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Classical Conditioning and Immune Reactivity in Rats

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CLASSICAL CONDITIONING AND IMMUNE REACTIVITY IN RATS

by

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Laura Anne Czajkowski

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ABSTRACT

Classical Conditioning and Immune Reactivity

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by

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Psychoneuroimmunology is an interdisciplinary area that examines the interaction between behavior, the central nervous system, and the immune system. Many investigations have utilized a taste aversion paradigm to examine the effects of classical conditioning on an immune response. The procedure generally consists of an animal ingesting a novel flavor, and then being made ill and immunosuppressed by injection of a pharmocological agent. The animal is provided access to that flavor at a later time. The rejection of the novel flavor on the test day is called taste aversion and the depressed antibody titer has been labeled conditioned immunosuppression.

The present research was designed condition a secondary immune response and expand the evaluation of such conditioning to include both antibody titer and affinity. The Enzyme Linked Immunoassay was also introduced as the procedure of choice to quantify immune

reactivity.

A depression in antibody titer and affinity was found following exposure to three of four test trials. Taste aversion did not correlate with the immune response as increased consumption of the novel flavor was exhibited on the third and fourth test trial.

In the second experiment, the dosage of cyclophosphamide was increased. A depression in antibody affinity was found after the third and fourth test trials, which was consistent with the results of the first experiment. Unlike the first experiment, a depression in antibody titer was not attained on test days. Although taste aversion was observed in the treatment group on three of the four test trials, it had extinguished by test four.

The results support the concept of conditioned suppression of an antigen specific immune response by exposure to the taste aversion paradigm. An important contribution of the present research was the use and modification of a precise and sensitive assay for quantification of titer and affinity; the demonstration of conditioned suppression in both antibody titer and affinity; and the demonstration of conditioned immunosuppression with a single component CS.

(183 pages)

CHAPTER I

INTRODUCTION

The conditioning of physiological activity has been a subject of continued interest in psychology since Pavlov's (1927) classic investigations of salivation. In classical Pavlovian conditioning an arbitrary stimulus, that is initially ineffective in eliciting a particular response, comes to produce that response after being paired with another stimulus that reliably elicits the response of interest (Terrace, 1972). The stimulus that produces a measurable response prior to conditioning is designated the unconditioned stimulus (US). The response elicited by a US is labeled the unconditioned response (UR). The stimulus that initially fails to produce a response until after it is paired with the US, is called the conditional stimulus (CS). The learned response to the CS is designated a conditional response (CR), and is measured in terms of latency and magnitude. Pavlov's original experiments examined the conditioning of salivation. Meat powder (US) placed on a dog's tongue elicited the UR of salivation. After a number of pairings between a bell (CS, a stimulus that did not initially produce salivation) and the meatpowder (US), the bell (CS) presented alone produced a conditional salivation response (CR).

Since Pavlov's classic experiments, many investigators have examined the variables involved in classical conditioning (e.g., Mackintosh, 1983), developed theoretical models to account for this learning (e.g., Mackintosh, 1983; Rescorla, 1978), delineated the

necessary and sufficient conditions for producing many conditioned responses (e.g., Damianopolous, 1982; Rescorla, 1967), and examined the conditionability of a variety of physiological responses (e.g., Davey, 1981).

The control and regulation of physiological responses through classical conditioning has included the conditioning of immune reactivity (Spector, 1987). Conditioning experiments on immune responses were initially conducted by Soviet investigators in the early twentieth century. These early investigators suggested that all physiological processes--if not directly regulated by the CNS--did in fact have CNS involvement. A variety of immunological responses were found to be influenced by classical conditioning.

Recent immunological as well as behavioral and brain science research supports the concept of an interactive process between the CNS and immune functioning (Ader, 1981). Evidence suggests a role for hormones and neurotransmitter substances in the modulation of immunolgical reactivity. Extensive documentation exists of sympathetic and parasympathetic innervation of lymphoid structures (Felten, Felten, Carlson, Olschowka, & Livnat, 1985; Williams, Peterson, Shea, Schmedtje, Bauer, & Felton, 1981). The involvement of endocrine and neurochemical influence in immune system function is further supported by findings that circulating neuropeptides such as Beta-endorphins influence immune responses (Smith, Harbour-McMenamin, & Blalock, 1985). Behavioral data also exists that supports the premise of an interaction between the brain and the immune system

(Bartrop, Lazarus, Luckhurst, Kiloh, & Penny, 1977; Monjan & Collector, 1977; Solomon, 1969). A significant amount of literature has been devoted to the study of stressful events and immune functioning in animals (Ader & Cohen, 1984; Solomon & Amkraut, 1981). Experiments which examined the effect in animals, who lacked control over aversive stimulation, resulted in depressed immune reactivity and increased tumor growth (Laudenslager, Ryan, Drugan, Hyson, & Maier, 1983; Visintainer, Volpicelli, & Seligman, 1982). Other studies have examined the association between personality factors, life events, and experimentally induced or spontaneously occurring disease processes in man (Fox, 1981; Plaut & Friedman, 1981; Solomon, 1987). Interest in this type of research has continued to expand and results suggest the existence of a complex interaction between the CNS and immune system. These experiments which directly condition immune reactivity have supported the position of an interaction between the CNS and immune functioning. Current interest in the regulation of immune reactivity has resulted in the labeling of this field as psychoneuroimmunology (Ader & Cohen, 1975).

Ader and colleagues have reported that the pairing of an initially neutral stimulus (CS) with a pharmacological agent (US) that produced immunosuppression, resulted in a conditional immunosuppressive response (Ader & Cohen, 1975; Ader, Cohen, & Bovbjerg, 1982; Bovbjerg, Ader, & Cohen, 1982; Rogers, Reich, Strom, & Carpenter, 1976). For this discussion, immunosuppression refers to the depression of immune function in terms of antigen specific

antibody production. The initial altering or suppression of the immune response is artificially induced by the administration of a cytotoxic drug used as the US (Webb & Winkelstein, 1980). The results of many experiments substantiate the finding of the conditionability of antibody production with a taste aversion procedure (Ader & Cohen, 1975, 1982; Ader, Cohen, & Bovbjerg, 1982; Bovbjerg, Ader, & Cohen, 1982; Bovbjerg, Cohen, & Ader, 1980; Cohen, Ader, Green, & Bovbjerg, 1979; Rogers et al., 1976; Wayner, Flannery, & Singer, 1978). The antibody titer was reduced when the animals were reexposed to the flavor CS after it had been associated with the cytotoxic US.

Taste aversion, or flavor conditioning, was initially reported by Garcia and Koelling (1966). The procedure consisted of pairing a novel flavor in a liquid (interoceptive stimulus) with an agent that produced gastrointestinal distress. In this classic experiment the animals subsequently avoided that flavor, and it was concluded that the rats had associated taste with illness. Experiments on conditioned immunosuppression frequently utilized the taste aversion procedure. A novel flavor (CS) is paired with a pharmacological agent that concurrently suppresses the immune response and induces gastrointestinal distress (US) (e.g., Ader & Cohen, 1975). The subjects are subsequently reexposed to the CS and tests are conducted to measure fluid intake (to determine taste aversion) and, more importantly, to measure antibody production.

Although the current research provides evidence in support of conditioned suppression of antibody titer, a thorough experimental analysis has not been completed. The conclusion of a causal relation between the conditioning and lowered antibody titer is perhaps premature due to the lack of procedural consistency across studies, frequent equivocal results regarding the taste aversion and conditioned immune response, and the questionable specificity of the measurement procedure for quantifying antibody titer. Whether the results of these investigations do in fact demonstrate classically conditioned immunosuppression remains to some extent an empirical question. The conditioning of an immune response is presumed to involve a complex interaction among neurochemical, endocrine, immune, and central neural systems (Ader & Cohen, 1985). Assuming that the immune response is conditionable, other investigations will be required to determine limits, parameters, and the necessary and sufficient conditions to produce the response.

The purpose of this research was to examine the effects of a one trial association between a novel flavor (CS) and cyclophosphamide (US, the chemical agent that induces both gastrointestinal illness and immunosuppression) on a secondary immune response elicited by injection of bovine serum albumin (BSA, the antibody inducing antigen). An alternative and reportedly superior procedure (Clark & Engvall, 1980) for accurately quantifing antibody was used to measure the conditioning effects on immune reactivity. One dependent variable consisted of a secondary immune response to

bovine serum albumin, which is a single protein antigen not used in prior investigations. BSA was selected to examine the specificity of the antibody response. A secondary immune response differs from a primary immune response in that the animal receives a second antigen challenge, the antibody response appears more quickly, and is more specific as it consists predominantly of IgG, a class of immunoglobulin (Roitt, Brostoff, & Male, 1985). In addition to antibody titer, (antibody at a particular serum dilution tested at a specific antigen concentration), antibody affinity (antibody binding strength at a specified serum dilution across a range of antigen concentration) was measured. The assessment of both antibody titer and affinity addressed the possible limits of conditioning to a specific characteristic of antibody. The subjects in both experiments were also exposed to multiple test conditions (reexposure to flavor) to more thoroughly evaluate the specificity of the response to the CS, and to investigate the association between the taste aversion response and immune reactivity. Finally, control groups were included, that were not employed in previous research, to demonstrate that the conditioned response was due to the CS-US parameters.

CHAPTER II

RELATED LITERATURE

The Immune System

This discussion is restricted to fundamental concepts of immunology as they relate to the present research. For a comprehensive review on the subject, the reader is directed to excellent texts by Cooper (1981), Fundenberg, Stites, Caldwell and Wells (1980), Kimball (1983), and Paul (1984).

The immune system is a complex network of genetic, cellular and molecular components that serves to maintain homeostasis and health (Katz, 1980). The term immune is derived from the latin word, immunis, meaning exempt (Guralnik, 1980). Immunity implies resistance to attack from infectious agents (Fundenberg et al., 1980).

The major cellular components of the immune system are lymphocytes, plasma cells, and macrophages (Gilliland, 1983; Paul, 1984). These cell types are found primarily in lymphoid tissue and organs, including the thymus, lymph nodes, spleen, bone marrow, tonsils, Peyer's patches, appendix and tissue along the gastro-intestinal tract (Cooper, 1981). Individual lymphocytes are committed to respond to a limited group of structurally related antigens (Paul, 1984). Receptors on the membrane of lymphocytes are specific for determinants on an antigen (Paul, 1984). The ability of an organism to respond to antigens is possible due to the existence of a large number of different sets of lymphocytes

existence of a large number of different sets of lymphocytes each bearing receptors specific for distinct antigens (Kimball, 1983). Lymphocytes differ from one another not only in the specificity of their receptors but in functional properties (Roitt, Brostoff, & Male, 1985). Two separate immune systems exist, cellular and humoral, for the differentiation of lymphoid cells that circulate throughout the body and are involved in immune reactivity.

T Lymphocytes

One set of lymphocytes are T cells, or thymus derived cells, and are effective in cell-mediated responses (Kimball, 1983). Thymus lymphocytes derive from stem cells within hemopoietic tissue (Eisen, 1980). The T lymphocyte precursors enter the thymus and differentiate as cells with distinct functions (Eisen, 1980). Once the T cell matures within the thymus, the cell joins the peripheral pool of T lymphocytes. Several distinct peripheral T lymphocyte populations exist and can be identified due to the characteristic antigen receptors on their membrane (Paul, 1984). T lymphocytes consist of a series of subtypes, including some that mediate important regulatory functions. For example, specialized T cells produce humoral mediators of immunity called lymphokines that promote the differentiation of B cells into plasma cells and the secretion of antibody (Calabrese, Kling, & Gold, 1987). T cells also confer immunity against viral and fungal infections, cause delayed hypersensitivity reactions, and reject grafts of foreign tissue (Paul, 1984). Two major subsets of T lymphocytes include T Suppressor and T Helper cells. Suppressor T cells are believed to

block the differentiation of B cells into plasma cells and inhibit the activiy of Helper T cells (Kimball, 1983). Helper T lymphocytes assist other T or B lymphocytes in responding to antigen stimulation (Roitt et al., 1985). The principal subtypes of T lymphocytes include: T Helper cells, T Suppressor cells, cytotoxic T cells, and natural killer cells (Roitt et al., 1985).

B Lymphocytes

Humoral immunity is thought rendered by B lymphocytes which produce antibodies (Kimball, 1983). B lymphocytes are precursors of antibody secreting cells, and are derived from hemopoietic stem cells (Paul, 1984). The pre B cell is the initial member of the B cell series, and is found to lack receptors on its membrane for particular antigens. However, within the cell is contained at least one of the chains of the antibody molecule, the heavy immunoglobulin Ig chain (Roitt et al., 1985). Pre B cells develop into immature B cells that do not contain cyotplasmic chains of antibody molecules, however these cells do exhibit surface immunoglobulin (Paul, 1984). Mature B cells result from immature B cells, express receptors for antigens on their membrane, and are activated as a result of the binding of the antigen to their receptors (Paul, 1984). Once activated, the B cell interacts with helper T cells (B cell growth factor) and proliferates so as to increase the number of cells capable of reacting against the antigen. Proliferation results in an increase in the number of cells that may differentiate into antibody secreting cells, and into an expanded number of B cells similar to the original precursor that are now called memory B cells

(Calabrese, Kling, & Gold, 1987). These memory cells are activated upon second exposure to the same antigen, and this results in the rapid differentiation into antigen specific antibody secreting cells (Paul, 1984).

Antibody Structure

The products of antibody secreting plasma cells are immunoglobulins (Ig), which are groups of glycoproteins with several similar structural features (Roitt et al., 1985). The basic immunoglobulin structure consists of two identical light polypeptide chains and two heavy polypeptide chains linked together by disulphide bonds (Roitt et al., 1985). Each immunoglobulin molecule is bifunctional, with one region of the molecule concerned with binding to the antigen, and a different region that binds to the host cell (Roitt et al., 1985). Five distinct classes of immunoglobulins are recognized that are determined by the heavy polypeptide chains. These classes include: IgG, IgM, IgA, IgE, and IgD. The immunoglobulins differ in structure, in their sites of origin, and in the mode of conferring immunocompetence (Spielberg, 1974).

The heavy and light chains of the immunoglobulin are composed of a series of domains consisting of amino acids. The aminoterminal domain or end is characterized by sequence variablity (V) in both the heavy (H) and light (L) chain and are labelled VH and VL regions (Paul, 1984). The rest of the molecule is thought to be a relatively constant structure (Paul, 1984). The sites at which the antibody binds to the antigen are located in the variable domains (Paul,

1984). Therefore, there are two antigen combining sites on each antibody molecule (Roitt et al., 1985). The determinants making up the antibody V region are termed idiotopes, and the determinants on the antigen molecule are called epitopes (Roitt et al., 1985).

Antibody and Antigen Interaction

The molecules that activate an immune response are called antigens. Once an antibody is produced, it binds to a particular part of the antigen called an antigenic determinant or epitope (Paul, 1984). The binding of antigen to antibody occurs by the formation of multiple non-covalent bonds between the antigen and the aminoacids of the binding site (Paul, 1984). The antigenic determinant (epitope) and the antibody combining site (idiotope) must have complementary structures in order to combine (Roitt et al., 1985). The total strength with which the site on the antibody molecule binds to the single antigenic determinant is termed the antibody affinity (Kimball, 1983). The serum that contains demonstrable antibody or antibody specific for one or more antigens is called antiserum (Stedman, 1982). The specificity of the antibody response refers to its ability to discriminate between antigenic determinants against which it was elicited, and other antigenic determinants or related structures (Roitt et al., 1985). The specificity of an antiserum is the summation of actions of the various antibodies in the total population each reacting with a different part of the antigen molecule (Kimball, 1983). The specificity of the antiserum can be increased by immunizing the

animal with a preparation that has been purified (Kimball, 1983).

Following antigen challenge, the antibody response consists of: (a) a lag phase - no antibody present; (b) a log phase -the antibody titer rises logarithmically; (c) a plateau phase - stabilization of antibody production; and (d) a decline - antibody is cleared, except for memory cells (Roitt et al., 1985).

After first exposure to a novel antigen, the immune response (whether cellular or humoral) is detected in several days. This initial response is termed a primary response and is generally a low level response that is sustained for only a limited time period, e.g., two weeks (Cooper, 1981). With a thymus dependent immunogen, IgM and IgG classes of antibodies are initially secreted. IgM is secreted first, followed by IgG as IgM concurrently decreases. The antibody response reaches a peak in approximately two weeks and then declines (Cooper, 1981). Following a second exposure to the same antigen, a more robust cell or humoral mediated response is observed. This has been referred to as a specificity of memory effect in terms of a secondary response (Cooper, 1981; Kimball, 1983). The latency of this response is brief compared to the primary response, requiring one to three days. A spectacular rise in the level and maintenance of the secondary immune response is also reported (Cooper, 1981). The production of antibody at this time can surpass that of the primary response, and is often 10 to 50 times greater. The antibody is of the IgG class and has a greater affinity for antigen than the antibody synthesized during the primary response (Cooper, 1981). In summary, the antibody level following a second antigen challenge

appears more quickly, presents for a longer duration, attains higher titer, and consists predominantly of IgG.

Regulation of the Antibody Response

The activation of an immune response requires a series of complex mechanisms that interact to protect the organism from the pathogen. The boundaries between humoral (B cell) and cellular (T cell) divisions of the immune response have become less distinct as each component has been shown to be dependent on the other (Calabrese et al., 1987). The interaction of T lymphocytes and B lymphocytes in the regulation of the immune response are exceedingly complex (Roitt et al., 1985). One of the most important regulatory functions of T lymphocytes is to cooperate with B cell activation in its proliferation, and differentiation into antibody secreting cells (Roitt et al., 1985). It is thought that B cell responses to most protein antigens are dependent upon T cell assistance (Paul, 1984). The extent to which B cells are involved with the activation of T cells is unclear (Paul, 1984).

For the purpose of this discussion, a basic model of immunoregulation is presented. The activation of a concerted antigen specific humoral response is contingent upon the initial recognition of the antigen by macrophages, T cells and B cells (Cooper, 1981). Initially, the macrophages process the antigen and display the antigen determinants to the T cells (Calabrese et al., 1987). The activated T helper cells assist B cells in the proliferation and differentiation of B cells into antibody secreting plasma cells. Additionally, T helper cells also mediate the production

of B memory cells that express receptors bearing a particular idiotope (Roitt et al., 1985). T suppressor cells are also activated and appear to inhibit the quantity of T helper cells that are available for the antibody response. T suppressor cells may also have a role in the regulation of B cells with the prevention of B cell activation or inhibition of B cell function after activation (Roitt et al., 1985). The T suppressor network also acts to suppress nonspecific T helper cells and T cells that participate in cellular immune responses. The subset of the T suppressor network appears to stop the immune response, after the organism has defended itself against the pathogen.

Cyclophosphamide and Immune Reactivity

Cyclophosphamide (CY) is a relatively potent immunosuppressive drug for T cell dependent antigens (Ghaffar, Sigel, & Huggins, 1985). The timing of the administration of CY and antigen stimulation appears to contribute to the overall suppressive effect, as less suppression is observed as the time interval between antigen exposure and cyclophosphamide injection is increased (Shand, 1979). CY has been reported to inhibit antibody synthesis, decrease delayed type hypersensitivity and T cell toxicity reaction, suppress natural killer cell cytotoxicity, and depress macrophage function (Shand, 1979). Suppressor T cells have been reported to be particularly sensitive to CY (Ghaffar et al., 1985). O'Reilly and Exon (1985) reported that the slow recovery of T suppressor cells from CY induced effects can also result in an enhancement of some immune responses. Although some study has been completed on CY and its

mechanisms underlying its action has yet to be identified (Shand, 1979).

Classical Conditioning

Classical conditioning has been viewed as the learning of relations between conditioned and unconditioned stimuli (e.g., Rescorla, 1981). The exact nature of the relation among stimuli that results in conditioning remains a controversial issue. Two models that attempt to explain the factors producing conditioning are the event contiguity (pairing) model (Gormenzano & Kehoe, 1975) and the contingency (correlation) model (Mackintosh, 1983; Rescorla, 1967, 1969, 1978, 1981). The contiguity model states that the temporal proximity between two stimuli is critical to conditioning. The contingency model emphasizes the informativeness or predictiveness of the CS-US relation. Damianopoulos recently (1982) reviewed both models and concluded that contiguity was the necessary and sufficient factor in classical conditioning, but a contingent relation between stimuli could serve as a modulating factor. Each model suggests specific control procedures to rule out nonassociative effects in classical conditioning. These controls are discussed briefly in the following section.

Control Conditions

Two nonassociative factors that may contribute to changes in responding to the CS are called sensitization and pseudoconditioning. In sensitization, the initial response to the CS is potentiated by prior exposure to the US alone (Mackintosh, 1974). In pseudoconditioning, the prior presentation of a US alone results in the subject responding to any stimulus as if it were a CS (Staddon, 1983). A variety of control procedures have been proposed and used to rule out these nonassociative effects to ensure that the recorded response is explicitly due to the CS-US relation. Standard control conditions include: CS-alone presentations; US-alone presentations; backward conditioning (US presented prior to the CS); explicitly unpaired (nearly random presentations of the CS with long intervals separating CS and US); and differential conditioning (two conditioned stimuli are available, a CS+ is paired with the US and a CS- is not). Proponents of the contingency model view the above controls as insufficient because a contingency between the CS-US is not explicitly removed. Rescorla (1967) favors the "truly random" presentation in which the CS and US are each randomly presented, yet no contingency exists between the stimuli although pairings may sometimes occur by chance. Most of these control procedures have been addressed in the literature in an attempt to support the explanatory models underlying classical conditioning. As the necessary and sufficient conditions (contiguity vs. contingency) for conditioning continue to constitute an unresolved issue, a pragmatic approach is to use as many controls as apply in

experiments that use classical conditioning procedures.

Taste Aversion Learning

Research in taste aversion learning was initiated by Garcia, Kimeldorf, and Koelling (1955), who demonstrated that when sickness was artificially induced in a rat following its ingestion of a novel substance (flavor), the animal would subsequently avoid that flavor. The taste aversion procedure consists of exposing the animal to a novel flavored substance and, after the animal has tasted the substance, inducing illness in the animal with an agent that produces gastrointestinal distress. Taste aversion is then determined by reexposing the animal to the flavored substance and measuring the amount of the substance consumed. This test consumption level is then compared to preconditioning levels or to consumption by control animals. A prominent characteristic of taste aversion learning is the consistency of the results across species. There is substantial evidence that a wide variety of species avoid ingestion of substances that have been paired with agents that produce gastrointestinal distress (e.g., Garcia, Rusiniak, & Brett, 1977). Taste aversion has been demonstrated with rats (Barker, Suarez, & Gray, 1974; Garcia & Koelling, 1966; Garcia et al., 1955; Miller & Domjan, 1981 and others); pigeons (Lett, 1980; Pounds, 1981; Westbrook, Clarke, & Provost, 1980); coyotes (Gustavson, Garcia, Hankins, & Rusiniak, 1974); opossums (Cheney & Eldred, 1980); quail (Wilcoxon, Dragoin, & Kral, 1971), as well as a variety of other organisms (e.g., Gustavson, 1977).

The interest in learning mechanisms in taste aversion was spurred by the findings of two investigations. Garcia, Ervin and Koelling (1966) reported that rats acquired an aversion to a novel flavor ingested prior to drug treatment or radiation exposure even when the interval between the taste and consequent malaise exceeded an hour. This apparent long-delay learning raised many questions as to whether the taste aversion paradigm represented actual classical conditioning. For example, optimal conditioning usually occurs when the time interval between the CS and US is very short (Fantino & Logan, 1979). The second finding unique to the taste aversion phenomena was observed by Garcia and Koelling (1966), who reported that flavor stimuli were more readily associated with toxicosis than with audiovisual cues, whereas audiovisual cues were more readily associated with peripheral pain produced by footshock than with flavor cues. These phenomena were consequently labeled the cue-consequence specificity effect, and challenged the idea that any arbitrary stimulus could serve as a CS. In this experiment, the authors paired saccharin flavored water with light and noise ("bright noisy water") with either x-irradiation (US), which produced gastrointestinal distress, or an exteroceptive foot shock (US). Flavored water and the x-irradiation are interoceptive stimuli, i.e., they impinge upon receptors monitored by the autonomic nervous system. Light, noise, and foot shock are exteroceptive stimuli; they impinge upon external receptor (Terrace, 1972). Garcia and Koelling (1966) examined which aspect of the multicomponent CS became

associated with the two types of aversive consequences. The findings clearly indicated that flavor was readily associated with the toxic x-irradiation (US). However, the audiovisual signal acquired no aversive properties following the single exposure to the x-irradiation US. When foot shock (US) was paired with the audio-visual CS, this CS acquired aversive properties; yet the flavor of saccharin was not associated with that US. Garcia and Koelling (1966) demonstrated that rats associated the interoceptive CS attributes (flavor) with the interoceptive US (x-irradiation that induced gastrointestinal distress); and conversely associated exteroceptive CS attributes (light, noise) with an exteroceptive US (foot shock). Opposite associations were not made. Other investigators (e.g., Domjan & Wilson, 1972; Garcia, McGowan, Ervin, & Koelling, 1968; Miller & Domjan, 1981) have also demonstrated the cue - consequence specificity effect in adult rats using single and multiple conditioning trials.

These findings generated debate as to whether the conditioning mechanism responsible for taste aversion adheres to the general laws of associative learning (Bitterman, 1976; Deutsch, 1978; Milkula, Leard, & Klein, 1977). Associations learned between the conditioned and unconditioned stimulus over extended intervals appeared to contradict the notion that optimal conditioning is obtained with close temporal contiguity between any arbitrary stimulus events (Gormenzano & Kehoe, 1981; Kimball, 1961). Additionally, the assumption that any stimulus can be conditioned to a specific unconditioned stimulus, the principle of Pavlovian equi-potentiality,

was also challenged by the findings of differential conditioning between specific cues and consequences in the taste aversion literature (Schwartz & Gamzu, 1977). Subsequently, some investigators suggested that a revision of the general laws was needed to accommodate these inconsistent findings (Best & Barker, 1977; Deutsch, 1978; Kalat, 1977; Kalat & Rozin, 1973; Logue, 1979; Rozin & Kalat, 1971; Seligman & Hager, 1972).

Presently, taste aversion learning is thought to involve associative processes, since many aspects of the phenomenon agree with existing general laws of learning (Domjan, 1980; Roper, 1983). It has also been demonstrated that taste aversion adheres to principles of conventional learning such as sensory preconditioning, higher order conditioning, and blocking (Dickenson, 1980). Numerous reviews of the issues are available (Logue, 1979; Revusky, 1977; Testa & Ternes, 1977). The present discussion is limited to issues regarding similarities between taste aversion and classical conditioning as they relate to mechanisms possibly governing conditioned immunosuppression.

Taste Aversion as Classical Conditioning

A question frequently addressed in the literature (Domjan, 1980) is whether taste aversion represents an association between the flavor (CS) and the aversive postingestional event (US). One approach examined the delay gradient between the CS and US and postulated that extensive delays between events will result in progressive decrements in taste aversion learning. Numerous investigations have reported orderly decrements in taste aversion

learning as a function of the interval between ingestion of a flavor and subsequent toxicosis (Garcia et al., 1966; Kalat & Rozin, 1973; Nachman, 1970; Revusky, 1968; Smith & Roll, 1967; Wright, Foshee, & McCleary, 1971). Such findings suggest the role of associative processes in taste aversion learning, because the association between the flavor (CS) and the illness-inducing agent (US) appears necessary for aversion learning (Domjan, 1980). Taste aversion experiments have demonstrated that "interfering" stimuli that are presented between the CS and US disrupt learning of the response as in traditional learning experiments (Revusky, 1977). Additionally, learning has been demonstrated without toxicosis using conventional visual signal stimuli over long delays between CS and US when the stimuli are highly salient and interfering stimuli are minimized (Lett, 1975). The data suggests that taste aversion learning is similar to classical conditioning in that optimal conditioning results when the CS-US interval is short, and no other stimuli are presented within the CS-US interval.

The observation that ingestion-related stimuli are favored as CS's in association with toxicosis and that audiovisual cues are favored as CS's in association with peripheral pain has been used to argue that taste aversion learning has unique properties. However, similar selective association effects have been observed in other experiments (Domjan, 1980). Testa (1975) observed that when the US was an air blast from the ceiling, a visual stimulus from the ceiling became more readily conditioned than a similar stimulus from the floor. LoLordo (1979) reported that pigeons' responses were

more readily conditioned to auditory stimuli than to visual stimuli in shock avoidance experiments. It was also reported that visual cues are favored over auditory cues in conditioning with food. In a number of second-order conditioning experiments, Rescorla and Furrow (1977) observed that stimuli that were similar in modality or visual characteristics became more readily associated than stimuli that were dissimilar. Domjan (1980) concluded that the cue-consequence specificity effects observed in ingestional learning with toxicosis are not unique, but appear to be a common characteristic of associative learning.

Other similarities between classical conditioning and taste aversion learning include: conditioned stimulus and unconditioned stimulus intensity effects; extinction; generalization; overshadowing; and blocking (Testa & Ternes, 1977). Research supporting these similarities is briefly discussed in the following section.

In classical conditioning the speed or strength of learning increases with the intensity of the CS as well as with the size of the reinforcing event, the US (Mackintosh, 1974). This has also been demonstrated in taste aversion, where aversion learning is an increasing function of the intensity of the taste CS (Nowlis, 1974) and the drug or radiation US (Nachman & Ashe, 1973). As with classical conditioning, the taste aversion response extinguishes when the flavor CS is repeatedly presented without aversive consequences (US) after conditioning (Garcia et al., 1955). Revusky and Bedarf (1967) observed that repeated exposure to the flavor CS without

aversive consequences prior to conditioning (the CS becomes familiar and safe) reduces the degree of aversion acquired. Preconditioning exposure to the US also interferes with subsequent aversion learning (Best & Domjan, 1979; Randich & LoLordo, 1979).

Gormenzano (1966) identified stimulus discrimination as one requirement necessary for associative mechanisms. The conditioned response (CR) occurs to the CS+ (paired with the US) and fails to occur to the CS- (the stimulus presented without the US). The evidence from the taste aversion literature results in differential aversion responses (Gillan & Domjan, 1977; Rozin, 1969). Stimulus generalization gradients were obtained where subjects who had been conditioned to avoid one taste did not avoid all other novel flavors (Domjan, 1975; Nachman, 1963).

Overshadowing is a phenomenon initially reported by Pavlov (1927). He conditioned a compound CS consisting of auditory and visual stimuli, then tested each component and found that only one component elicited a conditioned response. Pavlov concluded that control was dependent upon the relative intensity of the component stimuli. Other variables that have been reported to affect overshadowing include the predictive value of the components; that is, the degree to which each cue predicts the US and the amount of previous elemental training (Kamin, 1969). Overshadowing has also been demonstrated with taste aversion (Kalat & Rozin, 1973; Revusky & Garcia, 1970), and it appears that the overshadowing of one flavor by another is greater the more novel the overshadowing flavor.

Another observation of classical conditioning is that conditioning to one stimulus is blocked by the presence of other CS that are "better" predictors of the US (Rescorla & Wagner, 1972). This blocking phenomenon was initially reported by Kamin (1969). He found that when a previously established CS was compounded with another stimulus and paired with a US, the conditioned response was elicited only by the previously established CS on test trials with each element of the compound. That is, prior conditioning of a stimulus prevented a second stimulus from being established as an effective CS. One explanation for this effect is that a response does not condition to new stimuli when these stimuli provide no new information, i.e., are redundant (Rescorla & Wagner, 1972). Blocking effects have been demonstrated in taste aversion learning with exteroceptive cues and taste stimuli as blocking stimuli (Bateson & Best, 1979; Braveman, 1979; Domjan & Gemberling, 1980; Revusky, 1977). As in other classical conditioning work, taste aversion learning seems to be influenced by the extent to which the flavor CS is a reliable predictor of toxicosis relative to other tastes in the environment.

In summary, taste aversion learning remains a prominent field of investigation. Although some of the characteristics of this type of learning initially appeared to be unique, it has been demonstrated that taste aversion learning is more similar to classical conditioning than dissimilar. In a thorough review, Logue (1979) concluded that quantitative differences between taste aversion findings and results of conventional learning studies exist;

however, the mechanisms involved are not qualitatively dissimilar. Recent evidence suggests that the mechanisms in taste aversion learning can be incorporated into existing learning theory, and revisions are not warranted (Logue, 1979; Testa & Ternes, 1977).

CS and US Properties

One of the similarities between classical conditioning and taste aversion is that learning is a function of CS and US intensity (Testa & Ternes, 1977). Several studies have examined the effects of a variety of CS and US parameters on the strength of the conditioned taste aversion response. Bond and DiGiusto (1975) concluded that the strength of the aversion response was related to the amount of saccharin (CS) consumed prior to inducing illness. The dosage level of the US has also been studied by Wright, Foshee and McCleary (1971), who reported that animals receiving high doses of cyclophosphamide (an illness inducing drug) learned the aversion faster and their aversion response extinguished more slowly than animals receiving lower doses. Ader and Cohen (1981) examined the effects of changing the volume of sodium saccharin flavored solution (CS) on the acquisition and extinction of taste aversion. The US was cyclophosphamide (CY), which was administered by intraperitoneal (ip) injection thirty minutes after the rat subjects consumed one, five, or 10 milliliters (ml) of the flavored solution. The results were consistent with earlier findings in that the reduction in saccharin consumption following conditioning and resistance to extinction were related to the volume of saccharin consumed on the single conditioning trial. An unexpected finding related to the mortality

rates of some of the conditioned rats during extinction trials when the animals were re-exposed to the saccharin (CS). The first animals that died came from the group that received the largest volume of the flavored solution on the conditioning trial. Since CY is an immunosuppressive drug (it depresses the reactivity of the immune system), Ader and Cohen (1975) hypothesized that the taste aversion that had been conditioned by pairing saccharin and CY also resulted in the conditioning of the immunosuppressive effect of the drug. They speculated that the immunosuppression that occurred in response to the CS during extinction trials may have increased the susceptibility of the conditioned animals to pathogens in the environment. Based on this speculation, the conditionability of an immune response was, once again, a subject of research in psychology.

CHAPTER III

REVIEW OF THE LITERATURE

Introduction

One of the most puzzling relationships between behavior and the immune system is represented by the possible influence of classical conditioning procedures on immune responses (Ader & Cohen, 1981). The resurgence of interest in modifying immune reactivity has been directed towards clarifying the serendiptous observations of Ader and Cohen (1975). Conditioning of the immune response has been reported in terms of the enhancement of phagocytosis, increased nonspecific antiinflammatory responses, and in the suppression of antibody responses (Ader & Cohen, 1986; Ader & Cohen, 1982; Klosterhalfen & Klosterhalfen, 1983).

Conditioned Immunosuppression Protocol

The same general experimental design has been used in all conditioned immunosuppression studies. The protocol of the initial experiment by Ader and Cohen (1975) is described as the prototype example. Ader and Cohen's hypothesis was that the pairing of a novel flavor (CS) with a pharmacologic agent that both suppressed an immune response and produced gastrointestinal distress, would result in a depression of immune reactivity when the CS was subsequently presented.

Rats approximately three months of age, were individually housed under a 12h light/dark cycle. The animals had continuous access to food, but only 15 min of free water. This feeding and watering regimen was maintained throughout the experiment. Animals were randomly assigned to either conditioned, nonconditioned, or placebo groups. On the day of conditioning (Day 0), instead of tap water, the conditioned group received a 0.1% solution of sodium saccharin in tap water (CS), followed by an intraperitoneal (ip) injection of cyclophosphamide (CY), which was the US, 30 min after drinking. Each animal's dosage of CY was based on 50 mg per kilogram of body weight. Nonconditioned control rats were provided with tap water and injected with CY 30 min later. The animals in the so-called placebo group received plain tap water for 15 min and were injected with distilled water of a volume equal to the treatment injections of CY. All animals were exposed to the regular drinking format over the following two days. On the third day (a test day), all animals were administered an ip injection of sheep red blood cells, which served as an antigen to stimulate immune reactivity. Thirty min following this injection, randomly selected subgroups of conditioned and control animals were reexposed to either the saccharin or to plain tap water followed by an immediate ip injection of either CY or saline. The CS consisted of flavored water and an injection of saline, making the CS test a compound element. An illustration of the experimental procedures is provided in Table 1.

Table 1

Experimental Procedures (Ader & Cohen, 1975)

	Day 0-Cond				Day 3		Day 6	
Group	Sol	Inj	Subgroup	Antigen	Sol	Inj	Sol	Inj
Conditioned								
N = 67	SAC	CY	CS1	SRBC	SAC	SAL	Water	---
				SRBC	Water	---	SAC	SAL
			CS0	SRBC	Water	SAL	Water	---
				SRBC	Water	---	Water	SAL
			US	SRBC	Water	CY	Water	---
				SRBC	Water	---	Water	CY
			CS2	SRBC	SAC	SAL	SAC	---
Nonconditioned								
N = 19	Water	CY	NC	SRBC	SAC	SAL	Water	---
				SRBC	Water	---	SAC	SAL
Placebo								
N = 10	Water	DW	P	SRBC	Water	---	Water	---

Note. SAL - saline SAC - saccharin SRBC - sheep red blood cells

DW - distilled water CY - cyclophosphamide

The experimental groups included the following:

1. Conditioned animals: All 67 rats in this group were presented with a single drinking bottle containing the saccharin solution, followed by an injection of CY on the day of conditioning. Within this group there were four subgroups that were tested: CS1 animals were reexposed to the CS (saccharin + saline injection) on a single occasion either three or six days after conditioning; CS2 subjects were reexposed to the CS (saccharin + saline injection) on days three and six; CS0 animals were not reexposed to the saccharin flavor but received plain water followed by an injection of saline; and animals in the US group who received plain water followed by an injection of the CY.

2. Nonconditioned animals: The 19 subjects were presented with plain water followed by an injection of CY on the conditioning day. These animals were then exposed to saccharin followed by a saline injection as the CS on either day three or six.

3. Placebo: The 10 subjects in this group were exposed to plain water followed by an injection of distilled water on conditioning day. This group then received plain water during the fifteen minute drinking periods.

All animals were sacrificed on day nine, six days following antigen challenge, and trunk blood was collected for analysis. An aversion to the flavor was found in all conditioned animals reexposed to the SAC on one or two trials. A hemagglutination assay (e.g., Kimball, 1983) was used to determine the antibody level, and the presence of antibody was expressed in titers. Results indicated that

the placebo animals exhibited the highest antibody titer levels, which was expected since this group did not receive CY. The serum from the animals in the nonconditioned group who received a CY injection on conditioning day had a substantial titer level; however, probably due to the residual effects of the CY, the titer level was lower than that of the placebo group. This finding was statistically significant at the .05 alpha level, based on a two-tailed t test. There was no difference between titer level of the nonconditioned group and the conditioned sub-group (CSO) who were not reexposed to saccharin. No difference was observed between the conditioned animals exposed to saccharin on day 3 and those who were exposed on day 6, so this group was collapsed into a single group that received a single reexposure to the saccharin. The titer level of conditioned animals who received either a single or double exposure to saccharin was attenuated as compared to the NC and CSO groups. The lower titer level was significantly different from the above groups at the .05 alpha level. Ader and Cohen (1975) concluded that the association between saccharin (the CS) and cyclophosphamide (the US) enabled the CS to subsequently elicit a conditioned immunosuppressive response. The investigators also noted that the saline injection appeared to be an intergral part of the CS test complex. However, the failure to acquire a conditioned immuno-suppressed response in the CSO group suggests that the injection was not effective in eliciting a conditioned effect. Contrary to Ader and Cohen's (1975) conclusion of the necessity of the injection as part of the CS, their results demonstrate that the injection did not

acquire aversive properties. This finding is consistent with Garcia and Koellings' (1966) cue-consequence specificity effect, and supports the premise that flavor alone is associated with the initial toxicosis and subsequent conditioned effect.

Replications

Comparable findings of depressed antibody titer have been reported by Rogers, Reich, Strom, and Carpenter (1976), who replicated Ader and Cohen's (1975) initial investigation. However, in the former study the method for determining antibody titer was changed to a purportedly more sensitive procedure. The specifics of the hemagglutination microtiter assay were not described within the procedures. Their findings suggested that the antibody titer level of the conditioned group reexposed to the saccharin + injectin (CS) on one post-conditioning trial was slightly higher than the titers of nonconditioned animals and conditioned animals not reexposed to the CS (saccharin + injection). Only those conditioned animals presented with the CS on two post-conditioning trials showed a mean titer level that was lower than the mean titer level of the conditioned animals not reexposed to the CS and that of the nonconditioned group. This difference, using an analysis of variance and a one-tailed t-test, was statistically significant at the .01 alpha level. Taste aversion occurred in the three conditioned groups presented with the CS on one occassion, day 3. Mean consumption of SAC on test day was compared to baseline intake of SAC (day 0), and statistically significant differences were found. An interesting difference between Ader and

Cohen (1975) and Rogers et al. (1976) was that the conditioned animals in Ader and Cohen's study who were reexposed to the conditioned stimulus on two occasions continued to avoid the saccharin. Rogers et al. (1976) reported that the animals reexposed to the conditioned stimulus on two trials did not continue to avoid the saccharin. While Rogers et al. (1976) observed the taste aversion response to extinguish, the conditioned immunosuppressive response was only attained when the animal was presented with the conditioned stimulus on two occasions. An interesting question that had not been addressed is whether the behavioral aversion response is independent of the immune response.

To obtain additional information about the variables controlling a conditioned immunosuppressive response, Wayner, Flannery, and Singer (1978) conducted two experiments. In the first study, the experimental protocol was similar to Ader and Cohen (1975) with the following exceptions: the drinking period was extended to one hour; the male rats were three to four months of age; the animals were assigned to the various control/experimental groups based on weight; the sodium saccharin solution was changed to .125%; a group of rats exposed to the conditioned stimulus on three post conditioning trials was added; and this CS3 group was sacrificed nine days after the antigen challenge as opposed to six days. Results suggested that the aversion response to the saccharin was evident when the animals were exposed on one (day 3) or two (day 6) post conditioning trials. However, taste aversion extinguished for those animals who were reexposed to the CS on the third post-conditioning trial (day 9). The

mean SAC intake of the conditioned animals on test trials was compared to the baseline consumption of SAC on day 0. Statistical data were not provided to support the conclusions. In terms of the conditioned immunosuppressive effect, the titer levels of those animals exposed to the conditioned stimulus on one or two occasions were lower than that of the control groups. However, the titer level of those animals exposed to the conditioned stimulus on three post conditioning trials was similar to the control groups. These results leave open the question regarding the possible interaction of the taste aversion response with the conditioned immunosuppressive response. The results are similar to Rogers et al. (1976) in that conditioned suppression was attained after two presentations of the CS. The findings are also consistent with Ader and Cohen's (1975) results, as conditioned immunosuppression was found with one CS reexposure.

An experiment by Ader, Cohen, and Bovbjerg (1982) attempted to increase the effects of conditioning on immunological reactivity. The experimental protocol was similar to other investigations (Ader & Cohen, 1975; Rogers et al., 1976; Wayner et al., 1978). A thymus-dependent antigen, sheep erythrocytes was used, the CS consisted of a 0.1% sodium saccharin solution and the US was an ip injection of 75 mg/kg CY. The following factors were changed in this experiment: the CY dose level was increased; CS (saccharin + injection) reexposure test trials were presented in a preference context; antigen stimulation occurred either at ten, fifteen or twenty-five days after conditioning for specific groups; and serum

was collected from randomly selected animals from each group at four, six or eight days after antigen stimulation. The antibody levels were determined using the hemagglutination assay. The results showed that pairing of saccharin with CY was effective in conditioning an aversion to the flavor. The CS group consumed significantly less ($p < .01$) saccharin than the control groups (Placebo and CSO) on both test trials. In comparison with the nonconditioned animals, the conditioned animals reexposed to the CS had lower antibody titers, following antigen challenge, on days four, six and eight. An analysis of variance was not computed; however, a one-tailed t-test revealed that these results were statistically significant at the .01 alpha level on days four and six, and at the .05 alpha level on day eight. The investigators concluded that the findings supported the existence of conditioned immunosuppression. Although the findings are suggestive of a suppressive effect, the variables responsible for this effect cannot be identified. Experimental procedures were radically modified in this study in comparison to earlier studies, primarily in that the CS reexposure occurred before rather than at the time of, or after, antigen stimulation. Additionally, the CY level was increased and the interval between conditioning and antigen stimulation was expanded. Therefore, without further experimental analysis, the specific factors responsible for the effect cannot be determined.

In a second investigation, Wayner et al. (1978) used the standard experimental protocol except that a T-cell independent antigen, Brucella abortus, was administered to challenge the immune

system. The presentation of this particular antigen differed from the previous investigations since a T-cell independent antigen does not require T-cells to stimulate antibody production by B-cells. The purpose of this experiment was to further delineate the possible effects of conditioning primarily on B-cell function and to expand the generalizability of previous research by using a different antigen. A taste aversion response was reported; however, no difference was found in antibody titer levels between the conditioned and control animals. While the results suggested that conditioned immunosuppression may be limited to T-cell dependent humoral antibody responses, other variables such as the sampling time, the influence of the CY, and the antigen dose may have influenced results. A later experiment conducted by Cohen, Ader, Green, and Bovbjerg (1979) also examined the conditionability of a T-cell independent antigen in mice using hapten trinitropheny (TNP). The animals were exposed to a .15% saccharin solution followed by an injection of CY at a dosage level of 200mg/kg. The antigen was presented fourteen days after conditioning and all animals were sacrificed six days after antigen challenge. Additionally, the conditioned animals were reexposed to the saccharin solution on two occasions. The findings indicated that the CS2 group exhibited a taste aversion response as compared to the control groups during the first post-conditioning trial; that is, the first reexposure to saccharin. No data were provided on the saccharin intake levels during the second post-conditioning trial, so no conclusions regarding the interaction between the taste aversion response and a conditioned immunosuppressive response can be drawn.

The CS2 group antibody titer level was statistically different from control groups using a one-tailed t-test at the .05 alpha level. The authors concluded that the immune response to a T-cell independent antigen can be behaviorally conditioned in mice. Conclusions from this study cannot be confidently drawn as details about certain aspect of this experiment were not given (for example, sample size or assay procedures), nor were graphs included to illustrate experimental findings. The reported findings cannot be compared to those from the Wayner et al. (1978) investigation, since the experimental procedures and subjects were different.

O'Reilly and Exon (1985) investigated whether antibody production to T dependent keyhole limpet hemocyanin (KLH), delayed type hypersensitivity (DTH) to BSA, or natural killer cell toxicity to tumor cells could be altered by exposure to the conditioning of a flavorto an immunosuppressive agent. Of particular interest were the findings related to the conditioning of the antibody response to KLH. On the day of conditioning, rats received a .15% sodium saccharin solution followed by a subcutaneous (sc) injection of 50 mg/kg of CY. Fifteen days later, each rat was injected with KLH to induce a humoral immune response. The CS (SAC + injection) was presented 7 days after antigen challenge. A boost injection of the antigen was administered the following day to all animals. Three days following the antigen boost, the CS was again presented. The animals were sacrificed three days after the second presentation of the CS. The serum antibody levels was assessed using an ELISA, and antibody titer was the principle outcome measure. The results were indicative of a

taste aversion response on both presentations of the SAC in the treatment group. These findings were statistically significant at a .05 alpha level. However, the taste aversion response appeared to be undergoing extinction on the second test trial (day 26), as the consumption of SAC had increased. A conditioned immunosuppressive response was not found, as there were no differences between the treatment group and the relevant controls. The lack of conditioning was attributed to the residual effects of the CY on the control animals. This experiment is difficult to interpret as the graphs of the data on antibody titer do not match with the figure caption, nor with the text. However, it is interesting that the taste aversion response is present and the conditioned immune response is absent.

Taste Aversion and Conditioned Immunosuppression

The potential relation between the behavioral aversion response and the conditioned immune response has remained problematic. Bovbjerg, Ader and Cohen (1982) reported a disassociation between the conditioned taste aversion and the conditioned immune response of a graft versus host response, that was consistent with Rogers et al. (1976). A study that examined the effect of manipulating the interval between the CS and US (McCoy, Roszman, Miller, Kelley, & Titus, 1986), reported no differences in the magnitude of the conditioned suppression of plaque forming cell responses or in the taste aversion response. Bovbjerg, Kim, Siskind, and Wekslev (1987) hypothesized that the conditioned immune response would be stronger in those animals that exhibited a stronger taste aversion response.

Five week old mice served as subjects, and the antigen consisted of SRBC conjugated with 2, 4, 6 trinitrophenyl (TNP). A plaque forming cell assay was conducted on individual spleen cell suspensions. The experimental design consisted of a conditioned group presented with SAC followed (60 minute interval) by an ip injection of CY; nonconditioned animals given plain water followed by the injection of CY, and a placebo group given SAC followed by an injection of saline. The SAC + injection of saline (CS) was presented fourteen days later, followed two hours later by an the challenge with the antigen. Two and four days later all mice were again presented with the SAC + injection of saline (CS). The conditioned subjects were found to have significantly less plaque forming cells per spleen than the nonconditioned animals. The conditioned animals were also examined based on their total SAC consumption, and divided into two groups with weak and strong taste aversion. The results indicated that mice with the stronger taste aversion response exhibited less of a conditioned immunosuppressive response than the mice with the less robust taste aversion response. Statistical findings were not presented, nor were the results presented in graphs or in a tabular format. It was concluded that an interaction existed between the taste aversion response and conditioned immune response. The investigators speculated that the continued avoidance of the SAC masked the conditioned immunosuppressive response, as the subjects did not ingest adequate amounts of the SAC. This hypothesis is intriguing, however the empirical evidence to support this conclusion is lacking, and further research is warranted.

Summary

The resurgence in research efforts examining the conditionability of immune reactivity was generated by Ader and Cohen's (1975) initial publication on conditioned immunosuppression. The data accumulated on antibody mediated and cell mediated responses appears supportive of an interactive process between the CNS and immune processes (Ader, Grota, & Cohen, 1987). Subsequent research has replicated the findings on conditioning a humoral antibody response, however the generalizability of the findings is limited as the experimental conditions have varied across studies. As a relatively new area of research, the optimal experimental conditions and methodology that results in conditioned suppression or enhancement of immune functioning has not been identified or developed (Elkins, 1985). The effects of conditioning on the multiple components of the immune response have not been identified, nor have the pathways that may modulate the conditioned response been delineated. The measurement of the antibody levels has predominantly been restricted to an estimate of titer with the use of the hemagglutination assay. The limited sensitivity of this assay may also contribute to the reported small magnitude of conditioned effects (Ader & Cohen, 1981). There has been variability, within experiments across the critical comparison groups, on the number of CS presentations (Klosterhalfen & Klosterhalfen, 1985). A related issue is the continued use of a compound element CS (flavor + injection), as conditioned immunosuppression has never been reported in the CSO group who is presented with water + injection on test trials. It is speculated

that the conditioning is probably due to the flavor alone, and the injection may be redundant. The results have also been equivocal on the possible relation between the taste aversion and conditioned immune responses.

CHAPTER IV

STATEMENT OF THE PROBLEM

Introduction

Evidence exists that the immune system is integrated with other physiological systems such as the CNS and endocrine system (Neveu, Crestani, & LeMoal,1987). Substances under neural control such as hormones (Maestroni & Pierpaoli, 1981), neurotransmitters (Hall & Goldstein, 1981), and sympathetic nerve fibers (Williams et al., 1981) contribute to a neuro-immune regulatory network. Environmental events have been found to influence the activity of central norepinephrine (Anisman & Sklar, 1979; Cassens, Roffman, Kuruc, Orsulak, & Schildkraut, 1980), dopamine (Herman, Guillonneau, Dantzer, Scatton, Semerdjian-Rouquier, & LeMoal, 1982), acetylcholine (Hingtgen, Smith, Shea, Aprison & Gaff, 1976), and endorphins (Chance, White, Krynock, & Rosecrans, 1978). The findings of Ader and Cohen (1975) suggested that immunological responses were conditionable when the organism was exposed to a taste aversion paradigm. The possibility that learning processes modified immune responses provided additional evidence for the interaction between the environment, central nervous system and immune system (Ader & Cohen, 1985; Roszman, 1985). Since the immune system is modulated by CNS activity, then advances in psychoneuroimmunology require a more detailed analysis of the mechanisms by which environmental events influence CNS activity and induce changes in immune processes (Ader et al., 1987).

Experiments conducted in the area of conditioned immunosuppression of humoral immunity provide evidence that immune reactivity to an antigen can be depressed with a taste aversion paradigm (Ader & Cohen, 1982, 1975; Ader et al., 1982; Bovbjerg et al., 1982; Bovbjerg et al., 1980; Cohen et al., 1979; Rogers et al., 1976; Wayner et al., 1978). The experimental protocol in most studies consisted of a single pairing between a flavored liquid and an immunosuppressive agent, introduction of an antigen (generally an injection of sheep red blood cells), and subsequent measurement of antibody titer level following reexposure to the flavor + saline injection (CS). The hemagglutination assay was generally used to quantify antibody titer. This assay detects antibody to red blood cell antigens.

The reports of conditioned suppression of antibody titer have been replicated; however, as a relatively new area of research, controversy exists regarding the mechanisms producing the observed effect. For example, Kelley and Dantzer (1986) have generated a number of hypotheses to account for the the apparent conditioned change in antibody titer. These include: mediation by adrenocortical stress effects; interaction between brain and lymphoid cells that are activated during CS reexposure; or neuroanatomical pathways innervating cells producing hormones or neurotransmitters. The consistent recommendation included in any analysis of the CNS and immune system interaction (Ader & Cohen, 1985) has been the need for further research to: identify the components of the immune response

effected by conditioning, and to identify the neural pathways or mechanisms involved with the change in immmune reactivity.

Limitations

The extant research is limited by the following: the non-specificity of the immune response that was conditioned, the precision of the measurement, and the somewhat equivocal results. The following discussion reviews each of these factors.

Specificity. The effects of the conditioning protocol on the elements of the immune response and on the mechanisms that contribute to immune reactivity have not been defined. Based upon studies of humoral conditioning, it appears that the mechanisms regulating the conditioned immune response are perhaps non antigen specific (Ballieux & Heijnen, 1985). This assumption is supported by the observation that exposure to a CS-US combination prior to antigen stimulation leads to conditioned immunosuppression. The available research is inconclusive as to whether the effects of conditioning are confined to a direct effect on B lymphocytes. The study conducted by Wayner et al. (1978) did not find conditioned effects when the T cell independent antigen, Brucella abortus, was used. However, additional research on suppression of cell mediated responses (Bovbjerg et al., 1982; Gorczynski, Macrae, & Kennedy, 1982) has been more suggestive of the possible involvement of T and B lymphocytes.

The nonspecificity of the conditioned immune response is also observed with the measurement of a primary antibody response to an antigen composed of multiple complex proteins, as the dependent variable. The antibody titer measured, using a less sensitive assay, is most probably composed of classes of immunoglobulins (IgM, IgG, IgA, IgD) with the major proportion being IgM (Roitt et al., 1985). The specific immunoglobulin effected by conditioning has not been determined. The conditioning of a general immunoglobulin response is noteworthy, however it contributes little to elucidating the components of the immune response effected by classical conditioning.

Antibody Measurement. A procedural limitation to the accurate quantification of antigen specific antibody levels has been the use of the hemagglutination assay. This assay has been most frequently used in the existing literature to assess antibody titer to sheep red blood cells. Agglutination has served as a qualitative test, indicating the presence or absence of antibodies (Kimball, 1983). Because of its simplicity, hemagglutination has been widely used in research and clinical laboratories (Kimball, 1983). This measure is an easy technique to detect antibody in serum, however, it yields only a semiquantitative value for the interaction of antibody with antigen (Paul, 1984). Additionally, due to subjective estimates of the endpoint, the titer may vary by a factor of two (Paul, 1984). Therefore, the adequacy of this assay as a precise measure of antibody is questionable. Ader and Cohen (1985) have proposed that further study of the effects of conditioning on select aspects of immunity also requires a much more precise assessment procedure.

The development of the enzyme linked immunoassay (ELISA) provides a quantitative technique that is simple, extremely sensitive, precise, and allows for the assessment of multiple samples (Kimball, 1983; Paul, 1984). Due to its documented sensitivity, specificity, and quantitative accuracy, the ELISA was used for the measurement of antibody in the present research.

Reported Results. Research has supported the premise of conditioned suppression of antibody titer. However, the experimental conditions have not been uniform across studies, and therefore the conditioned immune response has at times appeared inconsistent. For example, the number of test trials which have resulted in a depression of antibody titer have been inconsistent. Ader and Cohen (1975) found a decrease in antibody titer following a single test exposure to the CS. However, Rogers et al. (1976) did not obtain conditioned suppression until a second exposure to the CS. With different experimental conditions O'Reilly and Exon (1985) were unable to acquire a conditioned immune response with KLH as the antigen.

The association between the taste aversion response and the conditioned immune response has been erratic. Conditioned suppression of antibody titer has been reported both in the presence and in the absence of the behavioral aversion response to the flavored liquid (Ader & Cohen, 1975; Rogers et al., 1976; Wayner et al., 1978). It is uncertain whether a conditioned alteration in immunological reactivity is contingent upon the initial demonstration of a taste aversion response.

Statistically significant differences between treatment and control groups have been small (Ader & Cohen, 1985), and there has been variability in immune reactivity within groups. It is hypothesized that the often observed marginal effects may be due to the magnitude of the standard deviation and possibly the accuracy of the hemagglutination assay. The inclusion of a more precise assay could result in substantial difference among the groups.

Summary

Prior research has demonstrated conditioning of antibody titer. However, the environmental factors that may produce optimal conditioning and attenuation of the immune response (e.g.,dose response relation of CS and US; interval between CS and US association); the specificity of the conditioning effect upon the immune response; and the multiple pathways by which conditioning may modulate the immunity have not been identified (Ader & Cohen, 1985; Ballieux & Heijnen, 1985). In addition, the sensitivity and accuracy of the measurement procedure used to quantify antibody levels remains an important issue. The use of the ELISA, that has been documented (Clark & Engvall, 1980; Kimball, 1983) to provide a rigorous and precise estimate of antibody presence, can potentially provide additional information regarding the magnitude and specificity of the conditioned immune suppression.

Research Design

The present research was designed to investigate the effects of conditioning on an antigen specific antibody response, that was T cell dependent. A repeated measures design (Ferguson, 1981; Winer, 1971) across groups was used to assess both immune reactivity and the taste aversion response. The dependent variables consisted of: the antibody titer (a single point measurement of IgG at a particular antigen concentration) and affinity (a measure of IgG across a range of antigen concentration) of a secondary immune response to BSA; and consumption of a liquid flavor as the measure of the taste aversion response. Data analysis consisted of Model I Anova for means and Scheffe' test to compare differences between all possible pairs of means.

Planned Experiments

Since taste aversion conditioning is a classical conditioning procedure (Logue, 1979), the pragmatic controls for classical conditioning (Rescorla, 1969) were included to demonstrate that the conditioned response was in fact due to the CS-US pairing. A single protein antigen (BSA) was used to generate an antigen specific antibody response. The secondary antibody response to BSA was monitored over six weeks to determine any pattern of response change. Antibody titer and affinity were quantified using the ELISA. The animals were exposed to multiple discrete trials to evaluate the effects of repeated CS presentations on the immune response over time, and to further investigate any interaction between the taste aversion response and immune reactivity. The CS

used in test trials was a single element (flavor) rather than a compound element (flavor and injection) often reported in the literature (Ader & Cohen, 1981).

The purpose of the following experiments was to evaluate the effect of a single trial association between a novel flavor (CS) and cyclophosphamide (US) on the generation of a secondary (IgG) immune response to bovine serum albumin (BSA). Lewis male albino rats (Charles River) were challenged with two injections of BSA to activate a IgG antibody response. The animals received a single pairing of a flavor and the cyclophosphamide, that suppressed immune reactivity and elicited gastrointestinal distress. The antibody titer and affinity of each subject was repeatedly monitored over a 42 day period. Test trials were initiated once the presence of antibody was established. Test trials were conducted at seven, fourteen, twenty-eight, and thirty-five days after the second antigen boost. Table 2 summarizes the general experimental procedures.

The principle differences between the present research and the existing literature include:

1. The use of a single protein antigen (BSA) instead of an antigen composed of multiple complexes of proteins, to examine the specificity of the response to the antigen.

2. The examination of a secondary antibody response as opposed to a primary antibody response, to evaluate the effects of conditioning on a specific class of immunoglobulin.

3. The monitoring of antibody titer and affinity in lieu of titer, to investigate the effects of conditioning on two parameters

Table 2

General Experimental Procedures

Event				Condit.		T1		T2		T3		T4
Days	0	7	10	14	17	21	24	28	31	35	38	42
BSA	250 ug chall.			125 ug boost								
Flav. (CS)				SAC or NaCL		CS		CS		CS		CS
CY (US)				inject.								
Serum Sample	+	+	+	+	+	+	+	+	+	+	+	+

Note. BSA = Bovine Serum Albumin

CY = Cyclophosphamide

CS = Conditioned Stimulus

US = Unconditioned Stimulus

SAC = Saccharrin

NaCL = Sodium Chloride

of the antibody response over time.

4. The use of the ELISA as opposed to the hemagglutination procedure, for the accurate quantification of antibody titer and affinity.

5. A single element CS (flavor) was used for the test trials instead of a compound element CS (flavor + injection).

Planned Tests

The following statements were formulated as guides to this research:

1. Following test trials (CS presentation), the antibody titer of the CS-US (treatment group) at an antigen concentration of 30 ng. will be significantly less than the US only group at the .05 alpha level.

2. Following test trials (CS presentation), the antibody titer of the CS-US (treatment group) at an antigen concentration of 30 ng. will be significantly less than the CS only group at the .05 alpha level.

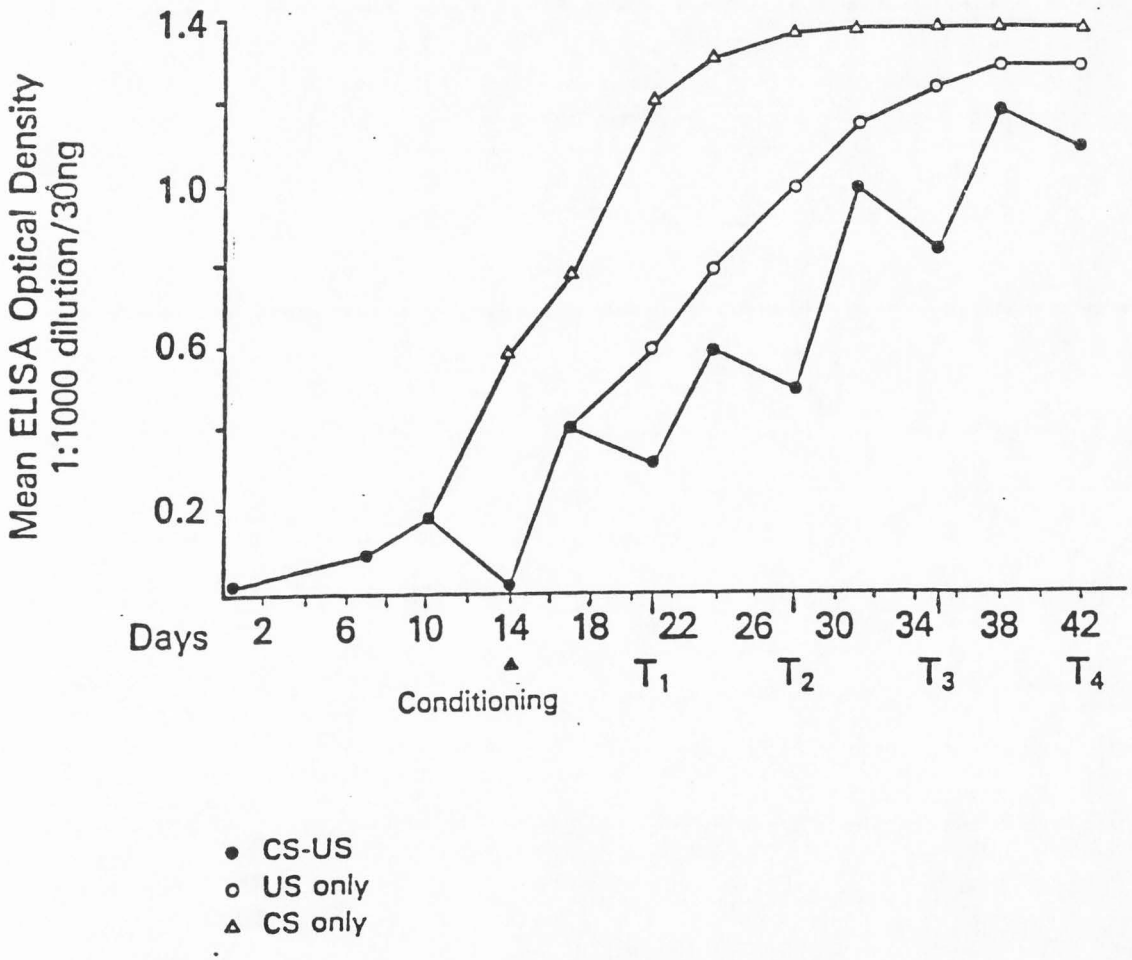
3. Following test trials (CS presentation), the affinity of the antibody response within the CS-US (treatment group) will be significantly less than the US only group at the .05 alpha level.

4. Following test trials (CS presentation), the affinity of the antibody response within the CS-US (treatment group) will be significantly less than the CS only group at the .05 alpha level.

5. A taste aversion response (reduced intake of saccharin) will be exhibited by the CS-US (treatment) group.

It was predicted that the CS Only group, who only received the injection of antigen, would exhibit a rapid increase of antibody. The US Only group who received the antigen and the CY, would show a lower antibody than the CS Only that was due to the suppressive properties of the drug. The treatment group (CS-US), who was presented with the pairing of the flavor and CY, would initally exhibit the same pattern as the US Only. However, with reexposure to the CS on test trials, the antibody level was predicted to decrease. Figure 1 provides an illustration of the expected outcome.

Figure 1. Expected Outcome - Antibody Titer.



CHAPTER V

GENERAL METHODS AND PROCEDURES

This section describes the conditions that were common to both experiments. A detailed description of the antibody assessment procedure, the Enzyme Linked Immunoassay (ELISA), is provided. Exceptions and additions to the General Methods and Procedures are described in the appropriate sections. The procedures differ in many ways from the research based upon Ader and Cohen (1975). The changes in methods were considered to be improvements in terms of basic immunolgy research (J. Rose, personal communication, April 1986).

Antigen

The antigen used to elicit the antibody response was bovine serum albumin (BSA) obtained from Calbiochem Laboratory (#126615, Albumin,Bovine, Purified-Lot # 506787). Purity was assessed by means of a SDS/PAGE electrophoresis and found to be greater than 98% pure. The BSA was dissolved in sterile distilled water (SDW) at 1 mg/ml; aliquoted and stored at -20 C. The same stock solutions were used throughout the immunization and ELISA testing portions of the experiments.

A critical component of this research was the identification of an immunization protocol that would result in a robust antibody response to BSA. The protocol chosen (Appendix A) was based upon pre-experimental findings that tested the BSA dosage and interval between injections with Lewis male albino rats.

Immunization consisted of an initial injection of 250 ug of BSA. The stock BSA solution was thawed and emulsified in an equal volume of Freund's Complete Adjuvant (Sigma Chemical Company). The addition of mycobacteria within the Freund's Complete Adjuvant (FCA) enhances the immune response (Maurer & Callahan, 1980). The total volume injected per rat was 0.50 ml. The injections were administered subcutaneously (sc) on multiple (3-4) sites of the back of each subject. An antigen boost (second injection) was administered fourteen days after the initial challenge injection. The stock BSA solution was thawed and 125 ug was emulsified in an equal volume of Freund's Incomplete Adjuvant (FIA). The injections were administered in the same manner as the initial challenge. This boost generated a secondary immune response that is characterized by a rapid increase in antibody levels consisting mainly of IgG. The total serum antibody levels attained with a secondary response are greater and more specific than that obtained in a primary response (Maurer & Callahan, 1980).

Flavor (CS)

In the first experiment, a 0.1% solution of saccharin (SAC) or a 0.2% solution of sodium chloride (NaCl) was used as the novel flavor. In Experiment 2, a 0.1% solution of SAC was used as the CS. Both flavors have been extensively utilized in the taste aversion literature (e.g., Barker, Best, & Domjan, 1977). The flavors were prepared in regular tap water and mixed by vortexing.

Immunosuppressive Agent (US)

Cyclophosphamide (CY) was the agent used to suppress antigen specific antibody production and induce gastrointestinal distress. This drug has been investigated extensively particularly with regard to its apparent selectivity for B lymphocytes (Turk & Poulter, 1972; Kerckhaerdt, Hofnois, & Willer, 1977; Shand, 1979) and certain T cell subsets (Turk, Parker, & Poulter, 1972). CY has been reported to suppress antibody production to BSA when given with a BSA challenge at a dose of 50 mg/kg (Koeller, Exon, Moore, & Watanabe, 1983). Preliminary research by this investigator identified the dosage of CY that would result in antibody suppression to BSA. The results are included in Appendix B, and show a depression of antibody titer seven days after the antigen boost.

The CY was obtained as a white crystalline powder from Bristol-Myers Oncology Division (Lot# 15-502). The CY was dissolved in sterile distilled water fifteen minutes prior to administration. The amount given was based on a dosage of 50 mg/kg of body weight. The total volume did not exceed 1 cc, and was administered via an intraperitoneal (ip) injection.

Serum Collection

A variety of techniques are available for obtaining blood samples from rats (Petty, 1982). Due to the multiple bleeding requirements and the small volume of blood required each time, the retroorbital plexus technique was selected. This procedure is reliable, safe, and conducive to procedures requiring small quantities of blood (Kraus, 1980). The procedure involved the

the following steps. Subjects were briefly and lightly anesthesized with ether. The rat was then manually restrained, and the skin to the eye tightened by the experimenter's fingers. The tension results in constriction of venous return and subsequent engorgement of the retroorbital plexus. The tip of a microcapillary pipette was inserted at the medial corner of the eye by gently rotating the pipette as it is advanced in order to rupture the venous plexus. Blood flow ceases when the pipette is removed and normal ocular pressure is restored. The blood collected in the pipette was then placed in microcapillary blood serum separators (B-D Microcontainers, #5960). The blood was spun in a Damon/IEC centrifuge for 15 minutes, and the serum withdrawn. The sera were then frozen at a 1:10 dilution in a phosphate buffered solution (PBS) and stored until analyzed.

Multiple time point serum samples were collected from each subject in both experiments. A pre-antigen serum sample was acquired to determine the presence of any antibody to BSA. This non immune serum served as a baseline measure and was also used as a negative control in the ELISA. A total of twelve samples were collected from each subject. A standardized schedule for bleeding was established from an earlier study, and used in both experiments. Although it has been reported that suborbital punctures could be taken on a daily basis, our findings indicated that multiple exposures to the anesthetic resulted in increased mortality, decreased blood volume, and increased scar tissue. Due to these limitations, blood samples were collected twice per week. Table 3 indicates the schedule of serum collection.

Table 3

Scheduled Serum Samples, Antigen Exposures, and Test Trials

Days	Event
-14	Subjects arrive, seven day acclimation to environment.
0	Pre-immune serum sample
	Initial challenge of BSA (250ug)
7	Serum sample
10	Serum sample
14	Exposure to experimental conditions
	BSA boost (125ug)
	Serum sample
17	Serum sample
21	CS Exposure - Test 1
	Serum Sample
24	Serum Sample
28	CS Exposure - Test 2
	Serum Sample
31	Serum Sample
35	CS Exposure - Test 3
	Serum Sample
38	Serum Sample
42	CS Exposure - Test 4
	Serum Sample

Measurement of the Immune Response

Immunoassays have replaced many other procedures to detect or quantify substances with biological and pharmacological properties (Clark & Engvall, 1980; Voller, Bartlett, & Bidwell, 1978). The high levels of sensitivity and specificity achieved with immunoassays result from the specific high affinity equilibrium binding of antibody to a low concentration of antigen and the use of sensitive detected labels to the antibody (Clark & Engvall, 1980; Kimball, 1983). The enzyme linked immunoassay (ELISA) was used in both experiments to quantify the antigen specific antibody response to BSA. Koeller et al. (1983) designated the ELISA as the method of choice for quantifying humoral immune responses due to its sensitivity, reliability, reproducibility, and automated quantification procedures.

The indirect method of ELISA was employed to measure antibody concentration. With this method the antigen (BSA) is attached by passive adsorption to the solid phase surface. A polystyrene plate was utilized (Dynatech Immulon-I) as it is reported to produce optimal binding (Clark & Engvall, 1980). The diluted test sera (rat serum against BSA) is then incubated on the plate and attaches to the antigen. The plate is then washed to remove unreacted serum components. A horseradish peroxidase enzyme conjugated to anti rat IgG secondary antibody is added and the plate is again incubated at room temperature. The immunoglobulin attaches to the antibody that is fixed to the antigen (BSA). The plate is washed again to remove any unreacted material and an enzyme color substrate (o-phenylenedia-

mine) is added. The degradation of the color substrate results in a color change that is an indication of the amount of peroxidase conjugated anti rat IgG that is bound to the rat antibody attached to the antigen on the plate. The color generation is therefore an indirect measurement of the amount of rat antibody bound to the BSA antigen. This procedure is illustrated in Figure 2.

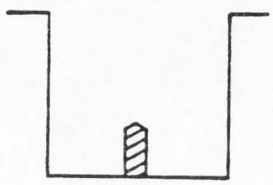
Extensive work was conducted over a six month period to systematize the above general procedure to attain a protocol that was replicable and optimal in its sensitivity to detect antibody titer and affinity. The initial objective was to determine antigen coating time and antibody serum dilutions in order to minimize non specific color generation. Since the ELISA is based on a color reaction, any variable that would contribute to extraneous color reaction was reduced. The following paragraphs address the steps that were adopted during this process.

A 96 well polystyrene microtiter plate was used as the solid phase carrier due to its reported reliability and adsorbability of proteins (Clark & Engvall, 1980). The binding capacity of the antigen to the plate was contingent upon: the ratio of the surface area to be covered to the volume of coating solution; the concentration of the adsorbing substance; the temperature; and the duration of the incubation period. It was determined that the maximal amount of antigen that adhered to the plate occurred after 72 hours at 4 degrees Centigrade. The optimal coating range of the antigen to generate a linear color development was below 125 mg. The antigen (BSA) was dissolved in Voller's Carbonate Coating Buffer and

Figure 2. Indirect Method For Assay of Antibody

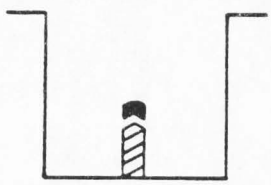
1. BSA (antigen) absorbed to plate

wash



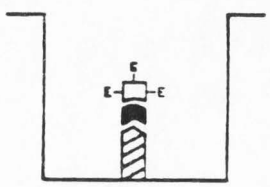
2. Add serum - 200 μl/well of antisera at 1:100 and 1:1000 dilution

wash

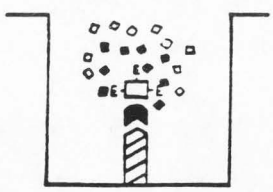


3. Add enzyme labeled antiglobulin - 200 μl/well GAR-HRP that attaches to antibody

wash



4. Add substrate - OPD 200 μl/well



5. Color = amount antibody present.

serially diluted to a coating range of 90 ng, 30 ng, 10 ng, and 3 ng. Each serial dilution of antigen was then pipetted onto the respective wells of the plate at a volume of 200 ul/well containing the stated antigen concentration.

A critical determinant of the reliability of the ELISA was the specificity of the antibody binding to the antigen. The inclusion of nonionic detergent in the antisera incubation and wash solution reduced the non specific binding of the rat antibody or the enzyme conjugated anti rat immunoglobin. PBS with 0.05% Tween 20 was the detergent that was used to control for non specific binding within the assay.

Preliminary testing indicated that antisera dilutions of 1:100 and 1:1000 yielded the most consistent antibody levels with the antigen coating range of 90 ng - 3 ng. On the day of the assay, each subject's 1:10 diluted serum was thawed and diluted with the PBS-Tween. The diluted serum was then pipetted onto the plate at a volume of 200 ul/well. Each subject's serum was evaluated in triplicate at each antigen concentration. The plate was covered and incubated for two hours at room temperature.

Three different commercially prepared enzyme conjugates to rat antibody were evaluated. Hyclone Affinity Purified Anti Rat IgG Horseradish Peroxidase labeled conjugate (GAR-HRP) was selected due to its purity and stability. The same lot number of GAR-HRP (lot# RDP 004) was used in both experiments. Conjugate was stored in concentrated form at 4 C, and diluted prior to usage in PBS-Tween. Optimal reactions were found between the rat antisera and enzyme

linked conjugate at a 1:2000 dilution. The diluted GAR-HRP was pipetted at a volume of 200ul/well. The plate was covered and incubated for two hours at room temperature.

The enzyme color substrate utilized to generate color change was o-phenylenediamine (OPD). Voller, Bidwell and Bartlett (1979) reported OPD as an optimal peroxidase substrate that yielded a high extinction coefficient at 492 nm. Optimal reactions were attained with 10mg of OPD + 1 ml methanol + 100 ml SDW + 10 ul of 30% hydrogen peroxide. A total volume of 200ul/well was added and incubated at room temperature for one hour. The color reaction was stopped with 50 ul of 8N H_2SO_4. Each plate was then read in a Micro Elisa Auto Reader (Dynatech) at 490 nm.

Variability in the amount of color generated on different ELISA testing days was reduced by including two columns of color reagent blanks on each series of ELISA assays. The reagent blank contained all the solutions except the rat sera. Any extraneous color is diminished by blanking the ELISA reader with these control wells, so that any day-to-day color fluctuations are controlled. Table 4 reviews the protocol for assessing antibody levels with the ELISA to BSA for both experiments.

Subjects

Lewis strain male albino rats from Charles River Laboratory, two months of age at the onset of each experiment served as subjects. Each rat was individually housed in a cage (10 in. by 8 in.) in a room with a 12 hour dark/light cycle. Room temperature was maintained between 66 and 69 degrees F. The subjects had continuous

Table 4

Bovine Serum Albumin ELISA Protocol

1. Coat plate with antigen (BSA). Antigen is diluted in Vollers Carbonate Buffer with initial concentrations of 90 ng/ul serially diluted x 3 to a final dilution of 3 ng/ul. Plate is incubated for three days a 4 degrees C.
2. Discard coating buffer solution and wash plate with 200 ul of PBS-0.05% Tween in each well. Repeat five times.
3. Add 200 ul of antisera diluted in PBS-0.05% Tween. Dilutions are at 1:100 and 1:1000.
4. Cover plate and incubate for two hours at room temperature.
5. Discard solution and wash as in #2.
6. Add 200 ul of GAR-HRP in PBS-0.05% Tween at a 1:2000 dilution.
7. Cover plate and incubate for two hours at room temperature.
8. Discard solution and wash as in #2.
9. Add 200 ul pf OPD substrate. Dissolve 10 mg of OPD in 1 ml methanol + 100 ml SDW + 10 ul 30% hydrogen peroxide.
10. Incubate one hour at room temperature.
11. Add 50 ul of 8N H2S04 to stop color development.
12. Read in Micro Elisa Auto Reader (Dynatech) at 490 nm wavelength.
13. Analyze and evaluate data.

free access to Wayne Rodent Blox provided in a container hanging on the front of each cage. Fluid was provided in an Oasis ball point bottle. Water availability was gradually reduced over a seven day period until the subjects consumed all their daily needs during a single 30 min period.

Water Presentation

The rats were individually housed and habituated to the environment for seven days with free access to food and water. Water availability was gradually reduced to a single 30 min period. The drinking period occurred at 7:00 AM throughout the experiment. All rats were presented with 100 ml of fluid each day. Daily fluid consumption was measured and recorded in milliliters.

Flavor Exposure

Rats were exposed to either a 0.1% solution of saccharin (SAC) or a 0.2% solution of sodium chloride (NaCl). Low concentration taste solutions were used to minimize flavor neophobia (Smith, 1978; Domjan, 1980). The novel flavor was presented for a 30 min period on the treatment day (Day 14, conditioning). The amount of fluid consumed for each subject was recorded in milliliters.

Cyclophosphamide Administration

In all experiments, CY was administered on the day of conditioning (Day 14) within 10 minutes after removal of the novel flavor. CY, diluted with sterile distilled water, was administered (ip) at a dosage of 50 mg/kg of body weight.

Conditioning

The single conditioning trial occurred 14 days after the initial BSA antigen challenge. Selected experimental animals (CS-US, treatment group) received a flavor CS during the 30 min drinking period. The bottles were removed at the end of the 30 min and within 10 minutes an ip injection of CY (US) was administered.

All rats received a second subcutaneous injection of BSA (125 mg) in order to generate a IgG antibody response. The antigen boost was administered on the same day approximately 60 min after the conditioning procedure.

Test Trials

The effects of treatment were evaluated by presenting the CS-US group (treatment) the same flavor as provided on the conditioning trial. Control animals were also offered the flavor. During these test trials, the CS was presented for 30 min at the usual watering time. Access to plain water for 30 min was provided 90 - 120 min after the test trial. This was done to reduce the possibility ofdehydration brought on by complete aversion to the flavor.

Serum samples were taken 8 - 10 hours after the test trial. A total of four test trials were administered, on days 21 (7 days after the antigen boost), 28 (14 days post boost), 35 (21 days post boost) and 42 (28 days post boost). The general experimental procedures are summarized in Table 2, page 52.

CHAPTER VI

EXPERIMENT 1

Purpose

The purpose of this experiment was to determine the effects of conditioning on an antigen specific antibody response, using a taste aversion paradigm. This experiment investigated the extent to which a previously neutral stimulus would elicit an immunosuppressive response following a single pairing with an unconditioned stimulus (CY).

This experiment differed from research procedures reported in the literature in a number of significant ways. First, the use of the antigen BSA, which is a single protein, as opposed to sheep red blood cells, which are a complex of proteins and glycoproteins. The reason for this alteration was to allow for an evaluation of the specificity of this type of immune conditioning. Second, the immune response elicited and evaluated was a secondary response to BSA (i.e., IgG), as opposed to a primary response utilized in the previous research (i.e., Ader & Cohen, 1975). Further, the affinity of the antibody response was examined in addition to the titer level. Third, the antibody response (titer and affinity) was monitored on a biweekly basis for six weeks in order to evaluate the time course of the effect. Fourth, the CS test consisted of a single flavor rather than a compound element (flavor + injection) used in prior research. Finally, the measurement of the dependent variable, the antibody levels, was completed using a more sensitive and precise

ELISA assay.

Subjects

Thirty-one Lewis male albino rats (Charles River Laboratory) approximately 60 days old served as subjects. They were individually housed as described in the previous chapter. A seven day habituation period was provided with free acess to water and food. The watering period was gradually reduced to 30 min daily. The rats were then assigned to the following seven groups: CS Only (1-5); US Only (6-10); CS+/CS- (11-15); 24 Hour Delay (16-20); No CS Test (21-22); Positive Control (23-24); and CS-US (25-31).

Serum Samples

Prior to group assignment, all subjects were evaluated for prior exposure to BSA. Procedures for serum collection were identical to that described in the previous chapter. All samples were collected between 19:00 - 23:00 hours, twice per week. A total of twelve serum samples was acquired from each subject.

ELISA

An ELISA was completed for each serum sample to determine antigen specific antibody levels. All assays were conducted at the Neuroimmunology Laboratory (Veteran's Administration Medical Center, SLC). The solutions used for the assay, and all assays were completed by the investigator. Rat sera that was positive and negative for antibody presence was used as an additional control for each assay. The procedures for the assay are reviewed in Chapter V and outlined in Table 4.

Briefly, a total of nine 96 well plates were run for each day's serum samples. Once each rat's antisera was diluted (1:100, 1:1000), the assay took approximately 10 hours to complete. A total of 21 assays were conducted for this experiment. Additional assays were completed in order to verify the results from the samples taken on Day 35. As a further reliability check, a random series of samples was rerun by qualified personnel within the laboratory.

Data Analysis

The planned comparisons or contrasts were analyzed using a Model I ANOVA for means with a correction in value for degress of freedom in order to account for unequal sample size and possible heterogeneity of variance (Hays, 1973). Post hoc comparisons were also conducted using a One Way ANOVA and Scheffe' test to compare differences between all possible pairs of means.

Statistical analyses were conducted using the SPSS/PC+ statistical and information analysis system (Version CP/X IBM/PC, SPSS Inc.). The immune response was measured by quantifying antibody levels to BSA using the ELISA. The optical densities for each subject at the serum dilution of 1:100 and 1:1000 were entered and coded. Each serum sample provided a total of 24 data points for each subject. This consisted of three reliability measurements at each antigen range (90 ng., 30 ng., 10 ng., 3 ng.) and across two serum dilutions (1:100, 1:1000). All analyses were conducted at 1:1000 serum dilution as the specificity of the immune response and linearity of the ELISA were best exhibited within this dilution.

The analysis that consisted of antibody titer at 30 ng/ 1:1000 serum dilution required that the three optical densities at 30 ng be entered for each subject within a group. The raw data were then analyzed and a mean titer response calculated for each group. An ANOVA of means across groups was then completed.

The affinity of the immune response was estimated from the slope of a regression plot of the binding capacity versus the log 10 antigen concentration (Hudson, 1986). The slope was attained for each subject for each test day by conducting a linear regression. The antigen range (90 ng., 30 ng., 10 ng., 3 ng.) was converted to a log 10 and fixed as the independent variable across time and subjects. The dependent variable was the optical densities attained at the serum dilution of 1:1000. It consisted of three replicate optical densities at each of the four antigen concentrations for each subject. Assumptions regarding independence, normalcy, and variance around the regression line were met. A mean slope was calculated from the regression lines for each subject in a group and a ANOVA of slopes across groups was performed. Additionally, post hoc comparisons were conducted on titer and affinity using a One Way ANOVA and Scheffe' test to compare differences between all possible pairs of means. The Scheffe' multiple comparison test was selected as it is reported to be a conservative measure (Norusis,1986); is applicable to groups of unequal size (Hays, 1981); and is relatively insensitive to departures from normalcy and homogeneity of variance (Hays, 1981).

Treatment (Conditioning)

On day fourteen, the following protocol was implemented. Instead of tap water, appropriate groups were offered water to which had been added 0.1% SAC or 0.2% NaCl. Following 30 min access to this fluid, rats in the treatment group (CS-US) and differential conditioning group (CS+/CS-), were each injected with CY (US). The dosage of CY was based on 50 mg/kg of body weight.

The CS Only group (1-5) was included to evaluate the effects of familiarity with the CS. On day 14, these rats were provided water to which SAC had been added. The US Only group (6-10) which was included to control for sensitizatiion by the US, was watered as usual followed by CY injection.

The CS-US Treatment group (25-31) was presented with SAC as the CS, followed within 10 min by an injection of CY, the US. The CS+/CS- group (11-15) served as a differential conditioning control. Saccharin flavored water was followed by a CY injection for rats 11, 12, and 13. Salt flavored water consumption was followed by CY injection for rats 14 and 15. The 24 Hour Delay group (16-20) was a control for the contiguity between the CS and the US. The flavor CS was SAC for this group and it was presented (Day 13) 24 hours prior to injection of the US (CY).

The No CS Test group of two subjects (21 and 22) was presented with the same treatment protocol as the CS-US treatment group. However, these subjects were not presented with the test condition. This group became a basis of comparison in terms of CS-US pairing and the subsequent nonstressed course of events. The

Positive Control (23 and 24) group received water on day 14 without presentation of the CS or US. These animals were included to determine the pattern of the antibody response to BSA without further experimental manipulation.

All subjects received an initial challenge (injection) of BSA on Day 0 and a boost (second injection) 14 days later. All injections were given subcutaneously as described in the General Procedures. All animals received the antigen boost approximately one hour after CY injections. Serum samples were taken ten to twelve hours after treatment. Table 5 presents the procedures for each group.

Test Trials

All rats received access to a single water bottle for 30 min each day (0700 hours), except on the treatment and test trials. The test trials occured on days 21, 28, 35, and 42. The first test was scheduled seven days after treatment because preliminary data indicated that the antibody response had increased by that time. Tests 3 and 4 were considered critical, as the residual effects of CY were reportedly eliminated by this time.

The test trials for CS Only, US Only, and 24 Hour Delay groups consisted of simple reexposure to the CS (SAC). The CS+/CS- group was offered either a CS+ or CS- in an alternating counterbalanced sequence across the four test trials. The animals in the No CS Test and the Positive Control groups were not given a flavor, but were offered regular tap water during the test trials. The CS-US treatment group was reexposed to the CS (SAC) across all test trials.

Table 5

Experimental Procedures in Experiment 1

		BSA				Condition				Test 1		Test 2		Test 3		Test 4	
	Days	0	7	10	13	14			17	21	24	28	31	35	38	42	
	Serum	+	+	+		+			+	+	+	+	+	+	+	+	
Group	N					Flv	CY	BSA		CS		CS		CS		CS	
CS Only	5	BSA				SAC	--	BSA		SAC		SAC		SAC		SAC	
US Only	5	BSA				WAT	CY	BSA		SAC		SAC		SAC		SAC	
CS+/CS-	5																
CS+ SAC	3	BSA				SAC	CY	BSA		SAC		NaCL		SAC		NaCL	
										NaCl		SAC		NaCl		SAC	
CS+NaCL	2	BSA				NaCL	CY	BSA		NaCL		SAC		NaCL		SAC	
										SAC		NaCL		SAC		NaCL	
24 HR.	5	BSA			SAC	WAT	CY	BSA		SAC		SAC		SAC		SAC	
No CS Tst	2	BSA				SAC	CY	BSA		WAT		WAT		WAT		WAT	
Pos Ctrl	2	BSA				WAT	--	BSA		WAT		WAT		WAT		WAT	
CS-US Trt	7	BSA				SAC	CY	BSA		SAC		SAC		SAC		SAC	

Serum was taken on test days between 19:00-23:00.

Dependent Variables

The dependent variables consisted of:

1. The consumption of CS fluid on test days measured in milliters.
2. The antibody titer response to BSA at 1:1000 serum dilution/ 30 ng antigen concentration.
3. The affinity of the antibody response as reflected by the slope at 1:1000 serum dilution/ 90, 30, 10, and 3 ng antigen concentration.

CHAPTER VII

RESULTS OF EXPERIMENT 1

Taste Aversion

All rats adjusted to the restricted water regimen and stabilized their intake and weight. Independent sample t tests were calculated to compare the mean consumption of saccharin flavored water between the CS-US treatment and the US Only group. The assumption of equal variances was met as tested by Hartley's F Max Test (Hays, 1973).

A significant aversion to the saccharin solution, (reduced intake), was observed in the treatment group (CS-US) on the first and second test trials. Statistical significance was attained between the CS-US treatment group and the US Only group at Test One ($t=4.84$, $df=10$, $p.<.001$), and Test Two ($t=5.822$, $df=10$, $p.<.001$). Statistically significant differences were not attained on Test Three (21 days post conditioning) or Test Four (28 days postconditioning). The increased consumption of saccharin on these tests indicated the extinction of the taste aversion response (Testa & Ternes, 1977). The mean intake of saccharin flavored water for CS-US group (treatment) and US only subjects is shown in Figure 3 and in Table 6.

Animals in the 24 Hour group (exposed to the conditioning protocol with a 24 hour interval between CS and US presentation), showed a mild decrease in the ingestion of the saccharin flavored water on Test 1, 2, and 3 (Figure 4). However, a statistically significant difference ($p.<.05$) between this group and the US only group was attained only on Test 2. The intake of water flavored

Figure 3. Mean Flavor Ingestion - Experiment 1

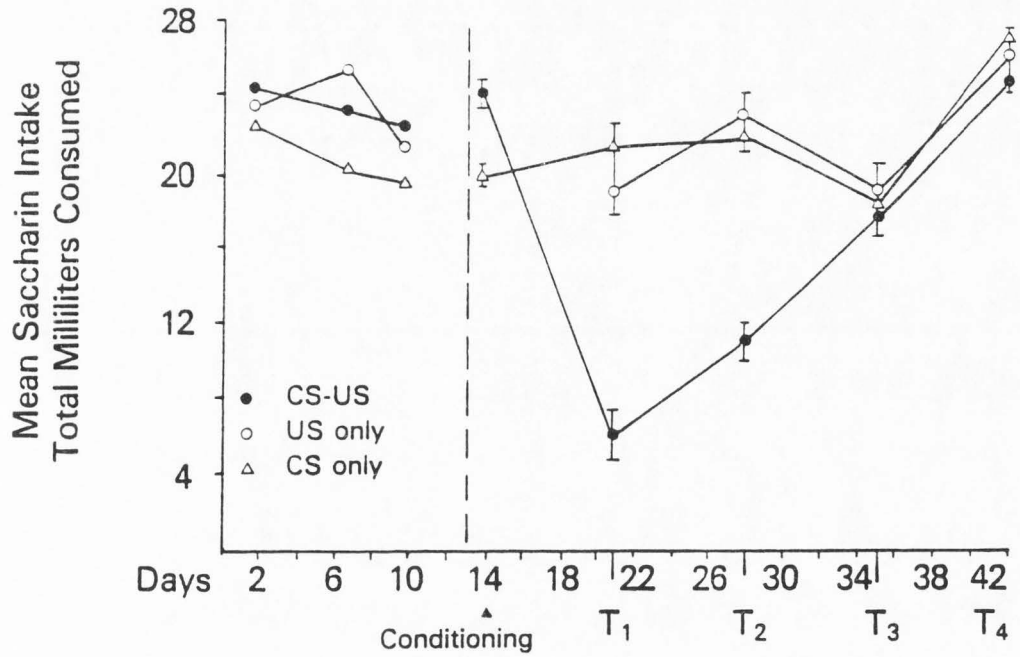


Table 6

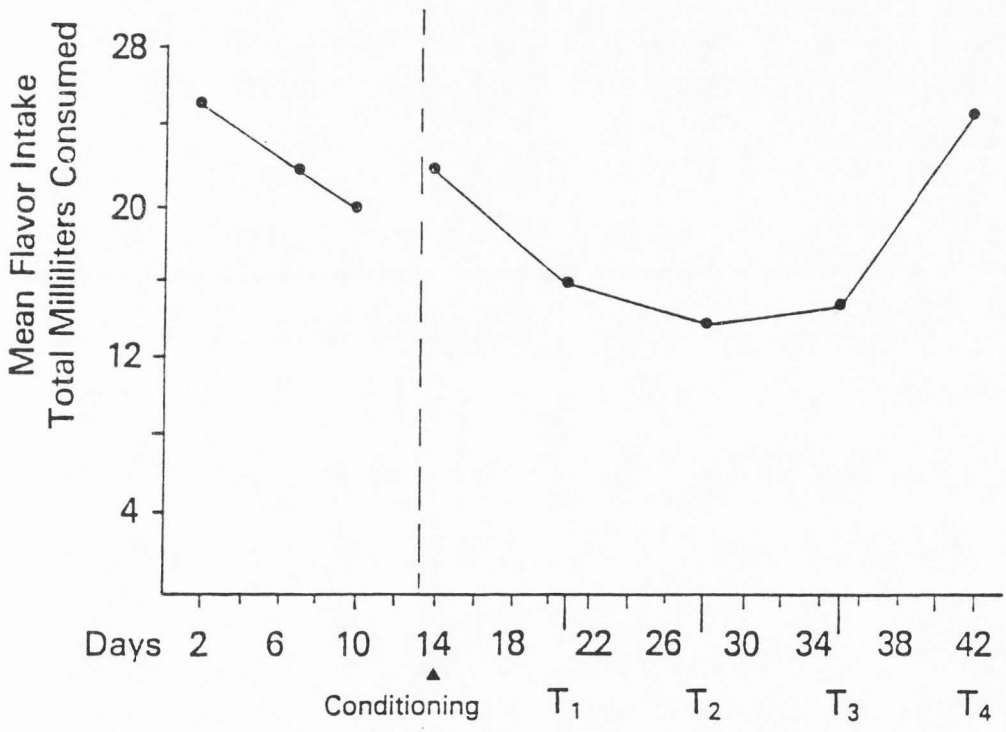
Mean Flavor Ingestion - Experiment 1

Group	N	Conditioning Day 14		Test 1 Day 21		Test 2 Day 28		Test 3 Day 35		Test 4 Day 42	
		X	SEM	X	SEM	X	SEM	X	SEM	X	SEM
CS Only	5	20	0.0	21	1.9	22	1.2	18	1.2	27	1.2
US Only	5			19	1.9	23	1.1	19	2.0	26	2.0
24 Hour	5	22	1.2	16	.98	14	.98	15	1.2	26	1.0
CS-US	7	24	.81	6	1.9	11	1.7	18	1.5	25	0.0

Note. X = Mean

SEM = Standard Error of the Mean

Figure 4. Mean Flavor Ingestion - 24 Hour Delay Experiment 1



with SAC for each animal within this group is shown in Table 7.

A formal analysis was not attempted with the CS+/CS- group because of the small number of subjects in each condition within this group (n = 3, n = 2), and the number of presentations of the CS+ was different from the other groups. The flavor intake data for this group is provided in Table 8, and visual examination suggests no consistent differential aversion. Decreased flavor intake is observed in animal 12 on Tests 1 and 3 compared to the preceding tests. Some aversion is also observed in animals 11 and 15 on Test 1, but not to the extent as animal 12. No aversion response is found on Test 3 (animals 11 and 15), nor on Test 4 (animals 11, 12 and 15). These data are graphed in Figure 5. Animals 13 and 14 were exposed to the CS- on the first test trial, and the observed decrease in flavor may be secondary to flavor neophobia (Figure 6). However, a clear aversion to the CS+ is observed in the second test trial in both animals. An increase in the consumption of the CS- is observed on the third test, and the aversion response to the CS+ is extinguished by the fourth test.

Antibody Titer

The means and standard deviations on antibody titer for each group in Experiment 1 are presented in Table 9. On Day 0, no statistically significant differences were present between the antibody titer of the CS-US group and various control groups. Differences

Table 7

Flavor Ingestion in the 24 Hour Delay - Experiment 1

Subject	Flv	Day 13	Test 1 Day 21	Test 2 Day 28	Test 3 Day 35	Test 4 Day 42
16	SAC	20	15	15	15	30
17	SAC	25	15	10	15	25
18	SAC	20	15	15	15	25
19	SAC	25	15	10	12	25
20	SAC	20	20	15	20	25
		$\bar{X}$ = 22	$\bar{X}$ = 16	$\bar{X}$ = 14	$\bar{X}$ = 15.4	$\bar{X}$ = 26
		sem = 1.22	sem = .986	sem = .986	sem = 1.25	sem = 1.0

$\bar{X}$ = Mean

sem = Standard error of the mean

Table 8

Flavor Ingestion in the CS+/CS- - Experiment 1

Subjects	Flv	Conditioning Day 14	Test 1+ Day 21	Test 2- Day 28	Test 3+ Day 35	Test 4- Day 42
11	SAC	20	10	15	15	25
12	SAC	27	5	15	10	25
15	NACL	20	15	10	20	25
			Test 1-	Test 2+	Test 3-	Test 4+
13	SAC	25	15	5	20	25
14	SAC	20	14	10	25	25

Figure 5. Mean Flavor Ingestion - CS+/CS-

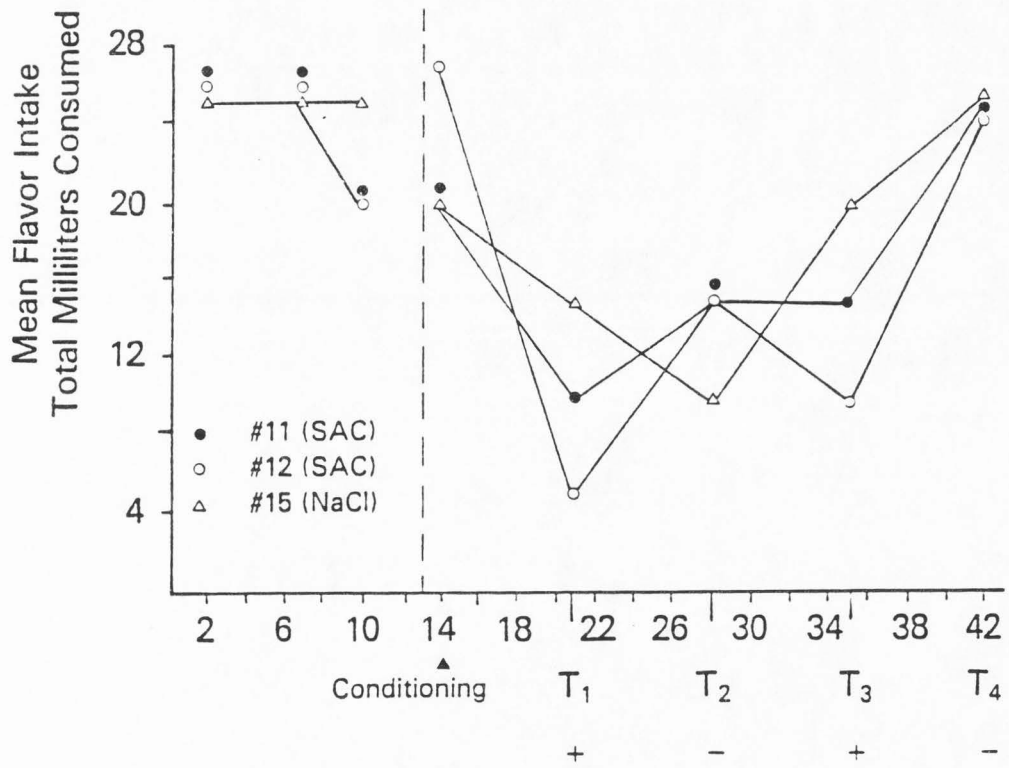


Figure 6. Mean Flavor Ingestion - CS+/CS-

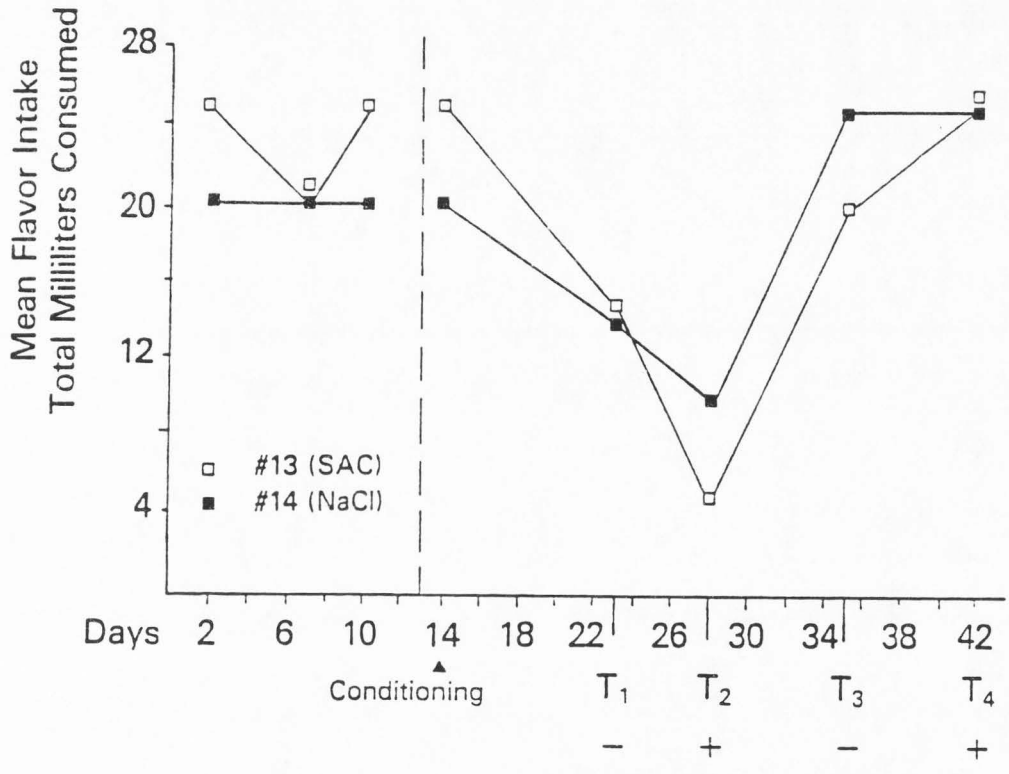


Table 9

Experiment 1 - One Way ANOVA Antibody Titer

	Boost Day 14				Day 17			
	N	X	SD	SEM	N	X	SD	SEM
CS Only	15	.0836	.0132	.004	15	.0607	.0143	.0037
US Only	15	.0962	.0156	.004	15	.0098	.0072	.0018
24 Hour	15	.1073	.0207	.006	15	.0100	.0103	.0027
Pos Control	6	.0948	.0054	.002	6	.0368	.0114	.0047
CS-US	21	.0826	.0129	.003	21	.0099	.0120	.0026
	F = 3.539				F = 43.80			
	df = 6				df = 6			
	p. = <.005*				p. = <.0001*			

	Test One Day 21				Day 24			
	N	X	SD	SEM	N	X	SD	SEM
CS Only	15	.4027	.1094	.0282	15	.5829	.1941	.0501
US Only	15	.2182	.0886	.0224	15	.6259	.2512	.0649
24 Hour	15	.2456	.0719	.0186	15	.8339	.1089	.0281
Pos Control	6	.5958	.1096	.0447	6	.9023	.1298	.0530
CS-US	21	.1940	.0681	.0149	21	.7197	.2742	.0596
	F = 28.4160				F = 8.2796			
	df = 6				df = 6			
	p. = <.0001*				p. = <.001*			

	Test Two Day 28				Day 31			
	N	X	SD	SEM	N	X	SD	SEM
CS Only	15	.6583	.1061	.0274	15	1.2785	.1278	.0330
US Only	15	.7483	.2434	.0628	15	1.1434	.1573	.0397
24 Hour	15	.7013	.1816	.0469	15	1.1135	.0377	.0097
Pos Control	6	1.0745	.0587	.0240	6	1.1765	.0838	.0342
CS-US	21	.8497	.1299	.0283	21	.9479	.2378	.0519
	F = 12.664				F = 16.8315			
	df = 6				df = 6			
	p. = <.0001*				p. = <.0001*			

	Test Three Day 35				Day 38			
	N	X	SD	SEM	N	X	SD	SEM
CS Only	15	1.0318	.1793	.0567	15	1.0265	.1821	.0420
US Only	15	.8651	.1325	.0419	15	.9431	.1984	.0512
24 Hour	15	.9243	.1257	.0397	15	.8875	.1607	.0415
Pos Control	6	1.2902	.1268	.0634	6	1.0450	.1078	.0440
CS-US	21	1.0555	.1467	.0392	21	.6569	.1162	.0254
	F = 23.413				F = 21.2571			
	df = 6				df = 6			
	p. = <.0001*				p. = <.0001*			

Note. * Statistical Significance

between the CS-US group and the CS Only group were expected after treatment, since the CS Only group did not receive CY, the immunosuppressive agent. There was a similar titer level between these two groups until day 17. The antibody titer in the CS Only group is graphed in Figure 7. Statistically significant differences were found between the CS Only and CS-US groups (Figure 7) on: day 17, CS Only greater than CS-US ($t=11.253$, $df=27$, $p.<.001$); day 21-Test 1, CS Only greater than CS-US ($t=6.539$, $df=22$, $p.<.001$); day 28, CS Only less than CS-US ($t=4.855$, $df=33$, p.,.001); day 31, CS Only greater than CS-US ($t=6.920$, $df=22$, $p.<.001$); and at day 42-Test 4, CS Only greater than CS-US ($t=5.749$, $df=31$, $p.<.001$). The results of the tests of significance are found in Table 10.

The critical contrast was between the the CS-US treatment group and the US Only group (Figure 7). It was hypothesized that the titer of the CS-US group would be suppressed following reexposure test to the flavor. Visual inspection of Figure 7 revealed a similar titer pattern between these two groups until day 24 (11 days post conditioning). At this point, the titer of the US Only subjects showed a rapid rise until day 31. A gradual rise in titer is observed in the CS-US group until day 35, when the titer decreased. Statistically significant differences were found on days 31, CS-US significantly less than US Only ($t=2.992$, $df=34$, $p.<.005$); day 35 - test 3, CS-US significantly greater than US only ($t=3.317$, $df=21$, $p.<.005$); day 38, CS-US significantly less than US only ($t=5.008$, $df=21$, $p.<.001$); and day 42-test 4, CS-US significantly less than US only ($t=3.963$, $df=31$, $p.<.001$). Table 11 summarizes the results of

Figure 7. Mean Antibody Titer in Experiment 1

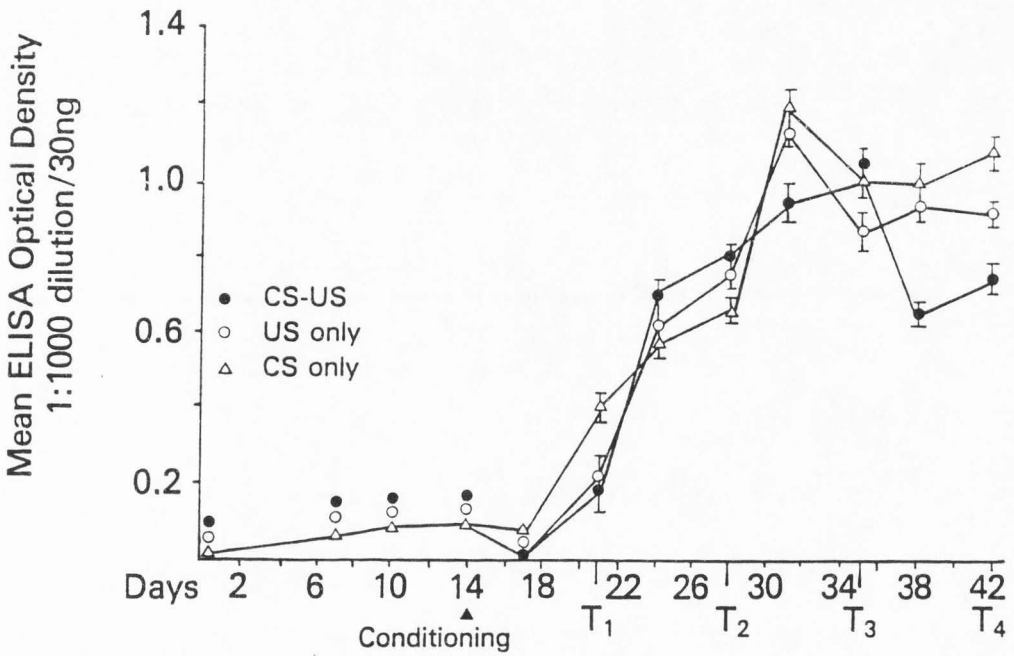


Table 10

Experiment 1

Results of Tests of Significance - Antibody Titer

CS Only and CS - US

	Boost Day 14	Day 17	Test One Day 21	Day 24
CS Only/ CS-US	t = .176 df = 19 p. = .862	t = 11.253 df = 27 p. = <.001*	t = 6.53 df = 22 p. = <.001*	t = 1.756 df = 34 p.= .088

	Test Two Day 28	Day 31	Test Three Day 35	Day 38
CS Only/ CS-US	t = 4.885 df = 33 p. = <.001**	t = 6.920 df = 22 p. = <.001*	t = .244 df = 17 p. = .735	t = 6.920 df = 22 p. = <.001*

	Test Four Day 42
CS Only/ CS-US	t = 5.749 df = 31 p. = <.001*

* CS only significantly greater than CS-US
** CS only significantly less than CS-US

Table 11

Experiment 1

Results of Tests of Significance - Antibody Titer

US Only and CS-US

	Boost Day 14	Day 17	Test One Day 21	Day 24
US Only/ CS-US	t = 2.246 df = 19 p. = <.05	t = .018 df = 33 p. = .986	t = .887 df = 25 p. = .384	t = 1.065 df = 32 p. = .295
	Test Two Day 28	**Day 31**	**Test Three Day 35**	**Day 38**
US Only/ CS-US	t = 1.471 df = 20 p. = .157	t = 2.992 df = 34 p. = <.005*	t = 3.317 df = 21 p. = <.005**	t = 5.008 df = 21 p. = <.001*
	Test Four Day 42			
US Only/ CS-US	t = 3.963 df = 31 p. = <.001 *			

*** CS-US significantly less than the US Only.**
**** CS-US significantly greater than the US Only.**

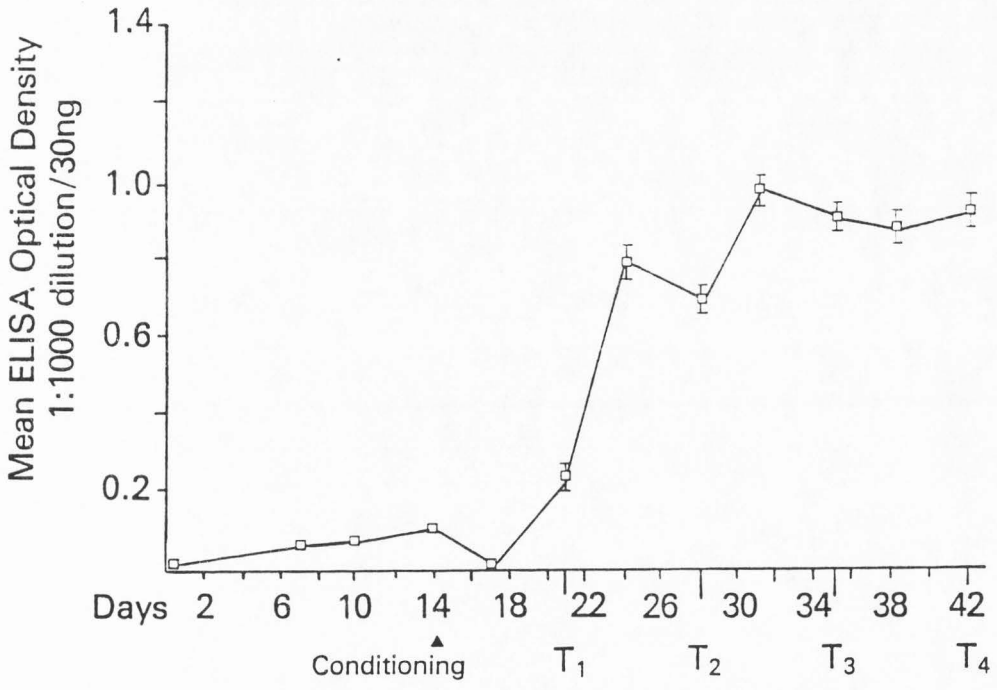
the ANOVA between the CS-US and US only groups. Figure 7 is a graphic comparison of the mean antibody titers of the CS Only, US Only, and CS-US treatment groups.

Post hoc analyses were also completed on titer using a One Way ANOVA and Scheffe' multiple comparison tests. The ANOVA indicated statistically significant differences among all pairs of means after day 14. Table 9 provides a summary of the ANOVA for titer in Experiment One. The Scheffe' test indicated statistically significant differences at the .05 alpha level between the CS-US and CS Only groups on day 17, day 21-test 1, day 31, day 38, and day 42-test 4. At all of these points, the antibody titer of the CS-US group was significantly less ($p.<.05$) than the antibody titer of the CS Only group.

Statistical differences between the antibody titer of CS-US and US Only groups were maintained at day 31, day 38, and day 42-test 4. At each of these time points the CS-US group was significantly less than the US Only at the .05 level of confidence. Significant differences were also attained between the antibody titers of the CS Only and US Only groups on days 17, 21, and 28. The antibody titer was greater in the CS Only group.

Statistical analyses were not completed with the two subjects in the No CS Test group. One of the subjects did not respond to the second antigen boost and no antibody production appeared to have been generated. Significant differences in titer between the 24 Hour and US Only groups were not found (Figure 8), even though a flavor aversion was observed on Test 2 (Figure 4).

Figure 8. Antibody Titer in 24 Hour Delay - Experiment 1



Each animal in the CS+/CS- group was presented with the CS+ a total of two times during the course of the experiment. Animals 11, 12, and 15 were presented with the CS+ on Tests 1 and 3; and animals 13 and 14 were presented with the CS+ on Tests 2 and 4. Figures 9 and 10 illustrate the titer pattern for each animal, and Table 12 summarizes the data. On day 17 all titers decreased to zero, then increased on day 21. Animals 11 and 12 when presented with the CS+, displayed a titer less than the mean of the US Only group. Taste aversion was observed in both of these animals (Figure 5). The titer of animal 15 was greater than that in the US Only group on day 21, Test 1. On Test 2, animals 11, 12, and 15 were presented with the CS- and a rise in titer was found. Test 3 showed a behavioral aversion response to the flavor in 11 and 12, but not 15. Titer in 11, 12, and 15 was decreased on day 35, however decreased titer was observed in all groups on day 35, Test 3. Animals 13 and 14 were initially presented with the CS- (Test 1), and a rise in titer was observed with a concomitant aversion to the flavor (Figure 6). On Test 2, an aversion to the flavor was found in both subjects, and the antibody titer was reduced below the mean titer of the US Only group and the CS-US group. Aversion to flavor was not seen on Test 3, and a rise in titer was found. By Test 4, the taste aversion response had extinguished in both animals. The titer in animal 14 rose, while little change in titer was observed in animal 13. The results from the CS+/CS- group are inconclusive, as the depression in titer that corresponds to CS+ presentation is observed on Test 3 where all animals display a decrease in titer.

Figure 9. Antibody Titer in CS+/CS- - Experiment 1

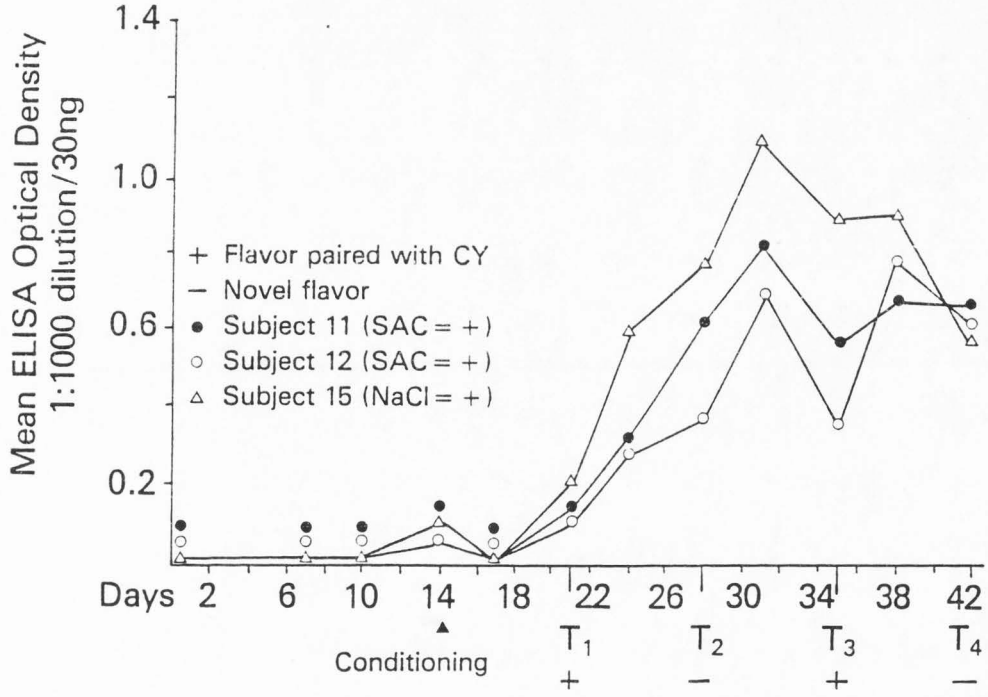


Figure 10. Antibody Titer in CS+/CS- - Experiment 1

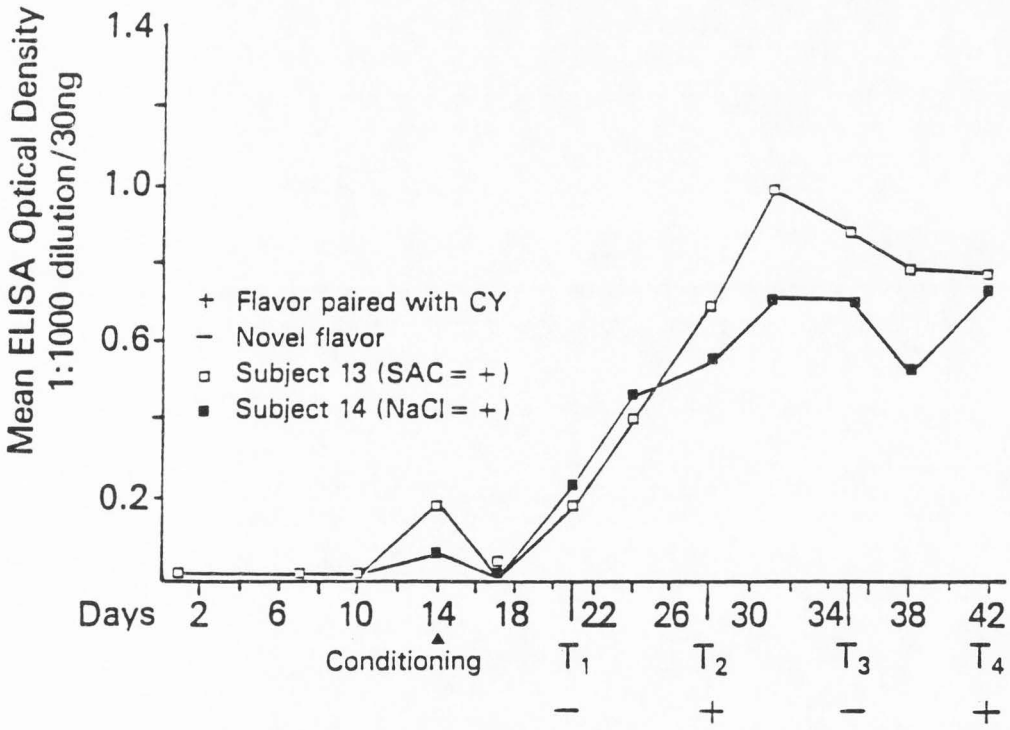


Table 12

Antibody Titer at 30 ng/1:1000 - CS+/CS-

Subject	Boost		Test 1+		Test 2-		Test 3+		Test 4-
	Day 14	Day 17	Day 21	Day 24	Day 28	Day 31	Day 35	Day 38	Day 42
+	$\bar{X}$ sd	$\bar{X}$ sd	$\bar{X}$ sd	$\bar{X}$ sd	$\bar{X}$ sd	$\bar{X}$ sd	$\bar{X}$ sd	$\bar{X}$ sd	$\bar{X}$ sd
11 SAC	.1285 .019	.0230 .004	.1533 .006	.3936 .011	.4613 .007	.8860 .009	.6115 .074	.6830 .013	.6743 .019
12 SAC	.0805 .000	.0000 .000	.1553 .003	.3130 .026	.3913 .015	.7090 .010	.6485 .038	.8053 .044	.6383 .022
15 NACL	.0930 .000	.003 .002	.2473 .004	.6090 .018	.7726 .017	1.191 .010	.9415 .013	.9036	.5926 .040

			Test 1-		Test 2+		Test 3-		Test 4+
	$\bar{X}$ sd	$\bar{X}$ sd	$\bar{X}$ sd	$\bar{X}$ sd	$\bar{X}$ sd	$\bar{X}$ sd	$\bar{X}$ sd	$\bar{X}$ sd	$\bar{X}$ sd
13 SAC	.2650 .0	.0330 .007	.2023 .005	.4240 .005	.6766 .005	1.033 .025	.9175 .010	.7623 .026	.7680 .039
14 NACL	.0935 .000	.0000 .000	.2406 .013	.4773 .000	.5026 .032	.7396 .015	.7400 .00	.4923 .013	.7450 .051

+ - Tested with flavor paired with the US
- - Tested with novel flavor

Antibody Affinity

The antibody affinity for the CS Only group is shown in Figure 11. Statistically significant differences were attained between the CS-US group and the CS Only group (Figure 11) on: day 17, CS Only > CS-US ($t = 5.472$, $df=8$, $p.<.001$); day 21-test 1, CS Only > CS-US ($t = 4.343$, $df=7$, $p.<.005$); day 38, CS Only > CS-US ($t = 4.555$, $df=10$, $p.<.001$); and day 42-test 4, CS Only > CS-US ($t = 2.570$, $df=8$, $p.<.05$). Table 13 contains a summary of the results of the One way ANOVA on affinity of the antibody response between these groups.

Visual examination of the contrast between the CS-US and the US Only groups revealed no differences between the groups until day 38, when the CS-US affinity decreased and the affinity of the US Only continued to increase (Figure 11). Statistically significant differences were found on day 38 with the CS-US significantly less than the US Only ($t = 4.055$, $df=6$, $p.<.01$); and on day 42-test 4, CS-US less than the US Only ($t = 2.312$, $df=10$, $p.<.05$). The results of the One Way ANOVA on antibody affinity between CS-US and US Only groups are presented in Table 14.

Post hoc analyses using a One Way ANOVA and the Scheffe' test were completed to determine differences between pairs of means. The .05 alpha level was established for statistical significance. The One Way ANOVA showed significant differences among means after day 17. Table 15 provides a summary of the exact values.

Figure 11. Antibody Affinity Experiment 1

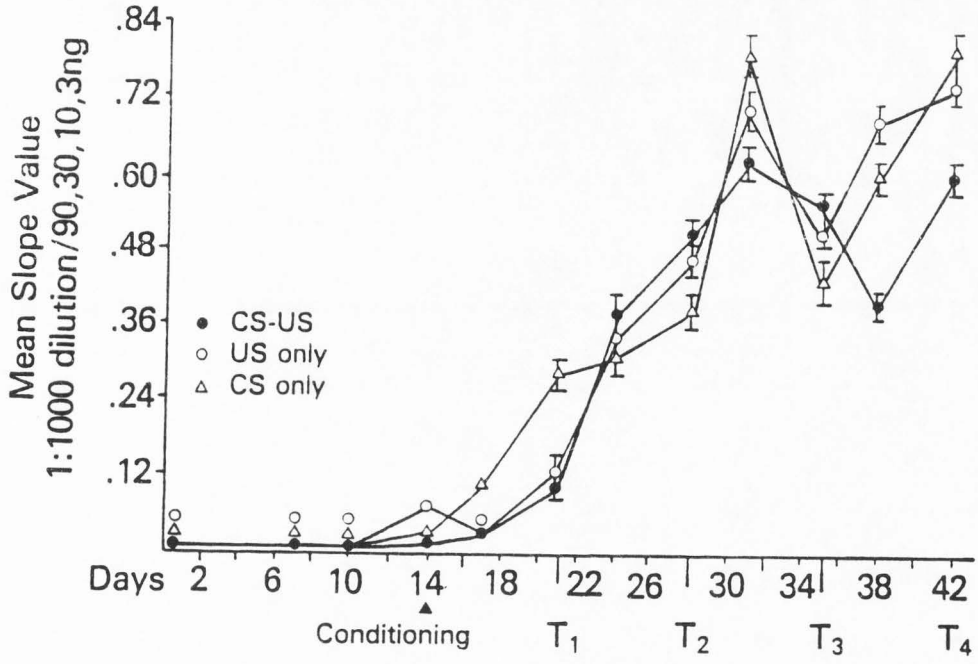


Table 13

Experiment 1

Results of Tests of Significance - Antibody Affinity

CS Only and CS-US

	Boost Day 14	Day 17	Test One Day 21	Day 24
CS Only/ CS-US	t = .942 df = 10 p. = .369	t = 5.472 df = 8 p. = <.001*	t = 4.343 df = 7 p. = .005*	t = .898 df = 10 p. = .390
	Test Two Day 28	**Day 31**	**Test Three Day 35**	**Day 38**
CS Only/ CS-US	t = 2.263 df = 9 p. = .051	t = 1.762 df = 9 p. = .111	t = 1.780 df = 10 p. = .106	t = 4.555 df = 10 p. = <.001*
	Test Four Day 42			
CS Only/ CS-US	t = 2.570 df = 8 p. = <.05*			

*** Antibody affinity of the CS Only is significantly greater than affinity of the CS-US.**

Table 14

Experiment 1

Results of Tests of Significance -Antibody Affinity

US Only and CS-US

	Boost Day 14	Day 17	Test One Day 21	Day 24
US Only/ CS-US	t = 2.467 df = 6 p. = .052	t = .001 p. = .999	t = .567 p. = .587	t = .412 df = 9 p. = .691
	Test Two Day 28	**Day 31**	**Test Three Day 35**	**Day 38**
US Only/ CS-US	t = .364 df = 6 p. = .728	t = 1.240 df = 10 p. = .245	t = .725 df = 7 p. = .492	t = 4.055 df = 6 p. = <.01*
	Test Four Day 42			
US Only/ CS-US	t = 2.312 df = 10 p.<.05*			

*** Statistically significant differences with antibody affinity. The affinity of the CS-US significantly less than affinity of US Only.**

Table 15

Experiment 1 - One Way ANOVA

Antibody Affinity

	Boost Day 14				Day 17			
	N	X	SD	SEM	N	X	SD	SEM
CS Only	5	.0362	.0179	.008	5	.1125	.0119	.0053
US Only	5	.0780	.0439	.0196	5	.0385	.0239	.0107
24 Hour	5	.0962	.0446	.019	5	.0482	.0235	.0105
Pos Control	2	.0679	.0346	.0245	2	.0916	.0163	.0115
CS-US	7	.0263	.0223	.0084	7	.0385	.0329	.0124
	F = 1.5693 df = 6 p. = .1992				F = 5.4679 df = 6 p. = .001*			

	Test One Day 21				Day 24			
	N	X	SD	SEM	N	X	SD	SEM
CS Only	5	.2868	.0757	.0339	5	.3170	.1100	.0492
US Only	5	.1358	.0630	.0282	5	.3482	.1506	.0637
24 Hour	5	.1606	.0627	.0280	5	.5382	.0370	.0165
Pos Control	2	.4722	.1037	.0733	2	.5291	.0677	.0418
CS-US	7	.1162	.0527	.0199	7	.3844	.1497	.0566
	F = 12.245 df = 6 p. = <.0001*				F = 3.5176 df = 6 p. = .0122*			

	Test Two Day 28				Day 31			
	N	X	SD	SEM	N	X	SD	SEM
CS Only	5	.3918	.0841	.0376	5	.7619	.1241	.0555
US Only	5	.4797	.1321	.0591	5	.7043	.0743	.0331
24 Hour	5	.4313	.1140	.0510	5	.7147	.0341	.0152
Pos Control	2	.6053	.0354	.0250	2	.7392	.0456	.0344
CS-US	7	.5042	.0859	.0325	7	.6281	.1371	.0518
	F = 2.6804 df = 6 p. = .0390*				F = 3.5256 df = 6 p. = .0121*			

	Test Three Day 35				Day 38			
	N	X	SD	SEM	N	X	SD	SEM
CS Only	5	.4388	.0562	.0385	5	.0675	.0654	.0292
US Only	5	.5153	.0349	.0156	5	.6926	.1411	.0631
24 Hour	5	.5667	.0469	.0210	5	.3920	.1358	.0687
Pos Control	2	.6102	.0198	.0140	2	.6693	.0883	.0625
CS-US	7	.5552	.1398	.0528	7	.4003	.0922	.0348
	F = 4.6598 df = 6 p. = <.01*				F = 8.0512 df = 6 p. = .0001*			

	Test Four			
	N	X	SD	SEM
CS Only	5	.7997	.1353	.0605
US Only	5	.7403	.0830	.0371
24 Hour	5	.6741	.0513	.0229
Pos Control	2	.7321	.0404	.0286
CS-US	7	.6093	.1101	.0427
	F = 5.406 df = 6 p. = .0012*			

Note. * Statistical Significance

Results of the Scheffe' multiple comparison tests indicated statistically significant differences between the CS-US group and the CS Only group on days 17 and 21 (Test 1). The antibody affinity was greater in the CS Only group. The affinity of the CS-US group was found to be significantly below the affinity of the US Only group on day 38. The affinity in the US Only group was significantly less than the affinity in the CS Only group on day 17. Significant differences between the CS Only and Positive Control groups were not found.

The antibody affinity of the 24 Hour group (Figure 12) was similar to the affinity of the US Only group, except for day 38. On that single day, the affinity of the 24 Hour group was significantly less than the affinity of the US Only. No aversion to flavor was found on day 38 within this group. The pattern of affinity of the 24 Hour group was similar to that observed in the CS-US treatment group. However, the magnitude of depression in affinity within the 24 Hour group was less than that found in the CS-US group. The minimal changes in the 24 Hour group are perhaps a result of the extended interval between the CS and US. Systematic changes in affinity were not seen in CS+/CS- group.

Summary

Taste aversion was obtained in Experiment 1, and it persisted for the first two test trials. A comparative decrease in both antibody titer and affinity was observed in the treatment group (CS-US) on days 38 and 42. A composite of the findings for the critical comparison groups in the first experiment is provided in

Figure 12. Antibody Affinity 24 Hour - Experiment 1

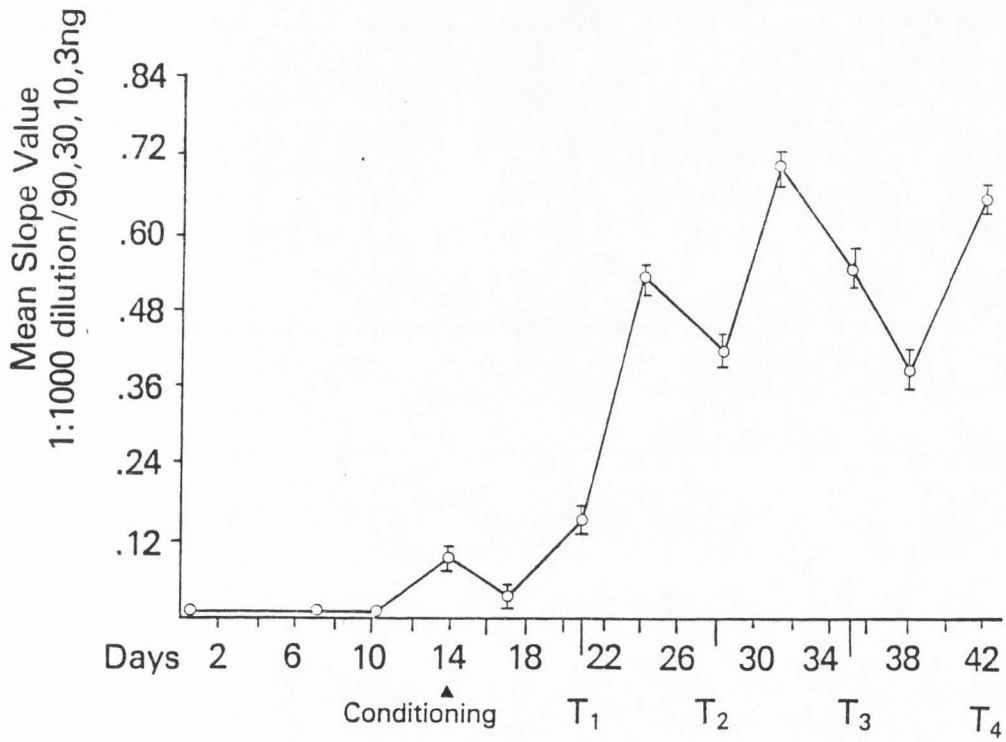


Table 16.

The results are consistent with the literature in that suppression was attained. The findings are unique in that suppression was found with both titer and affinity, and that the suppression was not evident until three test trials were presented. The decrease in titer and affinity was observed after the taste aversion response dissipated. An incidental but unexplained observation was the depression in affinity and titer across all groups on day 35 (Test 3).

Table 16

Composite Summary of Results - Experiment 1

Days		0	7	10	14	17	21	24	28	31	35	38	42
Groups							T1		T2		T3		T4
CS Only/	Titer					CS>	CS>		CS<	CS>		CS>	CS>
CS-US	Affinity					CS>	CS>					CS>	CS>
CS-US/	Titer									CS-US>	CS-US>	CS-US<	CS-US<
US Only	Affinity											CS-US<	CS-US<
Taste Aversion							+		+		0		0

CHAPTER VIII

EXPERIMENT 2

Purpose

The results of the first study showed a statistically significant depression in both titer and affinity in the CS-US treatment animals as compared to the US Only group. However, these differences occurred only on days 38 and 42. The purpose of Experiment 2 was to strengthen the possible conditioning effects on antibody titer/affinity to BSA.

It was hypothesized that the conditioned immune response could be improved by increasing the dosage of CY and thereby extending the taste aversion response (Wright, Foshee, & McCleary, 1971). The administration of the antigen boost was also changed to 24 hours after conditioning because the BSA boost on the day of conditioning may have disrupted the effects of CY by promoting antibody stimulation. The planned tests and dependent variables were identical to those in Experiment 1.

Subjects

Twenty-six Lewis male albino rats (Charles River Laboratory) approximately 60 days old served as subjects. The habituation and watering procedure was identical to Experiment 1.

Subjects were assigned to the following groups: CS-US (1-9); US Only (11-18); and Positive Control (19-27). The Positive Control was used in lieu of the CS Only group as no statistical differences were found between the CS Only and Positive Control in Experiment 1.

Antigen

For this experiment, the preparation of BSA for both the antigen challenge and boost was slightly modified. The process of emulsifying the BSA with the Freund's Adjuvant was improved by the use of a homogeneizer. All other aspects of the antigen administration remained identical to the first experiment. The initial antigen challenge was given on day 0 and the boost on day 14, one day after conditioning.

Serum Samples

All procedures were identical to Experiment 1, except the bleedings on days 7 and 10 were eliminated. These two serum samples were deemed unnecessary based on the findings from Experiment 1, that showed minimal presence of antibody.

Conditioning

On day 13, the CS-US treatment group received a 0.1% solution of saccharin at the regular 30 min drinking period. Ten minutes after termination of the drinking period, an ip injection of CY was administered to all CS-US animals at a dosage of 60 mg/kg of body weight. The US Only subjects received regular tap water during the drinking period, followed by an ip injection of CY at the above dosage. The Positive Control animals received regular tap water for the 30 min drinking period followed by an ip injection of SDW at a volume based on the CY dosage. Within three hours post conditioning, the CS-US and US only animals appeared ill with decreased motor activity and ruffled fur.

All animals received regular tap water on day 14. The antigen boost was administered three hours after the drinking period. The procedure was identical to Experiment 1. The serum sample was acquired on day 14 approximately 10 hours after the drinking period. Table 17 presents the procedures for this experiment.

Test Trials

The test condition in this experiment was identical to the procedures described in Experiment 1. Test trials occurred on days 21, 28, 35, and 42. The CS-US group was reexposed to the SAC (CS) on each test day. The US Only group was presented with SAC as the CS flavor also on each test day. The Positive Control animals received the regularly scheduled tap water.

ELISA

Procedures for the assay are described in Chapter V, and are identical to Experiment 1. A total of seven 96 well plates were run for each day's serum sample. Fourteen assays were completed, that included a separate reliability check.

Table 17

Experimental Procedures in Experiment 2

		BSA	Condition		BSA Boost		Test 1		Test 2		Test 3		Test 4
	Days	0	13		14	17	21	24	28	31	35	38	42
	Serum	+			+	+	+	+	+	+	+	+	+
Group	N		FLV	INJ	BSA		CS		CS		CS		CS
CS-US	9	BSA	SAC	CY	BSA		SAC		SAC		SAC		SAC
US Only	8	BSA	WAT	CY	BSA		SAC		SAC		SAC		SAC
Pos Ctrl	8	BSA	WAT	SDW	BSA		WAT		WAT		WAT		WAT

CHAPTER IX

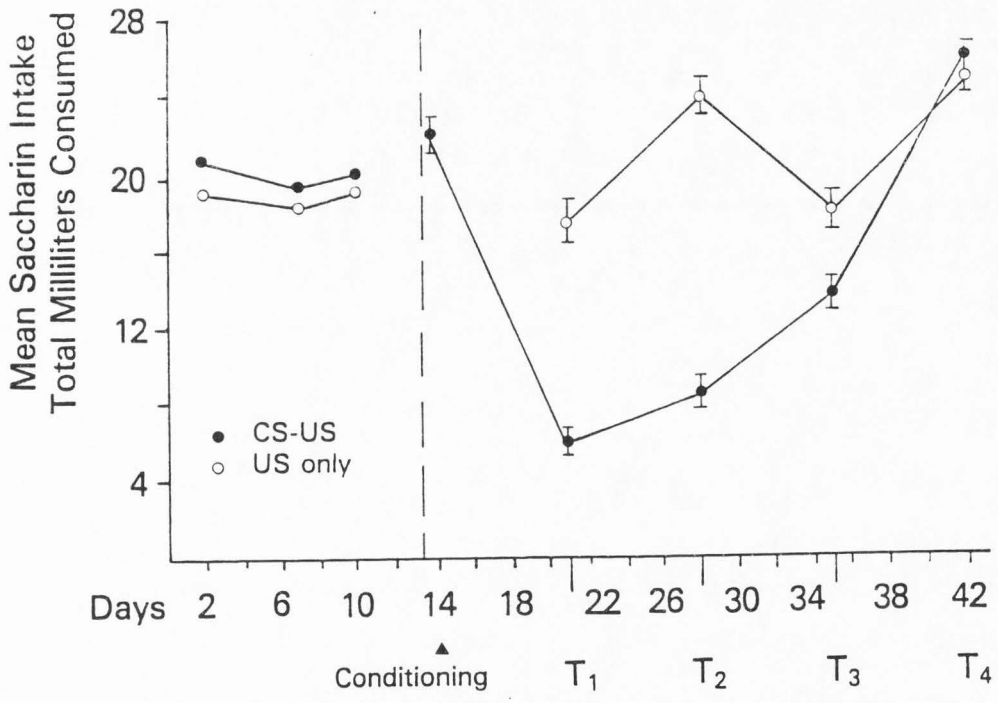
RESULTS OF EXPERIMENT 2

Experiment 1 resulted in a conditioned immune response with decreased titer and affinity on Days 38 and 42. It was hypothesized that the taste aversion response and possible conditioning of the immune response could be enhanced by increasing the CY dosage (from 50 mg/kg to 60mg/kg) and by administering the antigen boost 24 hours after the conditioning treatment session. Dependent measures used in this experiment were identical to the first experiment, and were analyzed using a t test for means. Post hoc comparisons were conducted using a One Way ANOVA and Scheffe' test to compare differences between all possible pairs of means. Procedures for statistical analyses were identical to Experiment 1.

Taste Aversion

An independent samples t test was calculated to compare the mean consumption of saccharin between the CS-US and US Only groups. Reduced intake of saccharin was observed in the CS-US group at day 21, day 28, and day 35 (Figure 13). Statistical differences were attained between the CS-US and US Only group on day 21-test 1 ($t = 9.296$, $df=15$, $p.<.001$); day 28 - test 2 ($t = 15.1$, $df=15$, $p.<.001$); and day 35 - test 3 ($t = 3.06$, $df=15$, $p.<.001$). No statistical differences were found on Test 4.

Figure 13. Mean Flavor Ingestion - Experiment 2

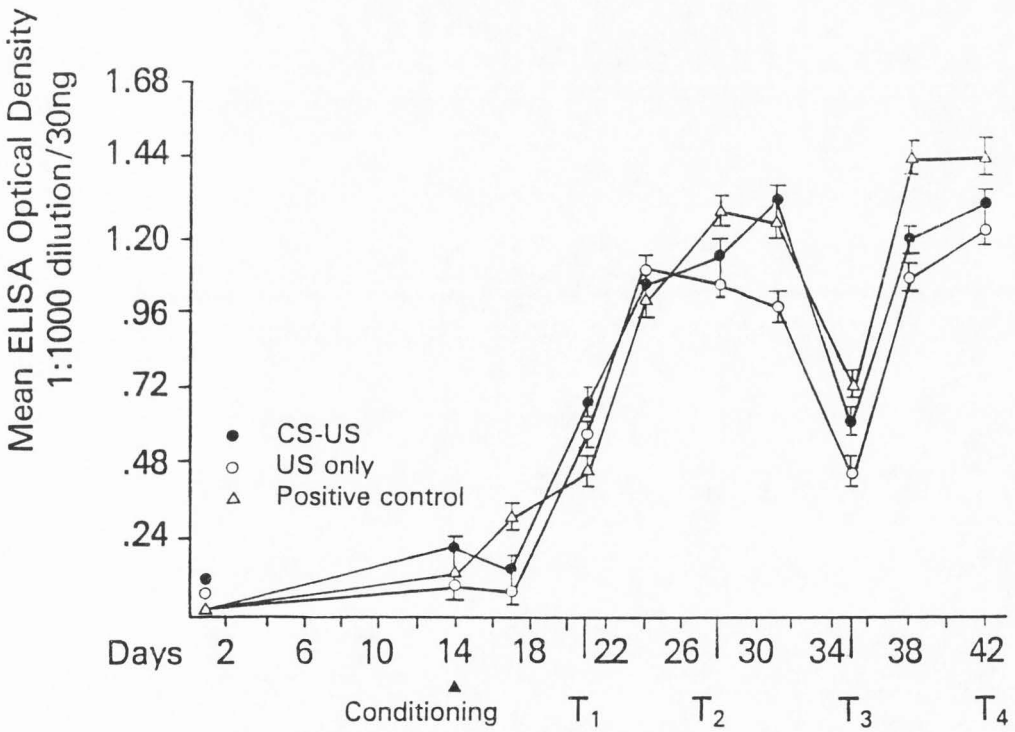


Experiment Two - Antibody Titer

The serum taken prior to antigen challenge showed no antibody titer to BSA across the three groups. A review of each subject's response across time revealed that subject 12 (US Only) appeared not to have been adequately immunized. The titer and affinity at both serum dilutions and all antigen ranges was significantly less than other subjects within that group (>2 std. dev.). A casewise residual plot (Norusis, 1986) was calculated and the results were consistent with the impression that subject 12 was an outlier. Therefore, the statistical analysis presented below does not include the data from this subject.

Differences between the CS-US and Positive Control were expected since CY was not administered to the Positive Control. The titer of the CS-US treatment group was significantly less than the Positive Control on day 17. The decreased titer in the CS-US group was secondary to the immunosuppressive effects of CY (Figure 14). Statistically significant differences were found between the titer of the CS-US and Positive Control on: day 14, CS-US greater than Positive Control ($t=3.597$, $df=44$, $p.<.001$); day 17, CS-US less than Positive Control ($t= 5.299$, $df=37$, $p.<.001$); day 21 - test 1, CS-US greater than Positive Control ($t= 5.167$, $df=47$, $p.<.001$); day 28 - test 2, CS-US less than Positive Control ($t= 3.530$, $df=43$, $p.<.001$); day 35 - test 3, CS-US less than Positive Control ($t= 3.258$, $df=32$, $p.<.005$); day 38, CS-US less than Positive Control ($t= 16.014$, $df=30$, $p.<.001$); and day 42 - test 4, CS-US less than Positive Control ($t= 9.824$, $df=30$, $p.<.001$). The results of the tests of

Figure 14. Mean Antibody Titer - Experiment 2



significance are presented in Table 18.

As in Experiment One, the critical contrast was between the CS-US and the US Only group. It was hypothesized that the CS-US would exhibit a lower titer than the US Only group following test trials. Visual inspection of the data is indicative of a similar response pattern until day 28 - Test 2 (Figure 14). At that point the titer of the CS-US increased, and continued to be greater than the US Only. Statistically significant differences were found on: day 14, CS-US greater than the US Only ($t= 4.041$, $df=43$, $p.<.001$); day 17, CS-US greater than US Only ($t= 2.901$, $df=44$, $p.<.005$); day 31, CS-US greater than US Only ($t= 6.890$, $df=38$, $p.<.001$); day 35 - test 3, CS-US greater than US Only ($t= 3.903$, $df=27$, $p.<.001$); and day 38, CS-US greater than US Only ($t= 3.958$, $df=41$, $p.<.001$). Statistical analysis did not support a suppressive effect (Figure 14). The results of the One Way ANOVA between the CS-US and US Only groups are presented in Table 19.

Post hoc analyses were conducted to determine differences among pairs of means. Results of the One Way ANOVA found statistical differences among means on all points after day 14, except day 24. Table 20 provides a summary of the data on titer for Experiment 2. The Scheffe' multiple comparisons tests were also completed, and statistically significant differences were found at the .05 alpha level. Statistical differences were found between the CS-US and Positive Control at all time points except day 28 - test 2. Statistically significant differences were also present between the CS-US and US Only groups at days 14, 31, 35 (test 3), and day 38.

Table 18

Experiment 2 - Results of Tests of Significance

Antibody Titer - Positive Control and CS-US

	Boost Day 14	Day 17	Test One Day 21	Day 24
Positive Ctrl./ CS-US	t = 3.597 df = 44 p. <.005**	t = 5.299 df = 37 p. = <.001*	t = 5.167 df = 47 p. = <.001**	t = .212 df = 38 p. = .833
	Test Two Day 28	**Day 31**	**Test Three Day 35**	**Day 38**
Positive Ctrl./ CS-US	t = 3.530 df = 43 p.= <.005*	t = 1.437 df = 49 p. = .157	t = 3.258 df = 32 p. = <.005*	t = 16.014 df = 30 p. = <.001
	Test Four Day 42			
Positive Ctrl./ CS-US	t = 9.824 df = 30 p. = <.001*			

** Antibody titer of CS-US significantly greater than titer of Positive Control.

* Antibody titer of CS-US significantly less than titer of Positive Control.

Table 19

Experiment 2 - Results of Tests of Significance

Antibody Titer - US Only and CS - US

	Boost Day 14	Day 17	Test One Day 21	Day 24
US Only/ CS-US	t = 4.041 df = 43 p. = <.001**	t = 2.901 df = 44 p. = <.01**	t = 1.997 df = 42 p. = .052	t = .453 df = 44 p. = .653
	Test Two Day 28	**Day 31**	**Test Three Day 35**	**Day 38**
US Only/ CS-US	t = 1.764 df = 31 p. = .087	t = 6.890 df = 38 p. = <.001**	t = 3.903 df = 37 p. = <.005**	t = 3.958 df = 41 p. = <.001**
	Test Four Day 42			
US Only/ CS-US	t = 1.530 df = 25 p. = .138			

**** Antibody titer of CS-US significantly greater than titer of US Only.**

Table 20

Experiment 2 - One Way ANOVA

Antibody Titer

	Boost Day 14			Day 17		
	N	X	SD SEM	N	X	SD SEM
CS-US	27	.2289	.1298 .0250	27	.1607	.0742 .0143
US Only	21	.1085	.0744 .0744	21	.1098	.0469 .0246
Pos. Ctrl.	24	.1219	.0790 .0790	24	.3114	.0205 .0246
	F = 10.881			F = 33.73		
	df = 2			df = 2		
	p. = <.0005*			p. = <.0001*		

	Test One Day 21			Day 24		
	N	X	SD SEM	N	X	SD SEM
CS-US	27	.6817	.0742 .0316	27	1.033	.2203 .0424
US Only	21	.5836	.1722 .0376	21	1.062	.2047 .0447
Pos. Ctrl.	24	.4772	.1168 .0238	24	1.024	.1041 .0212
	F = 11.404			F = .2517		
	df = 2			df = 2		
	p. = <.0005*			p. = .7782		

	Test Two Day 28			Day 31		
	N	X	SD SEM	N	X	SD SEM
CS-US	27	1.168	.1448 .0279	27	1.317	.1404 .0270
US Only	21	1.065	.2347 .0512	21	.9932	.1762 .0385
Pos. Ctrl.	24	1.294	.0942 .0222	24	1.265	.1193 .0244
	F = 8.881			F = 32.264		
	df = 2			df = 2		
	p. = <.0005*			p. = <.0001*		

	Test Three Day 35			Day 38		
	N	X	SD SEM	N	X	SD SEM
CS-US	18	.6286	.1085 .0256	27	1.204	.0880 .0169
US Only	14	.4709	.1171 .0313	21	1.098	.0951 .0247
Pos. Ctrl.	16	.7476	.1043 .0261	9	1.486	.0145 .0048
	F = 23.800			F = 66.814		
	df = 2			df = 2		
	p. = <.0001*			p. = <.001*		

	Test Four Day 42		
	N	X	SD SEM
CS-US	27	1.295	.0974 .0188
US Only	18	1.230	.1624 .0383
Pos. Ctrl.	9	1.487	.0170 .0057
	F = 14.776		
	df = 2		
	p. = <.0001*		

The CS-US was found to be greater than the US only at these time points. Statistical differences were also attained between the US Only and the Positive Control. The US Only was significantly less than the Positive Control on day 17, and days 28 through 42.

Experiment Two - Antibody Affinity

Prior to antigen treatment, no differences in antibody affinity were found between the groups. Visual inspection of the data revealed differences between the CS-US group and Positive Control on days 17, 33, 38, and 42 (Figure 15). The CS-US was less than the Positive Control on days 17, 33, 38 and 42. Data analysis resulted in statistically significant differences between the affinity of the CS-US group and Positive Control on: day 14, CS-US greater than Positive Control ($t= 2.567$, $df=10$, $p.<.05$); day 17, CS-US less than Positive Control ($t= 2.176$, $df=10$, $p.<.05$); day 21 - test 1, CS-US greater than Positive Control ($t= 2.909$, $df=15$, $p.<.01$); day 31, CS-US greater than Positive Control ($t = 3.332$, $df=8$, $p.<.01$); day 38, CS-US less than Positive Control ($t= 5.074$, $df=8$, $p<.001$); and day 42 -test 4, CS-US less than Positive Control ($t = 6.361$, $df=10$, $p.<.001$). The results on the contrast between CS-US and Positive Control on affinity is presented in Table 21.

Visual inspection indicated that the antibody affinity of the CS-US and US Only groups were similar until day 38 and 42 (Figure 15). A comparative depression in affinity was observed at that time in the CS-US group. Statistical analysis show significant differences between the CS-US and US only on: day 31, CS-US greater than US only ($t= 3.034$, $df=13$, $p.<.01$); day 35 - test 3, CS-US

Figure 15. Antibody Affinity Experiment 2

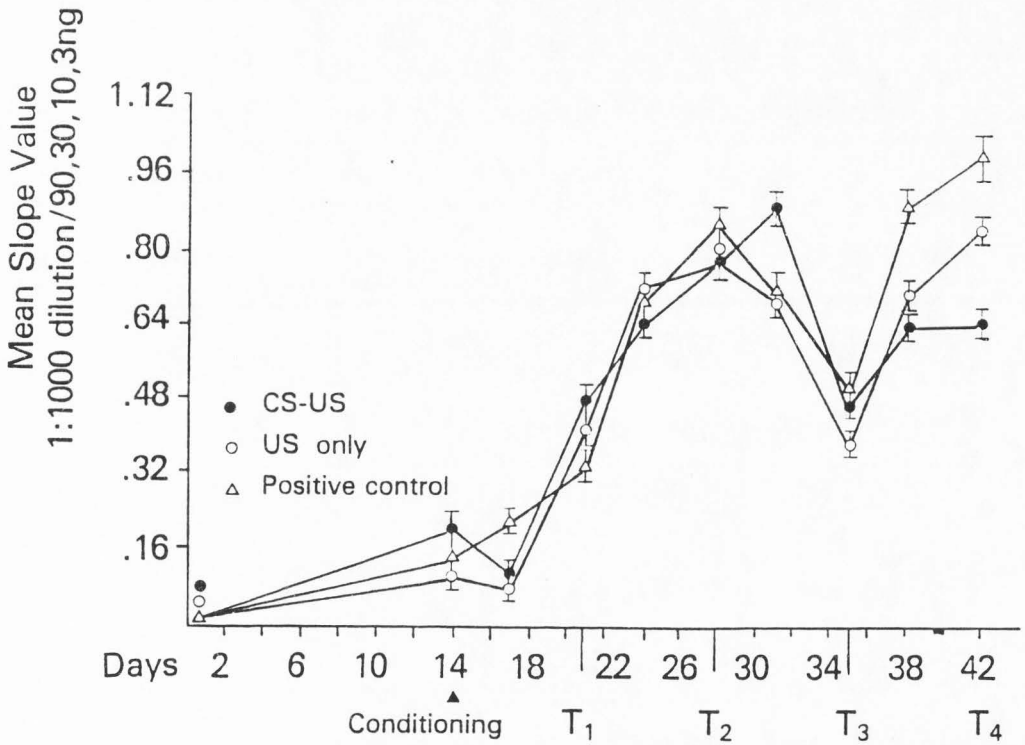


Table 21

Experiment 2 - Results of Tests of Significance

Antibody Affinity -- Positive Control and CS-US

	Boost Day 14	Day 17	Test One Day 21	Day 24
Positive Ctrl./ CS-US	t = 2.567 df = 10 p. = <.05**	t = 2.176 df = 11 p. = .05*	t = 2.909 df = 15 p. = <.05**	t = 1.282 df = 14 p. = .222
	Test Two Day 28	**Day 31**	**Test Three Day 35**	**Day 38**
Positive Ctrl./ CS-US	t = 1.751 df = 14 p. = .102	t = 3.332 df = 9 p. = <.01**	t = 1.374 df = 14 p. = .191	t = 5.074 df = 8 p. = < .005*
	Test Four Day 42			
Positive Ctrl./ CS-US	t = 6.361 df = 10 p. = <.001*			

* Antibody Affinity of the CS-US significantly less than affinity of the Positive Control.

** Antibody Affinity of the CS-US significantly greater than affinity of the Positive Control.

greater than US Only ($t = 4.109$, $df=10$, $p.<.002$); day 38, CS-US less than US Only ($t = 2.864$, $df=14$, $p<.01$); and day 42 - test 4, CS-US only less than US Only ($t= 5.380$, $df=13$, $p.<.001$). Unlike the findings on titer, statistical analysis revealed a suppression of antibody affinity in the CS-US group on days 38 and 42. Table 22 contains a summary of the statistical data on antibody affinity between CS-US and US Only groups.

Post hoc analyses were also completed to evaluate differences among all pairs of means at a .05 alpha level. Calculation of the One Way ANOVA resulted in statistically significant differences between pairs of means after day 14, with the exception of days 24 and 28. An overview of this analysis is provided in Table 23. The Scheffe' test showed statistical differences at the .05 level between the CS-US group and Positive Control group. The CS-US group was found to be greater than the Positive Control group on days 21 and 31. The CS-US group was less than the Positive Control group on days 38 and 42. The contrast between the CS-US and US Only groups also resulted in statistically significant differences on days 31, and 35. The CS-US treatment group was found to be greater than the US Only group. On days 38 and 42, the CS-US group was significantly less than the US Only group. These results are consistent with the findings from the planned test between the CS-US and US Only groups. Scheffe' analysis also resulted in statistical differences between the US Only and Positive Control groups on: day 17, 35, 38, and 42. The US Only group was less than the Positive Control group.

Table 22

Experiment 2 - Results of Tests of Significance

Antibody Affinity - US Only and CS-US

	Boost Day 14	Day 17	Test One Day 21	Day 24
US Only/ CS-US	t = 1.953 df = 14 p. = .07	t = 1.588 df = 12 p. = .138	t = 1.156 df = 21 p. = .261	t = 1.761 df = 13 p. = .102
	Test Two Day 28	**Day 31**	**Test Three Day 35**	**Day 38**
US Only/ CS-US	t = .086 df = 10 p. = .933	t = 3.034 df = 13 p. = <.01**	t = 4.109 df = 10 p. = <.005**	t = 2.864 df = 14 p. = <.01*
	Test Four Day 42			
US Only/ CS-US	t = 5.380 df = 13 p. = <.001*			

*** Antibody affinity of the CS-US significantly less than affinity of US Only.**

**** Antibody affinity of the CS-US significantly greater than affinity of US Only.**

Table 23

Experiment 2 - One Way ANOVA

Antibody Affinity

	Boost Day 14			Day 17		
	N	X	SD SEM	N	X	SD SEM
CS-US	9	.2006	.1241 .0414	9	.1194	.0580 .0193
US Only	7	.0942	.0938 .0355	7	.0844	.0277 .0105
Pos. Ctrl.	8	.1324	.0368 .0130	8	.2104	.1050 .0371
	F = 3.790			F = 6.293		
	df = 2			df = 2		
	p. = <.05*			p. = <.01*		

	Test One Day 21			Day 24		
	N	X	SD SEM	N	X	SD SEM
CS-US	9	.4845	.1003 .0334	9	.6426	.1141 .0380
US Only	7	.4277	.0957 .0362	7	.7221	.0641 .0243
Pos. Ctrl.	8	.3458	.0962 .0340	8	.7010	.0708 .0250
	F = 4.294			F = 1.782		
	df = 2			df = 2		
	p. = <.05*			p. = .192		

	Test Two Day 28			Day 31		
	N	X	SD SEM	N	X	SD SEM
CS-US	9	.7697	.1332 .0444	9	.8916	.1696 .0565
US Only	7	.7772	.1970 .0744	7	.6885	.0948 .0358
Pos. Ctrl.	8	.8657	.0980 .0321	8	.7018	.0232 .0082
	F = 1.118			F = 7.903		
	df = 2			df = 2		
	p. = .345			p. = <.005*		

	Test Three Day 35			Day 38		
	N	X	SD SEM	N	X	SD SEM
CS-US	9	.4788	.0360 .0120	9	.6405	.0452 .0151
US Only	7	.3811	.0543 .0205	7	.6954	.0314 .0119
Pos. Ctrl.	8	.5057	.0436 .0154	8	.9081	.1430 .0506
	F = 16.089			F = 20.854		
	df = 2			df = 2		
	p. = <.0005*			p. = <.0001*		

	Test Four Day 42		
	N	X	SD SEM
CS-US	9	.6452	.0687 .0229
US Only	7	.8348	.0708 .0268
Pos. Ctrl.	8	1.000	.1439 .0509
	F = 26.441		
	df = 2		
	p. = <.0001*		

* Statistical significance.

Summary

Taste aversion in this experiment was extended for three test trials. A conditioned immune response was not found with antibody titer. However, a suppression in antibody affinity was observed on days 38 and 42 that was consistent with the findings of Experiment 1 (Table 24). The depression in antibody titer and affinity was again observed across all groups on day 35.

Table 24

Composite Summary of Results - Experiemnt 2

Days		0	14	17	21	24	28	31	35	38	42
Groups					T1		T2		T3		T4
Pos. Ctrl/	Titer			PC>	PC<		PC>		PC>		PC>
CS-US	Affinity		PC<	PC>	PC<			PC<		PC>	PC>
CS-US/	Titer							CS-US>	CS-US>	CS-US>	
US Only	Affinity							CS-US>	CS-US>	CS-US<	CS-US<
Taste Aversion					+		+		+		-

CHAPTER X

DISCUSSION

Introduction

The immune system is intergrated with other physiological processes and is subject to regulation and modulation by the CNS (Ader et al., 1987). A large body of evidence supports the premise of a bidirectional relationship between the CNS and the immune system (Solomon, 1987). Communication between the immune system and the CNS is thought to occur by direct neuronal connections to immune related organs, and through the endocrine system (Schneider, Cohn, & Bullock, 1987). A variety of methodologic approaches have been used to elucidate specific pathways of communication between the CNS and immune system (Neveu et al., 1987). Electrical stimulation or lesions of different hypothalamic areas have been shown to modify immune reactivity (Cross, Markesberry, Brooks, & Roszman, 1984). Recent data also indicates that the relationship between the CNS and the immune system may be mediated by hormones from the hypothalamopituitary axis (Cosma, Leonhardt, & Weberle, 1982), and by the sympathetic nervous system through activity at the level of lymphocyte receptors (Besodovsky, Del Rey, Sorkin, Da Prada, & Keller, 1979). Immune system activity has also been shown to produce changes with corticosterone and norepinephrine levels (Shek & Sabiston, 1983).

Conversely, the immune system has been found to modify the activity of the CNS. Lymphocytes have been reported to produce

ACTH and endorphin like substances (Smith et al., 1985). Additionally, the activation of the immune system results in transient changes in the brain, such as the firing rate of neurons within the ventromedial hypothalmus (Besedovsky, Sorkin, Felix, & Haas, 1977). An extensive review of the neuro-immuno-regulatory system is beyond the scope of this paper. However, the current evidence supports the existence of a bidirectional relationship between the immune system and the CNS.

Psychoneuroimmunology is described as the study of interactions between the central nervous system and the immune system (Ader & Cohen, 1985). The influence of classical conditioning procedures on immune functioning can be traced to investigations by Metal'nikov and colleagues at the Pasteur Institute between 1920 - 1930 (Spector 1987). New interest in the conditioning of immune activity was generated by Ader and Cohen's (1975) publication on conditioned immunosuppression. The use of classical conditioning procedures to modulate cellular and humoral immune responses represents a single track of research devoted to the examination of the interaction between the immune and central nervous system (Ader et al., 1987).

The research on conditioned immunosuppression has generated many question such as: the factors contributing to the conditioned effect; the mechanisms underlying the conditioned alteration of immune reactivity; the effect upon cellular interactions that result in the synthesis and release of antibody; the relation between taste aversion and conditioned immune response; and the effect of

manipulating environmental factors (e.g., stimulus sequencing effects, extinction trials, dose response relation between CS and US) on the acquisition and retention of a conditioned immune response. The present research efforts addressed the following issues: the specificity of the antibody response, the accuracy and sensitivity of the ELISA to detect subtle changes in antibody production, and the effects of multiple CS test trials on the taste aversion and conditioned immune response over time.

The specificity of the antibody response was examined by conditioning an antibody response that was specific to a particular antigen, assessing a secondary antibody response predominantly consisting of IgG, and finally by testing for antibody affinity as well as titer. The sensitivity and accuracy of the assay was addressed by the use of the ELISA, which is an optimal procedure to quantify antibody production (Voller et al., 1978). An essential and important contribution of the present research was implementing and systematizing the ELISA protocol for the accurate and selective determination of a secondary response to BSA. The present investigation addressed the effects of classical conditioning in the following manner. First, the conditioned antibody response was evaluated over a six week period to identify the pattern of the response. Second, multiple CS test trials were presented to examine the taste aversion response and the conditioned immune response. Third, the effects of an extended interval between the CS and the US were investigated by inclusion of a 24 Hour Delay group. Finally, the presentation of the CS test trial consisted only

of flavor, rather than flavor and a superfluous saline injection.

Taste Aversion - Experiment 1

Rats that received the paired presentation of SAC and CY showed a significant flavor aversion ($p<.001$) on tests 1 and 2. As anticipated, conditioned rats (CS-US) showed a significantly reduced intake of SAC compared to control groups (CS Only and US Only).

Progressive decrements in taste aversion learning have been reported as a function of the interval between the ingestion of the flavor and subsequent toxicosis (e.g., Kalat & Rozin, 1973). The association between the flavor and illness inducing agent appears necessary for aversion learning (Domjan, 1980). The animals in the 24 Hour delay group did not show a significantly reduced preference for SAC as occurred in the CS-US group. This finding is consistent with the taste aversion literature, as the extended 24 hour interval inhibited the association between the CS and CY.

Animals in the CS+/CS- group were presented with a flavor paired with the US, and a novel flavor not associated with the US. Differential conditioning to the flavor (CS+) paired with the CY, was shown in one of five subjects. The other four subjects exhibited a decrease in consumption of the CS+ flavor only on the first test trial with their particular CS+. The increased consumption of the CS+ in those animals presented with the flavor on test 3, was probably secondary to the prior flavor exposures without aversive consequences (Garcia et al., 1955). Additionally, individual differences in the metabolism of the drug could have also produced

different associations (Bach, 1975). If drug effect onset varied, the different interstimulus intervals among rats could have occurred and produced response variability within the group. The animals exposed to the CS- on test 1, showed an aversion to that flavor that may be due to flavor neophobia (Domjan, 1977). The aversion to the CS+ on test 2 is due to the association of that flavor with the US on the conditioning day.

In summary, a conditioned flavor aversion was found in the treatment animals (CS-US). The response persisted for two test trials, prior to exhibiting an extinction pattern by test 3. A possible explanation for its extinction by test 3 was the repeated exposure to the flavor CS without aversive consequences, and the marginally intense CS or US used. Dragoin (1971) and Garcia, Ervin and Koelling (1966) have shown that the strength of a conditioned taste aversion is a direct function of the intensity of both the CS and US. A 0.01% solution of SAC, as used in the present study, has been frequently used in taste aversion research with a behavioral aversion exhibited. However, as a US, CY dosage has varied between 50 and 75 mg/kg with rats. It might be that the conditioned taste aversion response could have been enhanced by increasing the dosage of CY.

Antibody Titer - Experiment 1

It was hypothesized that CS-US and CS+/CS- groups would show a decrease in antibody titer compared to relevant control groups. Further, it was assumed that animals who did not receive the CY (CS Only, Positive Control) would exhibit a higher antibody

titer than those receiving the cytotoxic agent.

The CS Only exhibited a gradual rise in antibody titer with a plateau on days 35 through 42. The Positive Control subjects (n=2) showed a similar pattern in antibody production. However, a significant difference between these groups was found on day 28. The Positive Control animals had higher titer than the CS Only animals. Due to the small number of subjects in the Positive Control, it is possible that the increase in titer could have been due to sampling error or variability in the achievment of better immunization in these two subjects. Since this was the only data point at which significant differences were found, it is unlikely that the decrease in titer in the CS Only was due to the CS eliciting a response.

Rats not injected with CY, showed a higher titer than animals who received the CY injection. This finding was expected and observed on days 17 and day 21. This finding complements the results of prior investigations (e.g., Ader & Cohen, 1975). The present data also presented titer measured for 28 days following initial treatment. Such extended post treatment testing has not been reported previously in the literature. Although significant differences were not attained, the titer of the animals given CY (CS-US, US Only) was found to be higher than that of the CS Only on days 24 and 28 (Table 9, Figure 7). This finding could be partially accounted for by the degeneration of the CY induced suppression. The suppressive effects of CY terminate seven to ten days after CY administration (Shand, 1979). Therefore, the cessation of the effects of CY which

contributes to the inhibition of antibody production, could result in a fairly rapid increase of antibody titer.

The critical contrast, to ascertain any conditioned suppressive effects, was between the CS-US group and the US Only group. Since the residual suppressive effects of CY should have been cleared by day 28, the test trials after this point were of particular interest. The data revealed a suppression in titer in the CS-US group on days 31, 38 and 42 when compared with the US Only group. The attenuation of titer appears to be associated with exposure to the CS. This finding is consistent with results of previous research (e.g., Ader & Cohen, 1975). It extends the literature by demonstrating depression in titer following CS reexposure after the cessation of the residual effects of CY.

Of interest was the decrease in titer in the CS Only and US Only groups on day 35. As the antibody titer to BSA had not been previously examined beyond 14 days, it was thought that the decrease may be associated with the immunization procedure. By test 3, the antibody titer is outside the duration of CY suppressive effects. As the decrease in titer is also observed in groups not receiving CY, the change with titer is probably not associated with CY. There was no apparent changes in environmental conditions or experimental protocol at this time. It is reasonable to speculate that the decreased titer may be due to the time course of the immune response to BSA. However, until research is conducted on the subclasses of immunoglobulins involved in the response over an extended period, this conclusion remains tentative.

No attenuation of antibody titer was found in the 24 Hour group. These animals exhibited the immunosuppressive effects of CY on day 17 as compared to the CS Only. A depression in titer related to the CS was not observed in subsequent test trials. The failure to obtain conditioned suppression in the 24 Hour group was predicted. This failure is explained as a function of the extended interval between the ingestion of flavor and subsequent toxicosis. The pairing of the CS and US appears to require a shorter interstimulus interval.

The animals in the CS+/CS- group (Figures 9 and 10) also show an attenuation of titer on day 17 due to the immunosuppressive effects of CY. Animals that were presented with the CS+ on tests 1 or 2, do not show a depression in titer. Attenuation of titer is observed on test 3, in those animals presented with the CS+. However, these effects cannot be attributed to the CS as decreased antibody titer is found in the control groups. Therefore, the results appear inconclusive as depression in titer is only observed on day 35. The failure to attain systematic conditioned immune response might be explained by the marginal taste aversion found in this group. Animals only exhibited an aversion to the flavor paired with the US on the first presentation of that CS.

Antibody Affinity - Experiment 1

As with antibody titer, it was assumed that antibody affinity would be greater in those animals not given CY (CS Only, Positive Control). The affinity of the CS-US group was predicted to be less than the affinity of the US Only and CS Only following CS test trials.

The data on affinity was generally consistent with the findings on titer. As expected, no differences were found between CS Only and the Positive Control groups. This finding is consistent with results from prior investigations (e.g., Ader & Cohen, 1975), that reported no attenuation of titer due to flavor alone. The affinity was greater in the CS Only as compared to US Only and CS-US groups on days 17 and 21. This finding was expected since the CS Only was not exposed to the CY induced suppression. As with titer, the affinity of the CS-US and US Only groups was greater than the CS Only on days 24 and 28. It is argued that the increase in affinity was due to the cessation of the suppressive effects of CY.

The results from comparing the CS-US group and US Only were also similar to the findings with titer. A suppression in antibody affinity was found in the CS-US group on days 38 and 42, as compared to the affinity of the US Only group. As with titer, it appeared that the suppression was due to the CS exposures, that is a conditioned suppression was obtained.

A decrease in affinity was observed on day 35 in the CS Only, US Only, 24 Hour, and CS-US groups. These results complement the findings on titer, and, as noted earlier, may be associated with the immmunization protocol.

The affinity results in the 24 Hour group differ from the findings with titer in that affinity was significantly less than the affinity of the US Only group on day 38 (Table 15, Figure 12). The pattern of the affinity response in this group was similar to that observed in the CS-US group from day 31 through day 42, except that

the magnitude of the suppression was less. Statistical significance was only attained at this single time point, and the magnitude of the difference was not as great as the difference between the CS-US and US Only groups. The finding remains compatible with the evidence that optimal conditioning occurrs with short delays between the CS and US presentation (eg., Kalat & Rozin, 1973; Smith & Roll, 1967).

Summary - Experiment 1

This experiment demonstrated several findings of interest to this area of research. First, a taste aversion response was found in the CS-US group. The relation between taste aversion and the conditioned immune response remains unclear, as the depression in titer and affinity is found after the extinction of taste aversion. Second, a conditioned suppression in titer was found. This result complements the existing literature, and extends current knowledge by showing that a conditioned response could be attained after the suppressive effects of the CY had extinguished. Third, the conditioning effect was not limited to antibody titer, but was also expanded to the conditioning of antibody affinity. Fourth, the use of the ELISA was demonstrated to be effective in assessing an antigen specific response. Fifth, a conditioned suppression was found using a single element CS (test trials), which is different from previous studies. Finally, the findings of weak taste aversion and conditioned immune response in the 24 Hour group appear to be consistent with principles of learning. Areas that were problematic in this experiment included the failure to attain a taste aversion response and conditioned immunosuppressed response in the CS+/CS- group, and

the interesting finding of decreased titer and affinity on day 35 across all groups.

Taste Aversion - Experiment 2

Given the results obtained in Experiment 1, the dosage of CY was increased by 10 mg to 60 mg/kg of body weight. The purpose of this change was to increase and extend the duration of the taste aversion in the treatment group. An aversion to the flavor was found in the CS-US group on Tests 1, 2, and 3; and the response had extinguished by the fourth test. It appeared that the increase in the dosage of the US resulted in a prolonged aversion to the flavor in the treatment group.

Antibody Titer - Experiment 2

This experiment also differed from Experiment 1 as the antigen boost was given 24 hours after the single conditioning trial. The titer of the Positive Control was predicted to be greater than the titer of the CS-US and US Only groups. The CS-US group was also expected to have a decrease in titer as compared to the US Only group, following test trials.

A depression in titer was found on day 35 (test 3) in all groups independent of CY injection. The replication of this finding from Experiment 1 necessitates an extensive examination of the antibody response to BSA over time, and on variations of the immunization protocol.

The results on titer were different from the first experiment as suppression was not found in the treatment group. The titer of the CS-US group was greater than that of the Positive Control on Day 14 (conditioning day). This was unexpected as the increased dose of CY should have greatly suppressed antibody production. The increase in dosage could have resulted in a lysis of plasma cells with a massive release of antibody on day 14. The effect was short in duration as three days later (day 17) the titer of the CS-US group was less than the titer of the Positive Control. This result was expected, consistent with the findings of Experiment 1, and indicative of suppression induced by CY. On day 21, the titer is higher in the CS-US group as compared to the Positive Control. This rebound effect was possibly due to the effects of CY on the T lymphocytes (O'Reilly & Exon, 1985) in which the T suppressor cells are inhibited resulting in an enhancement of immune reactivity. Following day 24, the titer of the Positive Control was greater than the CS-US treatment group which is compatible with the findings from the first study. The US Only was also generally less in terms of titer than the Positive Control. As in the CS-US treatment group, increased titer on day 21 was found.

The critical comparison was between the titer of the US Only and the CS-US treatment group. Unlike Experiment 1, a decrease in titer was not found in the CS-US group. The titer of the treatment group was generally found to be greater than the titer of the US Only throughout this experiment. This finding was inconsistent with the assumption that the increased dosage of CY should have resulted in

marked suppression of antibody production. The variables accounting for the differences between the results of these two experiments in regards to titer remain obscure. The timing of CY administration is critical to attain maximum suppressive results (Dukor & Dietrich, 1970). The literature indicated that CY administration induced maximal suppression when antigen stimulation is given with CY until four days after CY administration (Bach, 1975). In the present study, the antigen was given one day following CY administration, and should have resulted in a robust immunosuppression. Therefore, the change in the timing of the antigen boost to 24 hours following exposure to the flavor and cyclophosphamide, was not thought to effect the change with titer.

The mechanisms explaining the changes seen in the CS-US group may have resulted from the increased dose of CY. Cyclophosphamide is one of the most potent inhibitors of antibody production in most species (Bach, 1975), including the rat (Harrison & Fuquay, 1972). Administration of CY shortly before or after antigen immunization inhibits subsequent antibody responses. Ghaffar, Sigel, and Huggins (1985) found that secondary immune responses were susceptible to CY administration, when given before the secondary challenge. The titer of the CS-US was generally found to be greater that the US Only from day 17 through 42, with significant differences on days 17, 31, 35, and 38 (Table 19, Figure 14). This finding could be related to the effects of CY on suppressor T cell functioning. Controversy exists regarding the mechanisms involved between CY and T lymphocytes (Shand, 1979). However, there is some evidence that CY affects T

suppressor cells that regulate humoral responses (Paul, Ghaffar, & Sigel, 1982; Shand, 1979). The inhibition of T suppressor cells could result in an elevation of antibody levels. Therefore, the higher titer in the CS-US treatment group could be explained by the initial effect of CY on the inhibition of T suppressor cells. The multiple exposures to the CS may extend this effect on subsequent test trials. This explanation is certainly speculative, and as such requires empirical evidence to examine the effects of various doses of CY on T suppressor cells and titer.

In summary, the results do not show conditioned suppression of titer in the CS-US treatment group following multiple presentations of the CS. A possible variable that may account for this change is the increased dosage of CY, with induced alterations on the T suppressor cell population.

Antibody Affinity- Experiment 2

As in Experiment 1, it was predicted that the affinity would be greater in the Positive Control than in animals receiving CY (US Only, CS-US). Antibody affinity was also postulated to be less in the CS-US treatment group, than in the US Only or Positive Control groups.

The affinity was found to be decreased on day 35 in all groups including the Positive Control. This finding is similar to the results with titer, and is consistent with the findings of Experiment 1. As described earlier, this depression on day 35 is thought to be related to the time course of the immune response and/or the immunization protocol.

The affinity was found to be greater in the Positive Control group on day 17, as compared to the CS-US and US Only groups. This finding is consistent with titer, and was expected. The affinity in the CS-US and US Only groups was larger than the Positive Control on day 21. As with titer, the increase in affinity could be due to the increased dose of CY and its effects upon T suppressor cells.

Differences between the affinity of the CS-US treatment group and the US Only were not found until days 31 and 35. The affinity was significantly ($p.<.05$) greater in the treatment group, and the result is similar to the findings on titer. Unlike the results with titer, an immunosuppressed response was found on days 38 and 42 (Figure 15). The affinity of the CS-US treatment group was less than the affinity found in the US Only group, following exposures to the CS. The result is congruent with the findings from Experiment 1. The suppresion in affinity but not in titer, can be resolved as affinity is more representative of the immune reaction. The assessment of affinity incorporates multiple data points across the entire range of antigen, while titer was limited to a measure of antibody production at a single antigen point. Therefore, the finding of no suppression with titer may be related to the limitations of examining a single aspect of antibody, rather than the complete range of the response.

Conclusions

The present investigation was significantly different from the prior research and produced several important outcomes to the area of psychoneuroimmunology. First, the results support the

premise that an environmental stimulus paired with an immuno-suppressive agent can subsequently elicit immunosuppression. A conditioned immunosuppressed response was found with titer and extended to affinity. Second, the effects of conditioning were broadened to include an antigen specific secondary response, not previously investigated. Third, the demonstrated immune suppression was extended to a response predominantly composed of IgG, and found after the residual effects of CY had been theoretically depleted. Fourth, the immunosuppression was shown following multiple exposures to a single element CS (flavor). The compound CS (flavor + injection) used in prior research is contrary to the findings of Garcia and Koelling (1966). Flavor stimuli have been shown to be more readily associated with toxicosis than exteroceptive cues. The cue-consequence specificty effect appears to apply in the present research, and it is suggested that the injection of saline does not acquire aversive properties. Fifth, the acquisition of taste aversion and conditioned immunosuppression appear to adhere to general learning principles. The response was not found by exposure to flavor alone, nor was it obtained in the condition with an extended interval between the CS and US. Sixth, the examination of the immune respone for an extended period resulted in findings not previously described. The attenuation of titer and affinity on day 35, across all groups, was unexpected. The change with titer and affinity may be secondary to the time course of the immune response to BSA, the immunization protocol, or the elimination of certain subclasses of immunoglobulins at this

time point. These explanations are tentative until additional research is completed on the lymphocyte response to different doses of BSA, and on the quantification of individual antibody producing cells to this antigen. Finally, the present research was inconclusive on the possible relation between taste aversion and the conditioned immune response.

Although a taste aversion was found in both experiments, it did not parallel changes in immune reactivity. Depression in antibody titer and affinity were observed after the taste aversion response had extinguished. Speculation on the relation between these two responses has received minimal attention within the literature. However, it could be important in identifying the experimental conditions that may result in optimal immune conditioning.

McCoy, Roszman, Miller, Kelley, and Titus (1986) varied the interval between the CS and US, and did not report changes in the magnitude of the taste aversion or conditioned immune response. Bovbjerg et al. (1987) found that mice with a weaker taste aversion, exhibited a stronger conditioned immunosuppressive response. It may be important to note that in the present study, depression in titer and affinity was shown only after the consummption of the CS increased. Bovbjerg et al. (1987) suggested that taste aversion and the conditioned immune response may be interdependent. It could be that the successful avoidance of flavor on early test trials, resulted in sufficient avoidance to reexposure to the CS such that only weak immunosuppression was elicited. Perhaps, the minimal intake of the CS flavor was not sufficient to

induce immune suppression. Additional investigations are needed to test such a concept, such as varying the intensity of the CS used in test trials.

A further contribution of this research was the demonstrated effective use of the ELISA to quantify antibody production. The adoption of this procedure allowed for the accurate and precise quantification of antibody titer and affinity. The continued use of this assay could result in a more accurate quantification of results and facilitate the comparison of results across investigations.

In conclusion, the present research supports the existing literature for an interaction between the CNS and immune system. This research has also generated additional questions regarding the variables controlling the conditioned immune response. As this field evolves, rigorous study is required to identify the necessary and sufficient conditions to elicit a conditioned immune response. Research efforts should continue to focus onelucidating the mechanisms that contribute to the response and on refining procedures to reliably quantify the effect.

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APPENDICES

Appendix A.
Immunization Protocol

IMMUNIZATION PROTOCOL

Antigen - Bovine Serum Albumin (Calbiochem 126615, lot 506787)

Procedure

The initial challenge of Bovine Serum Albumin (BSA) is 250ug emulsified in Freund's Complete Adjuvant. The total amount injected is .50ml and is a subcutaneous injection on multiple sites of the back.

The first boost is administered fourteen days later at 125ug emulsified in Freund's Incomplete Adjuvant. The total volume of the injection is .50ml and is a sc injection on multiple sites of the back.

Rationale

The above procedure is based upon data collected with Lewis male albino rats (Charles Rivers). In that experiment, the above immunization procedure was tested to determine optimal antibody response, time course of the response, and the effects of cyclophosphamide (50mg/kg) on the response.

Appendix B.
CY Induced Suppression

ELISA: Titer

Animal - Charles Rivers Antisera Dilution - 1:100 Antigen Range - 90ng

Condition	Animal	Non Immune		14 day chall		7day boost 21 day chall.		14day boost 28 day chall.	
		Opt. D.	X	Opt. D.	X	Opt. D.	X	Opt. D.	X
Immunized									
	CR 1	.084		.564		1.197		1.231	
		.081		.550		1.191		1.119	
		.080	.081	.544	.552	1.153	1.18	1.206	1.18
	CR 2	.078		.684		1.295		1.238	
		.080		.636		1.222		1.216	
		.086*	.079	.663	.661	1.298	1.27	1.212	1.22
	CR 3	.082		.579		1.071		1.039	
		.077		.557		1.068		1.037	
		.078	.079	.579	.571	1.044	1.06	.996	1.02
Immunized + CY									
	CR 5	.081		.236		.670		.610	
		.077		.242		.661		.658	
		.078	.078	.206	.228	.566	.632	.607	.625
	CR 6	.079		.313		.750		.816	
		.076		.282		.743		.826	
		.083	.079	.291	.295	.763	.752	.782	.808
	CR 7	.079		.333		.549*		.737	
		.081		.325		.521*		.709	
		.079	.080	.330	.329	.555	.555	.674	.706

ELISA: Titer + Affinity

animal: Charles Rivers Antisera dilution: 1:100 Antigen range: 90,30,10,3 ng

Condition	animal	Non-immune		14d challenge		7d boost 21d challenge		14d boost 28d challenge	
		slope	r^2	slope	r^2	slope	r^2	slope	r^2
IMMUNIZED									
	CR1	.0013	.008	.245	.91	.536	.97	.567	.96
	CR2	.0018	.002	.305	.87	.598	.96	.577	.96
	CR3	.0015	.00	.264	.93	.545	.99	.520	.98
IMMUNIZED + CY									
	CR5	.0022	.04	.087	.93	.337	.96	.328	.99
	CR6	.0022	.05	.114	.91	.379	.82	.396	.92
	CR7	.011	.21	.132	.93	.261	.91	.349	.94
immunized	MEAN (SEM)	.001 (±.002)		.271 (±.018)		.559 (±.019)		.555 (±.017)	
immunized + CY		.005 (±.003)		.111 (±.013)		.325 (±.034)		.357 (±.020)	

Probability: between antisera vs. time

immunized	p <	.00005	.00002	.4
immunized + CY	p <	.0007	.002	.2

Probability: immunized vs. immunized + CY

p <	.1	.00095	.0002	.0009

slope = multiple linear regression
r^2 = multiple r^2 (straight line= 1.00)
probability= students t-test (comparison of means)

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EDUCATION

Queensborough Community College	A.A. 1969	Liberal Arts
Hunter College	B.A. 1972	P.E./Health
Indiana State University	M.S. 1974	Adapted P.E.
Utah State University	Ph.D. Candidate	Psychology

PROFESSIONAL EXPERIENCE

Nov 1987 - Present

Clinical Instructor - Division of Behavioral Medicine, Department of Psychiatry, University of Utah Medical Center, Salt Lake City, Utah. Responsibilities - Provide behavioral/psychological evaluation and treatment of patients with health-related problems. Coordinate and develop behavioral medical consult-liason service for medical units. Coordinate chronic pelvic pain project.

Oct 1986 - 1987

Clinical Fellowship - Division of Behavioral Medicine, Department of Psychiatry, University of Utah Medical Center, Salt Lake City, Utah. Responsibilities: Provided psychological evaluation and treatment of patients with health-related problems, with an emphasis on the treatment and management of chronic pain. Included traditional psychological assessment and functional analysis of behavior. Intervention included cognitive/behavior therapy; relaxation/hypnosis/biofeedback; family systems, and psychoeducational approaches. Served as case manager for both inpatients, day-treatment patients, and outpatients as part of an interdisciplinary team. Behavior Medicine services provided on a consult basis to other medical units including: neuroscience, medicine, psychiatry and gastroenterology.

Oct 1985 - 1986	Clinical Predoctoral Internship - Consultation-Medicine/Geriatrics, Psychology Service, VAMC, Salt Lake City Utah. (APA Approved full-time internship.) Responsibilities: Provided psychological services to patients on medicine, neurology, or geriatric wards. The geriatrics rotation consisted of participation on an inter-disciplinary diagnosis/treatment team; psychological/cognitive assessment; and provision of brief individual/family therapy.
Sept 1984 - 1985	Psychology Intern (Clinical) - Outpatient Psychiatry, Primary Children's Medical Center, Salt Lake City, Utah. (APA approved program, 1000 hrs.) Responsibilities: Provided and developed therapy programs for children, adults, and families referred to the outpatient unit. Conducted psychological and educational assessments with written evaluation reports. Coordinate and implement research protocol for psychotherapy outcome studies.
July 1982 - 1984	Psychology Intern - Clinical Services, Division of Services, Exceptional Child Center, Utah State University. Responsibilities: Served as case coordinator or team member on interdisciplinary teams to evaluate developmentally disabled clients. Provided psychological and educational assessments, recommendations, and written evaluation reports for referred clients within the Exceptional Child Care Center and on Division of Services contracts for services outside of the Exceptional Child Center.
July 1981 - 1982	Research Associate - Social Integration Project (SIP), Division of Outreach and Training,. Exceptional Child Center, Utah State University. Responsibilities: Participated in the development and implementation of SIP monitoring system. Provided with SIP staff, training and consultant services to project sites. Designed and conducted a research project examining the social skill development of handicapped children.

Sept 1980 – June 1981: Research Assistant - Division of Outreach and Training, Exceptional Child Center, Utah State University.

July 1979 – June 1980: Project Director - Adapted Physical Education Project. Division of Training, Center for the Developmentally Disabled, University of South Dakota. Adapted Physical Education Project. Responsibilities: Directed/implemented BEH Grant activities.

July 1978 – June 1979: Coordinator - Life Skills Project, Division of Training Center for the Developmentally Disabled, University of South Dakota.

July 1975 – June 1978: Coordinator - Physical Education Project, Center for the Developmentally Disabled, University of South Dakota.

Sept 1974 – June 1975: Research Consultant - Indiana Special Olympics, Indiana State University.

Aug 1973 – July 1974: U.S.O.E. Fellowship Student - Indiana State University.

Nov 1972 – Aug 1973: Corrective Therapist - Veteran's Administration Medical Center, New York City.

ACADEMIC APPOINTMENTS

July 1976 – 1980: Adjunct Instructor - Department of Health, June Physical Education, Recreation, and Athletics, University of South Dakota.

July 1978 – June 1980: Clinical Instructor - Allied Health Sciences, University of South Dakota.

PROFESSIONAL TRAINING/PRACTICUM EXPERIENCE

Sept 1983 – 1984 Aug: Practicum Counseling/Therapy - Community Clinic, Psychology Department, Utah State University.

Supervisors: Elwin Nielsen, Ph.D., Professor of Psychology, Director of the Professional Scientific Program in Psychology, Utah State

University; William Dobson, Ph.D., Professor of Psychology, Utah State University, C. Foxley, Ph.D., Adjunct Professor Psychology, Utah State University.

Sept 1983 – May 1984: Practicum Biofeedback - Psychology Department, Utah State University.
Supervisor: Sebastian Striefel, Ph.D., Licensed Psychologist, Professor of Psychology, Director Division of Services, Exceptional Child Center, Utah State University.

March 1981 – June 1981: Child Practicum - Clinical Services Unit, Exceptional Child Center, Utah State University.
Supervisor: Phyllis Cole, Ph.D., Clinical Professor Psychology, Coordinator of Clinical Services, Exceptional Child Center, Utah State University.

Sept 1980 – March 1981: Child Practicum - Education Unit, Exceptional Child Center, Utah State University.
Supervisor: Frank Ascione, Ph.D., Associate Professor of Psychology, Chairman, Analysis of Behavior Program, Utah State University.

UNIVERSITY TEACHING

Jan 1983 – Mar 1983: Teaching Assistant - Human Development (undergraduate level, Psychology Department, Utah State University.
Supervisor: Frank Ascione, Ph.D., Associate Professor of Psychology.

Sept 1982 – Dec 1982: Teaching Assistant - Biological Bases of Behavior, Psychophysiology (graduate level), Psychology Department, Utah State University.
Supervisor: Carl Cheney, Ph.D., Professor of Psychology, Utah State University.

Sept 1979 – Dec 1979: Instructor - Seminar, Motor Activities for Handicapped, Physical Education/Special Education Department, University of South Dakota.

Jan 1976 – May 1976: Instructor - Adapted Physical Education, Physical Education Department, University of South Dakota.

PUBLICATIONS

Szykula, S.A., Czajkowski, L., and Laylander, J. (in press). Consciousness streaming: A single subject within subject analysis of relevant verbalizations. The Journal of Cognitive Psychotherapy: InternationalQQarterly.

Stowitschek, J., Czajkowski, L., Rule S., Striefel, S., and Innocenti, M. (1986). Systematic programming of social interaction through coincidental teaching. Technical Paper #59, Logan,: Utah State University, Development Center for the Handicapped.

Bernstein, G.S., Ziarnik, J.P., Rudrud, E.H., Czajkowski, L.A. (1981). Behavioral habilitation through proactive programming. Baltimore: Paul H. Brookes Pub. Co.

Czajkowski, L., Zawitkowski, A., and Rudrud, J. (1979). Directions in adapted physical education. Adapted Physical Education project (BEH Grant No. 6007900843): Center for the Developmentally Disabled, University of South Dakota, and S/D/Section for Special Education.

Czajkowski, L. (1978). Annotated bibliography on physical education of the handicapped. Division of Training, Adapted Physical Education Project: Center for the Developmentally Disabled, University of South Dakota.

RESEARCH INTERESTS

Psychoneuroimmunology

AIDS

Eating Disorders

Pain Management

PROFESSIONAL MEMBERSHIPS

Association for the Advancement of Behavior Therapy

Association for Behavior Analysis

American Psychological Association

Society of Behavioral Medicine