice in the unpaired group were injected with CY 24 hr after each Pb injection. Finally, mice in the CS alone group never received CY; rather they were injected with physiological saline 20 min after each Pb injection.

Following the conditioning phase, all mice were challenged with SRBC and reexposed to Pb on two occasions. Blood samples were then assessed for hemagglutinating activity. This initial assay constituted the pre-extinction assessment. The pre-extinction assessment provided an opportunity to evaluate the effects of conditioning on immunological activity in an experiment similar in design to other CY conditioning experiments (see MacQueen & Siegel, 1989). Based on the results of earlier

work, it would be expected that the group with no pre-assay history of CY (CS alone) would display normal immunoreactivity to the SRBC challenge, and the group with a history of CY, but not paired with Pb (unpaired), should display immunosuppression as a result of the residual effects of CY. The immunological CR is revealed in antibody titer levels of paired-group mice.

The extinction phase of the experiment started after pre-extinction assessment of antibody activity. Each of the three groups of mice was further divided into two subgroups, extinction and rest. Extinction subgroups received seven additional injections of Pb (one injection every three days). Rest subgroups were not injected during this period. Finally, all mice were again injected with SRBCs, reexposed to Pb, and antibody titers were again determined with a hemagglutination assay. This second determination of antibody activity (which was a measure of secondary antibody response) constituted the post-extinction assessment. The post-extinction SRBC challenge occurred 47 days after the last injection of CY. Comparison of hemagglutination activity of paired-rest, unpaired-rest, and CS alone-rest mice revealed the effects of conditioning manipulations long after the injection of CY, when the drug's immunosuppressive effect would presumably no longer be manifest. Evaluation of

hemagglutination activity of the extinction subgroups of the three experimental groups revealed the effects of repeated presentation of the CS on any conditional immunomodulation apparent in the rest subgroups.

Procedure

The procedures for the first replication of the experiment are described below. With the exceptions noted, the second replication was conducted in the identical manner.

Subjects and drugs. Wildstrain male mice, bred at McMaster University, were group housed in plastic cages (4-6 per cage). They had free access to food and water throughout the experiment. The mice ranged in weight from 11-31 gm at the start of the experiment (25-36 gm in Experiment 7). They were randomly assigned to paired, unpaired, and CS-alone groups. (In Experiment 6, 17 mice were assigned to the paired group, and 20 mice to each of the other two groups. In Experiment 7, 20 mice were assigned to each group).

All injections were ip. Pentobarbital was injected at a dose of 20 mg/kg, in a volume of .615 ml/kg. Cyclophosphamide was injected at a dose of 100 mg/kg (in a volume of 10 ml/kg). Physiological saline injections were equated volumetrically with CY injections.

Conditioning. Subjects received three conditioning trials, with a 7-day interval between trials. Each subject was injected twice on each trial. For subjects in the paired and unpaired group, the first injection consisted of Pb, and the second CY. For paired subjects, the Pb-CY interval was 20 min. For unpaired subjects, this interval was 24 hr. Unpaired subjects were injected with Pb on the day before paired subjects, thus both were injected with CY on the same day. Subjects in the CS alone group were treated like subjects in the paired group, except that the second injection consisted of saline, rather than CY.

After the third conditioning trial, all subjects received a 14-day recovery period. Immediately after the recovery period, antibody response was stimulated by injecting all mice with SRBCs (.6 ml of a 1% solution, approximately 1.5×10^8 cells/ml). Thirty min after administration of this antigen (20 min in Experiment 7), all mice were injected with Pb. Thus, in accordance with the procedure of others (see Ader & Cohen, 1975; MacQueen & Siegel, 1989), subjects were again presented with the CS on the day of the antigenic challenge. This constituted the first reexposure day. Three days later, subjects received their second reexposure day (i.e., they were again injected with Pb).

Pre-extinction assay. Three days after the second reexposure, mice were anesthetized (Pb, 65 mg/kg), and cardiac punctures were performed to obtain approximately .35 ml blood from each mouse. The blood was centrifuged, and the serum was heat-inactivated at 56°C for 30 min. Antibody titrations were performed using the technique described in Appendix A. Samples were assessed approximately four hours after the titrations were performed. Titers were recorded as $\log_2$ reciprocals of the endpoint dilutions.

Extinction. The extinction phase of the experiment started six days after blood was obtained for the pre-extinction assay. Although some animals did not recover from the cardiac puncture, those which did survive appeared to be fully active at this time. Surviving animals were randomly assigned to either rest or extinction subgroups. For Experiment 6, there were 7 mice in each subgroup during the extinction phase, except for unpaired rest, which had 8 mice. In Experiment 7, the number of subjects in each subgroup was as follows: paired rest, 7; paired extinction, 8; CS alone rest, 9; CS alone extinction, 10; unpaired rest, 9; unpaired extinction 9.

Extinction mice received seven further injections of Pb. In Experiment 6, but not Experiment 7, each Pb injection was followed 20 min later by injection of saline.

These extinction trials occurred at three-day intervals. Rest mice were left undisturbed during this period.

Four days after the last extinction-phase Pb injection, all mice were challenged with another SRBC injection, and were reexposed to Pb. Three days later, all mice received a final reexposure to Pb.

Post-Extinction assay. Two days after the final Pb reexposure (three days in Experiment 7), the mice were anesthetized, blood was collected, and a second hemagglutination was performed. The procedures for this post-extinction assay were the same as those for the pre-extinction assay.

Results

Similar results were obtained in each experiment. The results from each experiment are presented separately to emphasize the consistency.

Pre-Extinction Assay

The mean hemagglutination antibody titer values (± 1 SEM) obtained for each group on the pre-extinction assay in Experiments 6 and 7 are shown in Table 4.1. A 3 (group) X 2 (replication) analysis of variance of the data summarized in Tables 4.1 indicated that the groups differed significantly [$F(2,111) = 20.9$, $p < .001$]. Subsequent pairwise comparisons (Tukey's Tests) revealed that the unpaired group differed significantly from each of the

Table 4.1

Mean Antibody Titers For Each Group: Acquisition (Experiments 6 and 7).

Group	Titer (± SEM) (log_2)	
	Experiment 6	Experiment 7
Paired	5.5 ± 0.19	5.5 ± 0.23
CS alone	5.2 ± 0.14	5.3 ± 0.23
Unpaired	4.3 ± 0.19	4.3 ± 0.20

other two groups (all ps < .001), but the difference between the paired and CS alone group was not significant. Neither the effect of replication, nor any interaction involving this variable, was significant.

Post-Extinction Assay

The mean hemagglutination antibody titer values (+ 1 SEM) obtained for each group on the post-extinction assay in Experiments 6 and 7 are shown in Table 4.2. In both replications paired-rest subjects displayed the highest titers, with the remaining groups displaying similar levels of antibody activity.

The data summarized in Table 4.2 were subject to a 3 (conditioning treatment) X 2 (extinction treatment) X 2 (replication) analysis of variance. The results of this analysis indicated that titer values were significantly higher in Experiment 6 than in Experiment 7 [$F(1,83) = 19.6$, $p < .001$], but the replication variable did not significantly interact with any other variable. There was a significant interaction between conditioning and extinction treatments [$F(2,83) = 15.2$, $p < .001$]. Subsequent pairwise comparisons revealed the source of the interaction: paired rest subjects evidenced immunoenhancement, compared to subjects in all other groups. This finding was statistically significant in each replication (all ps < 0.01).

Table 4.2

Mean Antibody Titers For Each Group: Extinction (Experiments 6 and 7).

Group	Titer (± SEM) (log_2)	
	Experiment 6	Experiment 7
Paired-rest	9.9 ± 0.40	9.2 ± 0.10
Paired-ext	8.0 ± 0.24	7.7 ± 0.19
CS alone-rest	8.3 ± 0.16	7.5 ± 0.16
CS alone-ext	8.6 ± 0.28	7.4 ± 0.17
Unpaired-rest	8.0 ± 0.44	7.6 ± 0.25
Unpaired-ext	8.1 ± 0.35	7.7 ± 0.22

Discussion

Pre-Extinction Test

The results of the pre-extinction test in this experiment with mice are consistent with those reported by MacQueen and Siegel (1989) with rats. Unpaired subjects displayed immunosuppression compared to CS-alone subjects. That is, animals with a history of three CY administrations display residual immunosuppression in response to a SRBC challenge 14 days after the last injection of the immunosuppressant. This persistent immunosuppression was reversed in paired subjects; the usual effect of CY in compromising immunological functioning was attenuated or eliminated by reexposure to the pharmacological CS previously paired with CY. These pre-extinction test results are also similar to those reported by others with nonpharmacological CSs (Gorczynski et al., 1984; Krank & MacQueen, 1988).

Post-Extinction Test

The post-extinction tests provided an opportunity to evaluate immunological conditioning in animals receiving an antigenic challenge 47 days after the last CY administration. In rest-subgroup mice, left undisturbed during the extinction phase, no residual suppressive effect of CY was evidenced. That is, unpaired-rest mice (which received CY during the conditioning phase) displayed

antibody titers similar to those seen in CS alone-rest mice (which never received CY). However, even long after the last conditioning trial, the Pb-CY association was still apparent. Paired-rest mice displayed clear immunoenhancement; they responded to the Pb cue with significantly higher antibody titers than did the mice in the other rest subgroups. The results from these rest subgroups indicated that the effects of Pb-CY pairings, in common with other conditional responses (Mackintosh, 1974), display substantial retention. Moreover, the pairings establish a conditional immunoenhancement. In previous experiments (and in the pre-extinction test of the present experiment), in which there was a shorter interval between training with CY and assay of immunological activity, the effects seen in the paired group were compared with those seen in groups suffering residual CY-induced immunosuppression. In such experiments, the compensatory immunological CR was manifest as an attenuation of the immunosuppressive effect of CY. The results of the post-extinction test of the present experiment indicate that animals can actually learn to augment immunological processes in response to a cue paired with an immunosuppressive drug.

The performance of the extinction subgroups on the post-extinction test further indicate the associative basis

of the CS-elicited immunoenhancement. Paired-extinction mice, which were exposed to Pb on seven occasions prior to post-extinction assessment, no longer displayed immunoenhancement in response to Pb. Thus, although the factors that favor the development of an immunoenhancement CR are unclear, such a CR (in common with a an immunosuppressive CR, see Bovbjerg et al., 1984) can be extinguished. These results suggest that, in fact, associations formed between a sedative and CY are readily modifiable by traditional conditioning techniques. As suggested above, these results should be given consideration in view of the potential for drug-drug conditioning that is inherent in many treatment regimens involving immunomodulators. If it is the case that therapeutic effectiveness of immunomodulators might be adversely influenced by association with sedative or anti-emetic drugs, given in conjuncation with the immunomodulators, it may be necessary to consider manipulations such as extinction in order to reduce undesirable conditional immunological responses to non-immunological agents.

CHAPTER 5: Conditioned Secretion of Rat Mast Cell Protease II By Mucosal Mast Cells

The previous studies have been primarily concerned with the factors important in determining the strength of the association in conditioning with CY rather than the physiological mechanisms which produced the conditioned response. Given the complexity of antibody production, and the simplicity of the assay employed, questions of mechanism were not appropriate. The issue of the sorts of immune cells and nervous system components that are directly involved in the communication process that results in a conditioned immune response, is, however, critical. In order to address such issues, we employed a better defined, system of immunologic action in the next series of experiments. In effect, our previous studies relied on antibody production as a tool which permitted us to evaluate factors important in the formation of associations with CSs and CY. In the following studies we employed a standard conditioning paradigm as a tool which allowed us

to evaluate specific immunologic action and the consequences of this activity.

5.1: Support for a Nerve-Mast Cell Association

Several studies, and many anecdotal reports, suggested that anaphylaxis sometimes occurs in the absence of any antigenic stimulus or physical irritant (Khan,1973). Under these conditions, anaphylactic symptoms were termed reflexive or conditional responses to reflect the mechanism proposed to account for them (Khan, 1974). Given that a good deal is known about the immune cells of primary importance in anaphylactic responses, we thought that a conditional anaphylactic response might provide a starting point for examining the mechanisms involved during CNS-IS communication. That is, although many studies have provided evidence that immune and nervous system elements influence each other, the mechanisms whereby this interaction occurs remain unknown. Given the complexity of antibody responses such as those discussed above, it is unlikely that conditioning models of these sorts will soon be informative in suggesting potential communication mechanisms. Anaphylaxis in sensitized animals may be a somewhat simpler system, and we reasoned that if we could determine a specific immune cell capable of responding to CNS factors, we might have a starting point for examining

the nervous system factors that were responsible for influencing at least one form of IS response.

One of the most important triggers involved in the induction of anaphylaxis appears to be the mast cell (MC). There are several reasons to believe that MCs might be important in mediating anaphylactic responses. Increased numbers of MCs are associated with a wide range of inflammatory and allergic conditions (Befus, Bienenstock & Denburg, 1986; Ishizaka, 1984), and also with parasitic diseases, especially nematode infections, where, together with immunoglobulin E (IgE), they are thought to be involved in eradication of worms in the intestine (Miller, Woodbury, Huntley & Newlands, 1983; Woodbury, Miller, Huntley, Newlands, Palliser & Wakelin, 1984). Mast cells have high affinity binding receptors for IgE; when animals are challenged with antigen, the binding with specific IgE induces release of MC mediators and causes an immediate type hypersensitivity reaction. Thus we were interested to know if we could condition mucosal MC activity since allergic diseases of intestine and lung are initiated and/or perpetuated through their involvement. Two phenotypically distinct mast cell populations exist in the rat (Befus et al, 1986). One, known as the connective tissue type is thymus independent and found predominantly in the serosal cavity and skin. The other, called the

mucosal type, characterizes the MC in the mucosal lamina propria of the intestine and lung. The former contains heparin and a rat mast cell protease I, whereas the latter is characterized by another proteoglycan, choridiotin sulfate di-B and another enzyme, RMCP II, not found in other cells in the body. Thus much is known about the immune cell of primary importance in anaphylaxis. In addition to knowledge about the mast cell itself, a large amount of evidence suggested that MCs and nerves interact and have a functional relationship. This evidence, which will be summarized below, suggested that mast cells might be particularly appropriate for studies of immune-nervous system communication.

5.1.2: Morphologic Evidence for Mast Cell-Nerve Cell Interaction

Morphological studies have shown the presence of MC in peripheral nerves and autonomic ganglia (Olsson, 1971). Mast cells are also present in benign and nerve sheath tumors (Isaacson, 1976), in amputation neuromas in rodents and humans (Olsson, 1971, Pineda, 1965), and are associated with myelinated nerve regeneration (Olsson, 1968).

Recently Stead and his colleagues (Stead, Tomioka, Quinonez, Simon, Felten, & Bienenstock, 1987) documented an ultrastructural relationship between mucosal MCs and

enteric nerves in the rat intestinal lamina propria, both in the normal and infected states. Stead et al. demonstrated that approximately two thirds of intestinal mucosal MCs in the lamina propria were associated with neurone specific enolase-containing nerves. They reported frequent membrane-membrane contact, with distances of less than 20nM between the membranes, and dense core vesicles were often apparent at the site of interaction. Using immunohistochemical reagents, the nerves were shown to contain substance P (SP), a peptide which has been reported to cause the release of histamine from mucosal and connective tissue MCs in vitro (Shanahan, Denburg, Fox, Bienenstock & Befus, 1985). Other descriptions of mast cell/ nerve cell proximity can be found in the literature, but they are largely anecdotal in nature (Dimitriadou, Aubineau, Taxi & Seylaz, 1987; Heine, 1975; Newson, Dahlstrom, Enerback, Ahlman, 1983; Weisner-Menzel, Schulz, Vakilzadeh & Czarnetzki, 1981).

5.1.3: Functional Evidence for Mast Cell-Nerve Cell Interactions

Mast cells and SP containing nerves are thought to be involved in the vasodilatory response to noxious stimuli, the so called "neurogenic edema" (Kiernan, 1975; Lembeck & Holzer, 1979). Substance P injected into the

skin induces a response that mimics the response of local histamine injection or the mast cell degranulating agent 48/80 (Erjavec, Lembeck, Florjane-Irman, 1981; Hagermark, Hokfelt, Pernow, 1978). The effect of SP can also be abolished by antihistamines (Foreman & Jordan, 1982).

Further evidence for a functional relationship between nerves and MCs has been provided by the demonstration that vagal stimulation caused enhanced histamine release from MCs after challenge with ascaris antigen in natively allergic dog lung (Leff, Stimler, & Munoz, 1986). A decrease in MC granularity in rat ileum has been shown after electrical field stimulation (Bani-Sacchi,Barrattini, Bianchi, Blandina, Blandina, Brunelleschi, Fantozzi, Manaioni & Masini, 1986). As well, direct stimulation of the vidian nerve in humans caused degranulation of mast cells and a significant decrease in the tissue content of histamine. Antidromic electrical stimulation of the saphenous or trigeminal nerves may result in axon mediated inflammation of the skin and mucosal tissue of the hand (Jancso, Jancso-Gabor, Szolcsanyi, 1967). These studies suggest that neural activity can result in mast cell stimulation and secretion. Other studies have shown that nerves are sensitive to antigen; Weinreich and Undem (1987) demonstrated that antigen administration to neurones of the superior cervical

ganglia in sensitized rats leads to the generation of compound action potentials by these neurones.

5.1.4: Nerve Growth Factor and Mast Cell- Nerve Cell Interactions

Aloe and Levi-Montalcini (1977) demonstrated that injections of nerve growth factor (NGF), a factor responsible for promoting and mainaining nerves, to neonatal rats resulted in increased mast cell numbers. Bienenstock and his colleagues (Stead et. al., in press) extended this work by demonstrating that mast cell numbers increased in a dose dependent fashion after NGF administration. In animals given 5 ug NGF/gram for 14 days, connective tissue MC numbers were increased threefold, mucosal MC numbers were increased sixfold, and MC numbers in hematopoeitic tissues (spleen and liver) were increased by up to sixty three times normal numbers. The hyperplastic MC populations were not phenotypically different from MCs normally found in those sites (Stead, Perdue, Blennerhassett, Kakuta, Sestini, Bienenstock, in press).

NGF is also a known MC degranulator, and this suggested that a sort of feed forward mechanism whereby factors released during degranulation (interleukins 3 and 4, for example) might stimulate further MC growth. Further

support for this hypothesis has come from studies showing that sodium cromoglycate injections can abolish the *in vivo* effects of NGF. While only suggestive of a MC-nerve relationship, it is interesting that factors which affect nerves can also exert profound effects on the proliferation of MCs. Of particular interest is the hypothesis put forth by Stead et al. (in press) that NGF produced at sites of injury and inflammation may be involved in MC growth and differentiation at these sites. This hypothesis is supported by reports that NGF is produced by fibroblasts. The converse of this hypothesis, that MCs somehow affect the local innervation of a tissue, is also intriguing, however the evidence for such hypotheses is not yet well established. Preliminary evidence that a MC may produce trophic factors which promote neurite growth to the MC is discussed in the following section.

5.1.5: Co-culture Experiments Support Mast Cell-Nerve Cell Interactions

Blennerhassett and Bienenstock (submitted, and Bienenstock et al., in press) have conducted a series of co-culture experiments which have shown striking MC - nerve cell interactions. Sympathetic nerves from the superior cervical ganglia of neonatal mice have displayed selective and specific associations with rat peritoneal mast cells

and rat basophil leukemia cells (RBL 2H3, the homologue of rat mucosal mast cells).

Both MC types appear to have neurotropic effects on the neurites which grow from the ganglia. Within sixteen hours in culture, 60-80 % of the MCs appeared to have neuronal attachment, which persisted for the 144 hours of observation after contact was made. In contrast, fibroblasts and rat plasmacytoma cells failed to show any evidence of neuronal interaction.

Mast cells which were in contact with nerves exhibited electrophysiological changes; microelectrode probes indicated that innervated MCs had resistances of only 50% that of non-innervated MCs. In addition, a grerater number of dense core vesicles were observed in the innervated MCs and these MCs were inhibited in mitosis. Blennerhassett and Bienenstock have taken this evidence as support for specific functional MC-nerve interactions.

5.1.6: Physiologic Evidence for Mast Cell-Nerve Cell Interactions

Using Ussing chambers to study MC activity in vitro, several researchers have found evidence to support the view that mast cells and nerves interact as part of a homeostatic regulatory unit for intestinal epithelium (Harai, Russell & Castro, 1987; Perdue, Forstner, Roomi &

Gall, 1984). This evidence is reviewed in 6.1, and is simply mentioned here because studies of this sort have provided evidence to suggest that MC-nerve associations have physiological significance.

5.1.7: Diseases with MC and Nervous System Involvement

Numerous diseases that are thought to have mast cell involvement are also thought to have a nervous system component. Allergic asthma, for example, was reported to have a psychogenic component as long ago as 1886, when McKenzie noted that a woman allergic to roses developed asthmatic symptoms upon exposure to an artificial rose (McKenzie, 1886). Since that time numerous studies have examined a role for psychologic factors, particularly conditioned responses, in asthma and other hypersensitivity responses (see Table 5.1). While none of these studies have direct immunologic indices, responses which suggest immune system involvement have been shown to be conditionable in humans. As discussed above (section 1.8) psychosocial factors have long been hypothesized to be involved in disease. What is worth note here is that many of the diseases that have been thought to involve psychosocial factors are those with probable mast cell components (asthma, allergy, intestinal diseases) and those

Table 5.1

Human studies of conditional responses to stimuli associated with allergens

Reference	UR	CS	Response to CS Challenge
Dekker et al (1957)	Change in vital capacity to allergen	Apparatus	Decrease in vital capacity in response to appratus
McFadden et al (1968)	Decreased airway resistance to allergen	Nebulizer	Increased airway resistance to saline in nebulizer
Jordan & Whitlock (1972, 1974)	Itch response	Tone	Increased GSR & # scratches
Spector et al (1975)	Increase airway resistance to methacholine	Flavored diluent	Increased airway resistance Decreased specific airway conductance
Horton et al (1977)	Broncho-constriction	Flavored diluent	Increased bronchoconstriction Decrease in vital capacity
Khan & Olson (1977)	Brocho-constriction	Exercise situation	Deconditioned when EIB blocked
Smith & McDaniel (1983)	DTH to tuberculin	Environment & injection	Increased erythma & induration

with potential mast cell or inflammatory components (multiple sclerosis, rheumatoid arthritis).

5.2: Experiment 8

The body of evidence discussed above supports a role for a functional MC - nerve interaction. A large amount of evidence also suggests that psychologic factors may be involved in a number of diseases which also have MC involvement. Based on this evidence we designed a study to determine whether mucosal mast cell activity could be influenced by classical conditioning.

Methods

Many models of anaphylaxis employ a single sensitization and a single challenge, after which the appropriate responses are measured. This does not appear to be an especially relevant model of human atopic disease, as individuals usually encounter antigens many times after initial sensitization has occurred. We developed an animal model of repeated antigen challenge upon which we imposed a typical Pavlovian conditioning procedure (see Table 5.2). Male Sprague Dawley rats were purchased from Charles River, and weighed between 225-250 g at the time of sensitization. Animals were individually housed in metal hanging cages and were provided with ad lib food and water during the course of the experiment. On day 0, rats were sensitized with an

Table 5.2

Sensitization and Conditioning Protocol

Group	Day 0	Day 14	Days 34, 41, 48	Days 35, 42, 49	Day 60
Paired	Alum EA + pertussis	Nippo	NT	AV->EA	AV->PBS
Unpaired	Alum EA + pertussis	Nippo	AV	EA	AV->PBS
Negative Control	Alum EA + pertussis	Nippo	NT	AV->PBS	AV->PBS
Positive Control	Alum EA + pertussis	Nippo	NT	AV->EA	AV->EA

NT = no treatment

Alum EA = aluminum precipitated egg albumin

Nippo = Nippostrongylus brasiliensis

PBS = phosphate buffered saline

injection of aluminum-precipitated egg albumin (EA; 1mg/ml; sc) and Bordetella pertussis (1 ml; ip). Egg albumin (Sigma Chemical Corp., St. Louis, Mo.) and pertussis (Connaught Laboratories, Toronto, Ont.) were administered according to protocol described by Perdue, Chung, and Gall (1984).

This sensitization protocol results in high levels of EA specific IgE; both the aluminum precipitate and the pertussis injection act as adjuvants to promote IgE production. The animals were then infected with the larval form of the nematode Nippostrongylus brasiliensis on day 14 to produce intestinal MC hyperplasia and an increase in IgE anti-EA levels (see Table 5.2).

The US consisted of s.c. injections of EA (300 mg in 0.6/ml phosphate buffered saline, PBS). The CS consisted of an audiovisual cue (AV) similar to that employed in Experiment 4. Animals were removed from the colony room and placed in plastic cages inside concrete encased (soundproof) cabinets.

On days 35, 42 and 49, conditioned animals (Paired and Positive Control groups) were exposed to the AV cue for fifteen minutes, after which time they were injected with EA. They were then exposed to the AV cue for another fifteen minutes. Negative control animals received the CS and an injection of PBS. Another group of animals

(unpaired) was placed in the chamber for 30 minutes on days 34, 41 and 48. They received the EA injection on the following day, at the same time as conditioned animals, but they received the EA in their home cages. Thus the unpaired animals had equal exposure to both the CS and US, but the two were temporally dissociated by a twenty four hour interval. Other groups remained undisturbed on days 34, 41, and 48. All animals were left undisturbed for 10 days and then reexposed to the CS on day 60. Both unpaired and CS alone group animals were placed in the chamber and received a saline injection. Conditioned animals were divided into two groups: one group received only the CS at this time (paired) and another group received the CS as well as the US (the EA injection; positive control group). Blood was sampled 24 hr prior to reexposure to the CS, as well as at 1 and 5 hr after exposure to the CS. Exposure of sensitized animals to EA would be expected to cause MC stimulation and release of mediators. Thus the expected unconditional response to the EA injections received during training would be release of these mediators. We monitored release of a mediator which is specific to mucosal mast cells, RMCP II, by the use of a sensitive assay (see Appendix B). The experiment was designed to assess whether mucosal MCs from paired group animals would now release

mediators in response to exposure to the CS which had previously predicted the EA injection.

Results

Baseline RMCP II levels obtained 24 hours before the final challenge ranged from 250-400 ng/ml. There were no differences in the baseline levels between groups ($F < 1$). The results of the 1 hr samples are summarized in Figure 5.1. Although there were no differences in the baseline levels between groups, baseline levels were subtracted from the post-challenge level; differences reported are delta values, representing the change in serum RMCP II levels after challenge. At one hr post challenge, RMCP II levels differed significantly between groups [$F(3,39) = 15.58$; $p < 0.001$)]. The paired and unpaired group animals differed significantly in their response to the CS (8.8 $\pm$ 1.4 mg/ml paired versus 2.1 $\pm$ 0.4 mg/ml unpaired; Newman-Keuls, $p < 0.01$). The paired and unpaired group animals differed during training only in that the CS for paired animals predicted the occurrence of the US, while it did not predict the US for unpaired animals. Thus, since these groups were equivalent with respect to exposure to both the CS and the US, the difference between the paired and unpaired groups represents the effect of conditioning. There was no difference in the quantity of RMCP II released into the sera by either paired or positive control animals

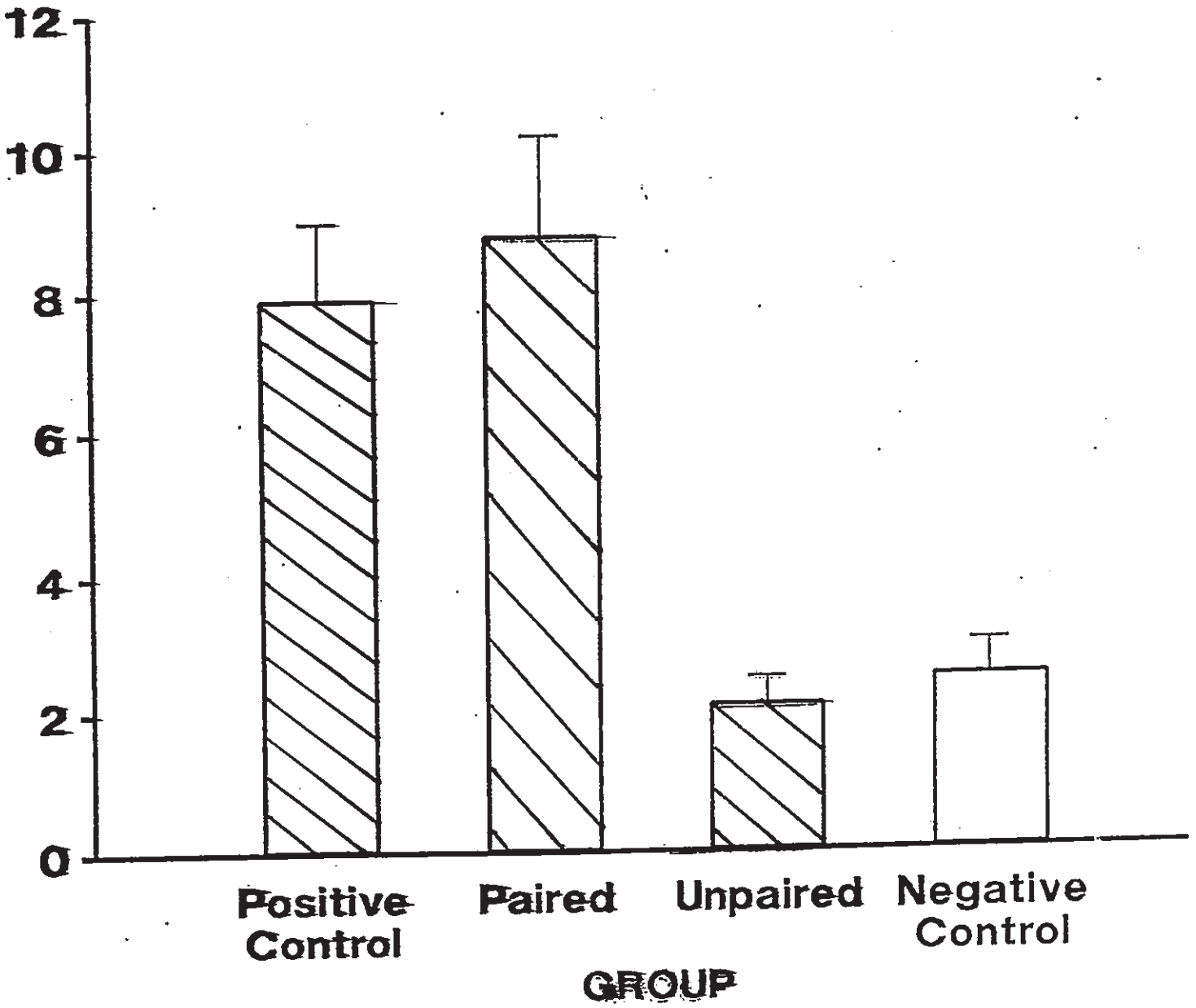

Figure 5.1: RMCP II levels in serum. One hour - pre challenge levels. Experiment 8

(8.8 + 1.4 mg/ml versus 7.9 + 1.1 mg/ml). This suggests that the CS elicited a CR from paired group animals that was equivalent to the unconditional release of RMCP II (from positive control animals that received the EA during the reexposure phase). At five hours post challenge, RMCP II in the sera of animals from all groups declined to approximately 1 mg/ml above baseline levels. Differences between groups were not significant [$F(3,39) = 1.55$; $p > 0.20$)].

Discussion

Our data clearly indicate that the CNS can regulate the secretion of a mediator known to occur only in mucosal mast cells. Our demonstration of conditional MC activity does not provide conclusive evidence for functional mast cell-nerve interactions, however, there is considerable evidence to support both morphological and functional nerve - mast cell interactions.

Communication between MCs and the nervous system may have a role in pathological intestinal conditions of uncertain etiology, such as Crohn's disease, ulcerative colitis, food sensitivity and irritable bowel disease, where mucosal MC numbers are increased and psychological factors have been incriminated. In allergic diseases of the lungs and upper respiratory tract, such as allergic rhinitis and asthma, these interactions between the nervous

system and mast cells may also be of pathogenetic significance, although this has been disputed (Pearson, 1988). The conditional response that we observed was equivalent in size to the unconditional response, suggesting that the conditional response was physiologically meaningful. Further studies were necessary to determine whether conditional responses were able to alter the physiology of tissues associated with mucosal mast cells. Experiment 9 was performed to address the issue of the physiological relevance of conditional mast cell activity.

CHAPTER 6: Physiological alterations after conditional mast cell activity.

6.1: Previous Studies of Mast Cell-Associated Physiological Alterations

Studies of intestinal and respiratory physiology have examined the role of mast cells in preserving homeostasis in the respiratory and immune systems. Many of these studies have employed Ussing chambers to examine intestinal and respiratory tissues *in vitro*. Ussing chamber experiments typically involve removing segments of intestine and examining transport parameters in flux chambers. The intestine is cut into flat strips, mounted like a gasket between two leucite chamber halves, and bathed in physiologic buffer. As the serosal side of the intestine is positive relative to the luminal side, an electrochemical gradient exists across the tissue. A current is introduced across the tissue to eliminate the spontaneous potential difference, and changes in this current indicate changes in the electrochemical gradient and provide an indication of net ion movement across the tissue. The current is monitored continuously; relatively large short circuit currents (Isc) indicate absorption or

secretion of large numbers of ions across the membrane and indicate that the tissue is in an active or secretory state.

Ussing chambers have been used to study the effects of alterations in mast cell activity on the local intestinal epithelium. It appears that an antigen-specific mechanism induces a net chloride ion secretion towards the lumen, and it has been suggested (Bienenstock, MacQueen, Sestini, Marshall, Stead, & Perdue, in press) that the antigen exerts its effect on these systems through interactions with IgE antibody on the surface of mast cells. Support for the hypothesis of mast cell involvement comes from studies in which the pharmacological agents cromoglycate (a connective tissue MC inhibitor) and doxantrazole (which inhibits both connective tissue and mucosal MCs) were added to Ussing chamber preparations of intestinal epithelium. Chloride ion secretion in sensitized rat intestine was eliminated by the addition of doxantrozole, thus supporting a role for mucosal MCs in the antigen response. These responses may also be inhibited by H1 antihistamines (diphenhydramine), serotonin antagonists (ketanserin), and neurotoxins (tetrodotoxin), further suggesting the involvement of both MCs and nerves in the typical secretory response to antigen.

Similar studies have been performed in Ussing chambers specially adapted for trachea (Bienenstock, Perdue, Blennerhassett, Stead, Kakuta, Sestini, Vancheri, & Marshall, 1988; Sestini, Dolovich,, Vancheri, Stead, Marshall, Perdue, Gauldie & Bienenstock, in press). Once again doxantrazole, but not cromoglycate, abolished the short circuit current changes observed after addition of antigen to chambers containing the trachea of previously immunized rats. In addition, rats which were capsaicin treated within twenty four hours of birth (rendering them deficient in SP) had diminished responses to antigen although the levels of IgE antibody to the immunizing agent was equivalent in capsaicin and non-treated animals. Once again a role for both mucosal MCs and nerves in the response to antigen was implicated.

It is evident that previous Ussing chamber studies have implicated mast cells in the changes associated with antigen challenge to the intestine. Inasmuch as Experiment 8 indicated that intestinal mucosal mast cells were activated by conditional stimuli for antigen, we wondered if the CR extended to the intestinal epithelium. That is, mast cells were activated by the CS, but we did not know whether this activation produced a physiologically detectable CR. Experiment 9 examined this issue.

6.2: Experiment 9

Methods

Thirty three male Sprague Dawley rats were individually housed and given free access to food and water. Animals weighed 250-300 grams at the time of sensitization. Animals were divided into three groups: paired (n=9), unpaired (n=12) and positive control (n=12). Animals were sensitized, infected, trained, and bled 24 hours prior to challenge in the manner described in Experiment 8. The animals were challenged, however, with the CS (paired and unpaired) or CS+US (positive control) at a slightly shorter interval after the last training day. All animals in Experiment 8 were challenged on Day 60 (post sensitization). Animals in Experiment 9 were challenged on days 55 (post-sensitization; 11 animals), 56 (11 animals), and 57 (11 animals) in order to facilitate performing the subsequent Ussing chamber tests. That is, it was not feasible to mount the tissues of more than three animals in Ussing chambers on any given day. Therefore, squads of animals were run; all manipulations were constant across squads. One animal from each group (paired, unpaired, positive control) was run on each day, with order of testing counterbalanced across groups.

On challenge days, animals were reexposed to either the CS or CS+US and returned to their home cage, as in

Experiment 8. Animals were anesthetized with urethane (50% in saline v/v, 1 ml/ 0.1 kg) approximately 30 minutes after antigen challenge. When animals did not respond to a foot-pinch, the intestinal cavity was opened and a length of jejunum (beginning 5 cm from the ligamnet of Treiz and extending for approximately 15 cm) was removed. Blood was drawn via cardiac puncture immediately after removal of the intestinal segment. The intestine was rinsed in oxygenated mannitol buffer, mounted on a glass rod, cut along the mesenteric border and stripped of muscle and myenteric plexus. Segments of tissue from each animal were then mounted in leucite chambers (approximately 4 chambers per animal), and the tissues were bathed in oxygenated, 37^{o}C, mannitol (luminal) and glucose (serosal) isotonic electrolyte buffers. Chart recorders monitored the short circuit current necessary to eliminate the potential difference across the tissue.

Several measures were obtained from the tissues mounted in the Ussing chambers. Conductance (G) of the tissue was monitored throughout the time the tissue was mounted. As well, basal Isc was monitored. Antigen (100 ug) was added after the tissue had stabilized (conductance constant across time, small fluctuations in short circuit current) or at twenty-five minutes post mounting (if the tissue had not stabilized by this time). Sensitized tissue

responds to *in vitro* antigen challenge with a relatively rapid increase in Isc. Absolute response to antigen was recorded by subtracting the pre-challenge Isc from the post-challenge Isc. Response to transmural stimulation (square wave current, 10Hz, 10mAmp, 5 sec duration) applied across the tissue at fifteen minutes after antigen challenge was also recorded.

Results

The results are summarized in Table 6.1. Criteria for inclusion required that tissues have conductances less than 50 mS/ 0.6 cc^2 tissue, and demonstrate responsiveness to transmural stimulation indicated by a change of at least 5 uAmp in the Isc. Of the tissues included, there were no significant differences between groups in response to TS or in conductance of tissues ($\underline{F}s < 1$). This suggests that the patterns of results described below are not the result of tissue damage or of general changes in quality of the tissue.

Anaylsis of variance indicated that basal short circuit current levels were not statistically significantly different between groups ($\underline{F}[2,25] = 1.13$, $\underline{p} = 0.33$). Not surprisingly, however, unpaired animals had the lowest basal short circuit currents (64 $\pm$ 4.3 uAmp/cc^2), while positive control animals had higher Isc levels (76 $\pm$ 4.8 uAmp/ cc^2), and paired group animals had intermittent basal

Table 6.1: Experiment 9. Responsiveness of intestinal segments after conditioning with EA.

Group	N	Baseline Isc (uA/cm^2)	EA Peak (Δ Isc)	G (mS/cm^2)
Positive	9	76 $\pm$ 4.8	23 $\pm$ 4.2	36 $\pm$ 1.9
Paired	9	69 $\pm$ 5.7	58 $\pm$ 5.2	37 $\pm$ 2.5
Unpaired	11	65 $\pm$ 4.3	40 $\pm$ 6.3	36 $\pm$ 1.5

Isc levels (69 $\pm$ 4.7 uAmp/cc^2). Unpaired group animals did not receive antigen *in vivo* on the test day, and the CS which they did receive had no signal value. Therefore tissues from these animals provided baseline information of Isc levels in a non-secretory state. The positive group animals indicated the effect of *in vivo* EA challenge, which is an increased secretion, translated into increased Isc levels (using a t-test, the difference between the unapired and positive control animals is significant, t = 2.67, p = 0.05). Tissue from paired group animals appeared to be in an intermediate state of secretory activity.

Analysis of variance indicated a significant difference in responsiveness to EA added *in vitro* ($F[2,25]$ = 6.7, p = 0.005). Independent t-tests indicated that the positive control group animals were less responsive to EA *in vitro* than either the paired or unpaired group animals (t = 2.16, p= 0.05). This suppression of responsiveness is not surprising in view of the fact that the Isc levels indicate that the tissues from positive control animals were already in a secretory state. What was surprising was that paired group animals had greater changes in Isc to EA than unpaired group animals (t = 2.03, p = 0.05). That is, the tissue responded to antigen more vigorously than tissue from unpaired group animals. Thus the effect of the CR was not to reduce responsiveness of the tissue (the

unconditional effect) but to increase tissue responsiveness (CR opposes the unconditional effect). It appears that the effect of the CS in the paired animals was to over-compensate for the unconditional reduction in responsiveness which EA *in vivo* produces.

Discussion

These results support the hypothesis that the conditional response observed after exposure to a cue for EA is physiologically significant. Alth ough further studies will be necessary to ascertain the importance of the apparent dissociation of the conditional immunological response (CR mimics UR, RMCP II released into blood) and the conditional physiological response (CR opposes UR, tissue responsivness supramaximal), we have shown that a physiological CR is present after conditioning with the cue and EA.

Further work with this model may provide critical insights into the ways in which the nervous and immune systems interact to induce physiological alterations. Many questions remain to be addressed. First, studies are needed to ascertain whether chloride ion secretion or sodium ion absorption is responsible for the observed alterations in Isc. Ion flux studies, in which radioactive sodium and chloride are added to the Ussing chambers would address this question. Although chloride ions have been

shown to be largely responsible for alterations observed in other studies, those results are not generalizable to this model. A further question concerns the optimal time to observe the conditional response. In previous pilot studies, we obtained very different results in the paired group at short and long delays. An obvious question is whether increasing the interval of time between CS reexposure and *in vitro* antigen challenge would enhance or reduce the sensitivity of the tissue to antigen challenge *in vitro.* Another question concerns the role of nerves in the observed alterations. Addition of nerve blockers, such as tetrodotoxin, may be useful in elucidating the role of nervous activity in this system.

Our results indicate that a consideration of mast cell - nervous system communication may be important for understanding and effectively treating a range of diseases. Future studies to determine the molecular bases responsible for the communication between the nervous system and the mast cell may provide clues to understanding the variety of diseases mentioned. Although we have not determined what nervous system factors are acting in our model, we do have a system in which it may be possible to ask these sorts of questions. This approach may ultimately serve as a template for future studies into the mechanisms mediating CNS-IS communication.

CONCLUSIONS

The previous chapters have described a number of studies in which classically conditioned responses have been shown to exert effects on IS activity. Chapters three and four described studies in which the unconditional stimulus was a drug, the immunosuppressant cyclophosphamide. Contrary to several prior reports, we have shown that a variety of CSs can elicit CRs which attenuate the immunosuppressive effect of CY. Studies of this sort primarily address issues which are important for conditioning theories. The contrasting results from our studies compared to those of others highlight, but in no way solve, one of the central issues in conditioning today: the nature of the conditional response. Certainly other studies of physiological conditioning have attempted to address this problem (see Eikelboom & Stewart, 1982; Siegel, in press), but the issue has remained difficult to resolve.

The issue of the nature of the CR is an important one from more than an empirical perspective. If this work is to become integrated into clinical practice, it will be important to have some power to predict when a CR will

mimic or oppose the UR. Arguably the most important implication of this work is that procedures of this sort (pairing neutral cues with immunomodulating agents) may someday be used to enhance or attenuate the immunomodulatory effects of therapeutic drugs. In order for conditioning to become an integral part of chemotherapy, however, it will clearly be important to have some indication whether the CR will act to oppose or enhance the drug effect. Either augmentation or attenuation of the drug effect may be the desired response depending upon the reason for administration of the immunomodulatory agent. Ideally, we will be able to determine the conditions necessary (nature of the CS, perhaps) to produce a CR in the desired direction. As yet, however, there remains no theory with the power to reliably predict whether the CR will act to oppose or mimic the UR.

The studies in chapters 5 and 6, in which conditioned mast cell activity was examined, tell us less about conditioning phenomena, and more about the ways in which the CNS and IS may communicate and affect surrounding tissues. Further studies will be necessary to determine the precise form of messenger used by the CNS to communicate to the IS, but our studies have at least provided an immune cell which is clearly receptive to CNS signals. Studies may provide information indicating the

nerves involved in any hardwired communication system that may exist between the CNS and mast cells. Selective nerve blockers such as atropine and guanethidine, as well as the substance P depletor, capsaicin, may isolate the nerve type, if any, involved in communicating with the mast cells. These studies may allow us, for the first time, to analyze the mechanisms employed by the CNS to communicate with one component of the IS.

The studies in which antigen was employed as a US may be important in understanding and treating a variety of diseases. The results of Experiment 9 supported the hypothesis that the CR was physiologically significant. Although early reports have suggested a role for the nervous system in a variety of mast cell-associated diseases, the empirical data to support the clinical observations was lacking. Evidence that, in fact, nervous system processes are important in a variety of immune-associated diseases suggests that a behavioral approach to the treatment of these diseases might be beneficial. Claims that behavioral approaches can "cure" diseases, or that behavioral treatment should substitute for medical intervention are extreme. A reasonable hope is that behavioral approaches may be employed in conjunction with standard treatment to reduce the severity of diseases such as asthma, allergy, and a host of others.

Studies examining the role of immune cell - nerve communication may also suggest directions for research into understanding and treating a variety of diseases which have previously been viewed as primarily immune system disorders. In effect, diseases which have long been viewed as autoimmune diseases might be better perceived as nervous system disorders. A "normal" immune system may appear to be functioning improperly if it is receiving abnormal signals from the CNS. If it is the case that aberrant nervous system messages activate immune cells, then a reasonable therapeutic approach might be to block the lines of communication between immune cells and nerves. To fully understand and treat certain autoimmune diseases, increased attention will have to be paid to the nervous system factors which may be playing a role in the pathogenesis of these diseases.

While studies of the sort described in chapters 3-6 do not provide much insight into the workings of the IS itself, they do highlight several questions about this system which remain to be addressed. If the IS is subject to the same influences of conditioning as many other systems (gastrointestinal, thermoregulatory), is it the case that this system is under the same sort of homeostatic control as other physiologic systems? Questions concerning the regulation of the immune system as a system remain to

be addressed. It is likely that the elements which resulted in the view that the immune system was "autonomous" have also prevented the system from being subjected to the sorts of general analyses of homeostatic regulation that have physiologists have imposed upon other systems. As evidence now indicates that the immune system is not fundamentally isolated and different from other systems, further knowledge of this system may be obtained by subjecting it to these sorts of analyses. While many details are known about the cells and components of the immune system, little is known about the way the system functions as a whole; for example, does one component of the system become depleted if another is very active? Are atopic individuals more or less susceptible to infection? In addition, little is known about the influence of factors such as metabolic state of the organism on immune system functioning. Further work on the interaction of the IS with other systems may provide new insights into the immune system itself.

It has become clear that much remains to be learned about the systems considered by psychoneuroimmunologists. The studies described above have highlighted two main approaches to further studies of conditional immunomodulation. First, factors which influence the CS-US association, and resultant direction of the CR, must be

determined. That is, psychology has much to contribute to psychoneuroimmunology. As well, the physical mechanisms responsible for the CNS-IS communication that allows for conditional immune responses must be elucidated. Thus, neuro-immunology also has many questions to address. It may be that demonstrating that the CNS and IS communicate was in fact the easy part; what remains to be done are the technically difficult studies which will tell us precisely how these systems influence each other. It is not yet clear whether psychoneuroimmunology will emerge as a discipline capable of making important contributions to empirical knowledge and applied areas, or whether researchers in the area will find themselves suffering from the same difficulties as Dolin and his colleagues faced thirty years ago. At this point the area clearly has more questions than answers; it may be reassuring to note that other emerging areas have the same problems:

> *At every stage in the past and at every stage in the future, the advancing edge of knowledge in every field has been and will be in a state of confusion. There are phases when the emergence of a new technique or, more rarely, of a fertile generalization allows a swift development of a new area in which ignorance and confusion can be replaced by understanding and the possibility of control and utilization for the satisfaction of human desires. But the edge where ignorance lies beyond the zone of ad hoc hypothesis and inadequate experimental techniques is always there. Speculation and tentative generalization as well as the search for and development of new technical approaches are the*

legitimate weapons to take us further toward the always receding periphery.

F. MacFarlane Burnet (1958)

Enzymes, Antigen and Virus

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Appendix A:

Hemagglutination Technique

Citrated sheep blood was obtained from Woodlawn Farms (Guelph, Ont.). The red blood cells were separated and washed three times in saline. For inoculations, a 1% solution of sheep red blood cells (SRBCs) in normal saline (approximately 3 x 10 7 cells/ ml) was prepared. For use in the assay, a 10% solution of SRBC : normal saline was prepared.

Samples of blood were collected via cardiac punctures and were placed in heparin-rinsed test tubes. The blood was centrifuged and the serum removed. The serum was heated in a water bath for approximately twenty minutes at 56^{o} C. Serial dilutions were then performed in microtitre plates (Becton Dickinson), using a start volume of 100ul serum and 100ul normal saline in each well as diluent. Fifty microlitres of the 10% SRBC was then added to each well. The wells were covered with parafilm and left at room temperature for 3-5 hours, at which time the hemagglutination reaction was judged. Values were reported as log base 2 of the endpoint dilution values.

Appendix B

ELISA for RMCPII

An enzyme linked immunoassay (ELISA) for detecting RMCP II was modified from Miller et. al. (1984). Rats were anesthetized with ether, and blood was obtained from the retro-orbital plexus. Sera were collected and stored at -20° C. The wells of a tissue culture microtitre plate (Nunclon Delta) were coated with a 0.5 mg/ml solution of RMCP II in a 0.2 M carbonate buffer, pH 9.6. Samples and standards were diluted in PBS containing 0.3% w/v bovine serum albumin (Boeringher Mannheim) / 0.02% v/v polyoxyethelyne sorbitan monolaurate (Tween 20) / 0.02% w/v sodium azide and incubated for 16-24 hr with a diluted specific rabbit anti-RMCP II antiserum from which all activity against RMCP I had been removed by immunoabsorbtion. After extensive washing of the plates, 100 ml of samples and standards were placed in duplicate wells and incubated for 16-24 hr. Plates were again washed several times and rabbit antibody bound to the plate was detected using an alkaline phosphatase conjugated goat-anti-rabbit antibody (ICN) and a Na-p-nitro-phenyl phosphate substrate (Sigma). The absorbance in each well was measured on a Dynatech MR600 ELISA reader. Results

were calculated on the basis of a standard curve constructed using known concentrations of purified RMCP II. Both RMCP II and anti-RMCP II were generous gifts from Dr. R. Woodbury (Seattle).