Tolerance to Drug-Induced (Poly I:C) Natural Killer Cell Activation: Effects of Partial Reinforcement

by

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ABSTRACT

Natural Killer (NK) cells are large granular lymphocytes that are considered to be the putative effector cells in immune surveillance against incipient neoplasia (Herberman & Ortaldo, 1981). Previous research has shown that tolerance to drug-induced Polyinosinic Polycytidylic Acid (Poly I:C) NK cell activation is attenuated by extinction and CS preexposure conditioning manipulations (Dyck, Greenberg & Osachuk, 1986). The thesis further evaluated the role of associative processes in the development of tolerance to NK cell stimulation by examining the effects of a known decremental Pavlovian conditioning training parameter - partial reinforcement (PRF). Fifty-six, female, DBA/2J mice were subjected to a conditioned tolerance training protocol involving repeated pairings of a complex environmental CS (exposure to peppermint odour and drug injection cues) with intraperitoneal injections of either Poly I:C (an unconditional stimulus - UCS for NK activity) or saline (placebo). Interspersal of nonreinforced (CS + placebo) trials between reinforced (CS + Poly I:C) trials constituted PRF. It was hypothesized that relative to continuous reinforcement, PRF would result in less tolerance to Poly I:C induced NK activity, and, that leaner PRF schedules would accentuate this effect. The design permitted

comparisons of a standard tolerance trained group (100% continuous reinforcement - CRF) with three PRF groups (55%, 38%, and 29% PRF), and, with additional controls receiving a single CS-UCS pairing at test. One of these latter controls (Handled-Injected Stimulated Control) was given prior exposure to the complex CS and placebo before the test drug injection while the other (Handled Stimulated Control) was not. Tolerance was observed in the CRF group relative to stimulated controls. Compared to the CRF group, PRF significantly increased NK activity only in the 29% PRF condition. Finally NK levels in the Handled-Injected Stimulated Controls were significantly elevated relative to Handled Stimulated Controls suggesting that one source of increased NK activity is increased numbers of injections. These results tentatively confirm experimental hypotheses and provide partial support for a conditioning analysis, however, they also suggest the need to isolate effects of decremental conditioning manipulations and number of injections in future investigations.

INTRODUCTION

There has been a longstanding belief in medical and psychological circles of a relationship between psychological processes and the onset and progression of disease. Anecdotal examples abound of people dying of a 'broken heart' following the loss of a spouse or loved one or becoming ill after a series of traumatic or stressful situations. In recent years folklore has been bolstered by empirical evidence which supports the influence of psychological/psychosocial variables as contributing factors to infectious diseases (Irwin & Anisman, 1984; Jemmot & Locke, 1984; Laudenslager, in press; Locke, 1982; Palmblad, 1981; Plaut & Friedman, 1981; Rogers, Dubey & Reich, 1979; Solomon & Amkraut, 1981; Stein, 1981) and to onset and prognosis of cancer (Borysenko, 1982; Eysenck, 1987; Fox, 1978, 1981; Irwin & Anisman, 1984; Levy, Herberman, Maluish, Schlien & Lippman, 1985; Sklar & Anisman, 1981; Solomon & Amkraut, 1981). Initial attempts to study the relationships between psychological processes and disease involved the correlation of life events, individual differences and disease onset. A typical example of this is the work of Holmes and Rahe (1967) who attempted to study the effect of various life stresses (as measured by the Social

Readjustment Rating Scale) on concurrent and subsequent illness development.

The immune system has recently been proposed to be an important mediating link between the various psychological and disease processes. The immune system can be thought of as a complex collection of various cellular and non-cellular humoral factors that act in concert to maintain the integrity of an organism by distinguishing components of self from non-self, and, destroying or eradicating non-self components (foreign bodies) when they have entered or are present in the organism. Psychological factors would then have their effect upon disease development by somehow compromising immune system functioning thereby rendering an organism more susceptible to disease (Fox, 1981; Irwin & Anisman, 1984; Jemmot & Locke, 1984; Locke, 1982; Palmblad, 1981; Stein, 1981).

Traditional immunology has considered the regulation and functioning of the immune system to be autonomous, self regulated and relatively uninfluenced by other factors (Ader, 1980; Ader & Cohen, 1985). This autonomy premise has delayed immunological researchers' appreciation of the potential contribution of psychological processes in modulating immune system activity and subsequent susceptibility to disease. It is only relatively recently that an interdisciplinary approach taking into account psychological, neurological and neuroendocrine influences

upon immune system functioning and illness development has emerged (Ader, 1980, 1981a, 1981c; Ader & Cohen, 1981, 1985; Cunningham, 1981; Fox & Newberry, 1984; Irwin & Anisman, 1984; Solomon & Amkraut, 1981; Stein, 1981). This new area has been coined "Psychoneuroimmunology" (Ader, 1981c) and is concerned with investigation of the role of the central nervous system (CNS) in the co-regulation of immune responses.

Evidence of bi-directional interactions between the CNS and immune system are provided by studies manipulating CNS activity and producing changes in immune functioning and vice versa. A fairly extensive body of evidence suggests that various central neurohormones and neurotransmitters effect immune functioning (Besedovsky & Sorkin, 1981; Hall & Goldstein, 1981; Irwin & Anisman, 1984; Sklar & Anisman, 1981). In addition to CNS influences on immune activity, it has been observed that stimulated immune cells affect CNS activity. For example, Besedovsky, Sorkin, Felix and Haas (1977) showed that there was more than a twofold increase in rat ventromedial hypothalamic neuronal firing rates at peak immune response to 2 different antigens. Subsequent research (Besedovsky, delRey, Sorkin, DaPrada, Burri & Honegger, 1983) demonstrated that rats having high immunological responses to sheep red blood cells exhibited significantly greater hypothalamic noradrenaline turnover rates compared to low responders. In addition, this effect

was mimmicked by intraperitoneal injections of supernatants from immune cells stimulated in vitro leading Besedovsky et al. (1983) to postulate that soluble products released from stimulated immune cells (lymphokines) might be acting in a feedback loop to induce the observed hypothalamic changes (Besedovsky, delRey & Sorkin, 1983, 1984). Additional support for this view is provided by recent evidence demonstrating that IL1 and rIL1 (immunoregulatory cytokines) produce changes in blood levels of ACTH and glucorticoids (Besedovsky, del Rey, Sorkin & Dinarello, 1986) suggesting that IL1 acts as an afferent signal to the CNS, while glucorticoids act as an efferent hormonal signal to the immune system.

Although the aforementioned studies provide evidence for the hypothesis of CNS - immune system interactions, the bulk of the research investigating the reciprocal communication between these two systems has relied upon two methods. The first method is an indirect strategy examining the effect of behavioral parameters and stress on host resistance to tumors and immunity (See Irwin & Anisman, 1984; Sklar & Anisman, 1981 for reviews). The second approach attempts to influence immune processes directly by conditioning of immunobiologic responses via Pavlovian conditioning procedures (See Ader & Cohen, 1985 for a review).

Behavioral Parameters, Stress and Immunity

The typical "stress" model used in studying CNS - immune system interactions has involved subjecting organisms to various aversive stimuli (i.e., stimuli that produce physiological changes, c.f. Sklar & Anisman, 1981, pp.369) and then monitoring various measures of immune functioning and/or resistance to tumors. Generally these procedures have led to immunosuppression which has been attributed to increases in glucorticoid steroids (Ader & Cohen, 1985, Irwin & Anisman, 1984; Sklar & Anisman, 1981). However, these effects have not consistently been observed and depending on parameters of the stressful situation, an aversive stimulus may enhance, suppress, or produce no changes in the same parameter of immune functioning. Not all of these aforementioned changes have been identified as being adrenocortically mediated (Ader & Cohen, 1985).

Some of the empirical inconsistencies in this literature have been reconciled by Sklar and Anisman's (1981) integrative review of the relationship between stress and cancer. They reviewed human and animal studies and concluded that aversive stimuli may produce either increased or decreased resistance to tumors depending upon two parameters - chronicity and controllability of the stressor. Specifically it was concluded that acute uncontrollable stress typically exacerbates tumor growth while this effect

is not seen with controllable or with chronic uncontrollable stress. In a representative study Sklar and Anisman (1979) found that DBA/2J mice receiving a single session of acute inescapable shocks grew larger tumors and had higher mortality rates than escapably shocked or non-shocked controls. On the other hand, increased tumor growth was no longer seen after five sessions of similar treatment and was actually inhibited after ten such sessions.

More recent studies have focused on the immunosuppressive effects of acute inescapable but not escapable shocks on measures of tumor rejection or direct measures of cellular immune function. In a study of tumor rejection, Visintainer, Volpicelli and Seligman (1982) found that male Sprague Dawley rats receiving threshold injections of tumor cells (Walker 256 sarcoma) prior to inescapable shock showed lower tumor rejection rates than escapable or no-shock controls. These results are consistent with the effects of acute inescapable shock on tumor growth (Sklar & Anisman, 1979). Results paralleling tumor rejection data using <u>in vitro</u> measures of cellular immune functioning were reported by Laudenslager, Ryan, Drugan, Hyson and Maier (1983). These investigators subjected rats to inescapable tail shock, escapable shock or no-shock and 24 hours later after all animals had been primed with a short series of mild inescapable footshocks, lymphocyte proliferation was assessed. They found that lymphocyte proliferation was

significantly suppressed in previously inescapably shocked animals relative to escapably shocked controls. In a similar vein, Shavit, Lewis, Terman, Gale and Liebeskind (1984) found suppressed Natural Killer (NK) Cell activity in Fischer 344 rats receiving intermittent (inescapable) footshock compared to continuously shocked controls. The former treatment is reversible by opioid antagonists thereby implicating endogenous opioids in the immune response. Still other research (Greenberg, Dyck & Sandler, 1984; Greenberg, Dyck, Sandler, Pohajdak, Dresel & Grant, 1984) has shown that the in vivo elimination of NK cell sensitive tumors (murine lymphomas) is suppressed following exposure to acute inescapable tail electric shock while chronic exposure to inescapable shock actually augmented tumor elimination.

The aforementioned results in general are consistent with the analysis of acute inescapable but not escapable aversive events being immunosuppressive, while, chronic exposure to these same stressors either shows no effect or immunoenhancement. Ader and Cohen (1985) stress that parametric analysis of the effects of stress, coping factors and immunocompetence need to be done to further advance our understanding of the contribution of these factors to infectious and neoplastic disease and provide an appropriate summary by which to end this section. They state that the contribution of stress on immune function depends upon:

(a) the quality and quantity (intensity,

> frequency, and duration) of stressful stimulation and the availability of means for coping with the environmental demands; (b) the quality and quantity of immunogenic stimulation; (c) the temporal relationship between stressful stimulation and immunogenic stimulation; (d) the parameters of immunological reactivity and the time(s) at which measurements are made; (e) the social (e.g., housing) and environmental (e.g., temperature, time of day) conditions on which stressful and immunogenic stimulation are superimposed; (f) a variety of host factors such as species, strain, age, gender and nutritional state; and (g) the interaction among these several variables. (p. 380)

Clearly, we are still a long way from defining the mechanisms by which stress and immunological interactions take place.

Conditioning of Immunobiologic Responses

Historical Overview of Conditioning of Immune Responses

The earliest research in conditioning of immunobiologic responses was conducted in the Soviet Union beginning in the late 1920s by followers of Pavlov.[1] One of the first investigations of the interaction between conditioning and immunity is thought to nave been conducted by Metalnikov and Chorine (1926) (c.f. Ader, 1981b). It had already been established at that time that injection of foreign material into the peritoneum of guinea pigs unconditionally elicited a nonspecific defense reaction characterized by an increase in polymorphonuclear (PMN) leukocytes[2] as well as the formation and secretion of antibodies. Using this

[1] See Ader's 1981b and Ader and Cohen's 1985 detailed reviews of the early Soviet research in conditioning of immunobiologic responses.

[2] By definition, an antigen is a molecule that stimulates immune responses (e.g. antibodies) by activating only those lymphocytes that bear surface receptors for what antigen. Like surface receptors, the antibodies that are elicited will react only with the antigen that induced their production. Such reactions are referred to as immunologically specific. In contrast, antigenic as well as nonantigenic materials can also elicit defense responses characterized by the production of nonantibody humoral factors. These factors (e.g. chemotactic, mitogenic, lytic, macrophage activating) interact with a variety of leukocytes in a nonantigen-specific way and thereby effect elimination of any foreign material that happens to be in the vicinity. Such reactions are referred to as "nonspecific". (This quote is taken from Ader and Cohen, 1985, pp. 395).

information they endeavoured to condition elevated levels in PMNs by Pavlovian conditioning techniques. Guinea pigs received ip injections of tapioca (bacillus anthracis) or staphylococcus filtrate (UCSs) in association with CSs of heat or a scratch applied to an area of the skin. Animals received single CS-UCS pairings daily for 18-25 days followed by a 12-15 day rest interval to allow the peritoneal exudate to return to baseline levels. One animal that received 21 CS-UCS pairings and was reexposed to the CS 13 days later showed a .6 to 62% increase in polynucleated cells in a 5 hour period. Two other animals showed similar responses providing additional support for conditioned increases in PMNs.

Metalnikov and Chorine then conducted a second study to determine if conditioned stimuli could be used to combat infection. Twelve CS (scratching of skin) - UCS (ip injection of staphylococcus filtrate) pairings were administered daily to two guinea pigs. Ten days after conditioning the CS was presented alone several times. The following day the two experimental guinea pigs and an additional untreated control animal were given a lethal ip dose of vibrio cholera. The control animal died while the two experimental animals survived. Two subsequent experiments repeated this exact same procedure with one modification. In these experiments only one of the two experimental animals was reexposed to the CS before

receiving the lethal vibrio culture. Thus two animals were conditioned one of which was reexposed to the CS; one animal received no conditioning treatment; all animals received lethal levels of vibrio culture. In these cases only the experimental animal reexposed to the CS lived or survived longer than the other two animals.

In another study changes in a specific antibody titer[3] in rabbits was measured (Metalnikov & Chorine, 1928, c.f. Ader 1981b). A group of three rabbits was exposed to CSs of heat to the ear or scratching of a flank followed by UCSs of 2 cc of vibrio cholera emulsion injected ip. These daily CS-UCS pairings occurred for 12-15 days. Conditioning of antibody titer was assessed by reexposure to the CS three weeks later (when antibody titer was still high). The two animals reexposed (rabbits 92 and 93) exhibited elevated antibody titers relative to the third animal (rabbit 96) not reexposed to the CS. Subsequent reexposure to the CS two months later in animals 93 and 96 again showed elevated titers while rabbit 92 who was not reexposed showed no change in antibodies. The results of these experiments suggested that levels of specific antibodies could be conditioned.

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[3] A haemagglutination antibody titer is a measure of antibody activity in blood serum. Different dilutions of serum are mixed with a specific antigen and placed in wells of agglutination trays. The term titer itself refers to the highest serum dilution giving an unequivocally positive reaction or antibody response. (Adapted from Roitt, I.M., 1977, pp. 134-135.)

The potential importance of the aforementioned studies by Metalnikov and Chorine was the impetus for further investigations. One of the superior studies of its time was conducted by Ostravskaya (1930) (c.f. Ader 1981b, pp. 326-328) who increased both the use of control conditions and the number of subjects. A CS of kinesthetic stimuli (either heat, scratching or electrical stimulation) was presented for 3-5 minutes followed by an ip injection of antigen (UCS). Conditioned subjects (guinea pigs) received CS-UCS pairings once daily for three weeks. Nonconditioned subjects received the CS without the UCS or the UCS without the CS. On the test day, 10-15 days later, peritoneal exudate[4] was examined at different intervals before and after UCS exposure and before and after CS presentation. Sixty seven percent of conditioned subjects reexposed to the CS exhibited an increase in polymorphonuclear leucocytes in their peritoneal exudate while only 23% of controls showed this change.

Numerous other studies followed Metalnikov and Chorine and Ostravskaya in further attempts to document conditionability of immune responses, but most were fraught with methodological and procedural difficulties. These

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[4] Peritoneal exudate is a fluid removed from the peritoneal cavity of an animal; it has a high concentration of protein and cellular debris which has escaped from blood vessels and has been deposited in tissues or on tissue surfaces, usually as a result of inflammation. (Adapted from Dorland's Pocket Medical Dictionary, 1982, 23 ed., Toronto, Ontario: W.B. Saunders Company.)

problems are aptly summarized by Ader and Cohen (1985):

> ...by 1960 several Russian studies had indicated that it was possible to condition alterations in specific immune responses (i.e., antibody production). However, the failure to confirm these observations in some laboratories and the lack of any notion as to how conditioned immunomodulation might occur left the issue open. Also, the variety of experimental paradigms made it difficult to discern the nature of any functional relationships between parameters of the conditioning process and immunological changes. For example, the nature of the antigen as well as the dose, route of inoculation, frequency of application, and the temporal relationship among conditioning, antigenic stimulation, and reexposure to conditioned stimuli are all relevant parameters that could influence the observation of conditioning effects. Similarly, the qualitative and quantitative characteristics of the CS, the CS-UCS interval, and the number of conditioning trials varied among experiments or, in a single experiment had been found to influence the conditioned response. (p. 382)

Contemporary Research in Conditioning of Immune Responses

Despite the difficulties inherent in earlier attempts to document conditioning of immune responses much progress has been and is currently being made. Among the forerunners in contemporary "psychoneuroimmunology" are Robert Ader, Nicholas Cohen and their research colleagues at the University of Rochester.

Conditioning and Humoral or Antibody Mediated Immunity

Ader and colleagues rediscovered the conditioning of humoral[5] immune responses when some of the animals involved in taste aversion learning experiments began to die. The animals (rats) were being exposed to saccharin flavoured water and an immunosuppressive drug (cyclophosphamide - UCS) which reliably produced a taste aversion to saccharin. Upon re-exposure to the CS (saccharin) during extinction trials, some of the animals died. Further examination revealed that

[5] When a foreign body (antigen) enters the body two types of immune responses can occur. The first, humoral or antibody mediated immunity (the subject of this section) is characterized by the synthesis and release of free antibody into the blood and other bodily fluids. Antibodies are non-cellular factors carried as soluble protein in the blood and bodily fluids. Some examples of antibodies are the various classes of immunoglobulins and complement. These antibodies act by direct combination with and neutralization of bacterial toxins by coating bacteria to enhance their phagocytosis and so on. The second type of immune response, cell mediated (T-cell mediated immunity) will be dealt with in a subsequent section. (Footnote adapted from Roitt, I.M., 1977, pp. 47.)

the animals which died first were those that had ingested the largest volume of saccharin i.e., those which received the largest amount of exposure to the CS. Suspecting that the death of the animals might be due to a conditioned immunosuppression which developed to the CS (saccharin), Ader and Cohen (1975) designed an experiment to test this possibility (Ader, 1981a, Ader & Cohen, 1985).

The standard protocol used by Ader and Cohen (1975) (and in many subsequent experiments) utilized placebo, nonconditioned and conditioned groups. After animals (male Charles River rats) were adapted to drinking their total daily water intake during a 15 minute period, conditioning treatments began. Conditioned animals received saccharin (0.1 % sodium saccharin solution) in their drinking water (CS) followed 30 minutes later by the UCS (ip injection of 50 mg/kg cyclophosphamide, CY). Nonconditioned (NC) animals received plain tap water followed by cyclophosphamide 30 minutes later. Placebo (P) animals received tap water followed by an equal volume of vehicle injected ip. Animals received plain water for the next two days during their 15 minute drinking period.

On the third day after conditioning all animals received ip injections of antigen (sheep red blood cells, SRBC). Thirty minutes later animals were re-exposed to saccharin or water followed by injections of CY or saline. Conditioned animals were divided into three subgroups: 1.

Group CS which received a single drinking bottle of saccharin followed by saline either 3 days, 6 days or on days 3 and 6 after conditioning; 2. Group UCS which received plain water and an injection of CY to determine the unconditional immunosuppressive effects of CY; 3. Group CSo which received only plain water and a saline injection to control for prior conditioning effects. During this phase conditioned animals were counterbalanced so that they received either plain drinking water with or without saline injections or saccharin and saline injections to control for fluid consumption and injection treatments over all groups. Nonconditioned animals received saccharin and saline injections to control for saccharin consumption and ip injections. Placebo animals were unmanipulated and only had access to plain drinking water during their 15 minute drinking periods. Nine days after conditioning all animals were sacrificed and trunk blood was collected for haemagglutinating antibody assay.

The results substantiated Ader and Cohen's (1975) initial suspicions. The placebo group had the highest antibody titers. The nonconditioned and CSo groups did not differ from each other but were both significantly lower in immune activity than the placebo group. Group UCS which received CY after antigen totally suppressed immune activity. The two critical experimental groups to assess conditioned immunosuppression were group CS1 (receiving one

CS exposure) and group CS2 (receiving two CS exposures). Antibody titers in Groups CS1 and CS2 were significantly lower than the placebo, nonconditioned and CSo groups. In addition, Group CS2 had titers below CS1 but the differences were not significant. These initial results suggested that conditioned immunosuppression of humoral immune responses were possible.

Replications of this initial experiment have been performed with male Sprague Dawley rats (Rogers, Reich, Strom & Carpenter, 1976) and male Wistar rats (Wayner, Flannery & Singer, 1978) using essentially the same procedures as Ader & Cohen (1975). Both of these studies found significantly lower haemagglutinating antibody titers in groups receiving two CS re-exposures compared to the other groups, while the single CS re-exposure group did not exhibit significant suppression of antibody titer. These experiments are consistent with Ader and Cohen (1975) in demonstrating the conditioned immunosuppression phenomenon.

Further research has attempted to replicate and extend the generalizability of the conditioned immunosuppression phenomenon by varying conditioning parameters within Ader's taste aversion protocol. For instance, the dose of CY has been increased from 50 to 75 mg/kg (Ader & Cohen, 1981), the UCS to produce immunosuppression has been changed from CY to methotrexate (Ader & Cohen, 1981), the CS in the taste aversion model has been changed from saccharin to sucrose

solution (Ader & Cohen, 1981) and to a chocolate milk solution (Ader & Cohen, 1985), the antigen used to produce antibodies has been changed from SRBC to 2,4,6-trinitrophenyl coupled to lipopolysaccharide (Cohen, Ader, Green & Bovbjerg, 1979), or Brucella Abortus (Wayner et al. 1978), the time between conditioning and CS reexposure has been varied (Ader, Cohen & Bovbjerg, 1982), as has been the number of CS reexposures before antigenic stimulation (Ader et al., 1982), differential fluid intake has been controlled (Ader et al., 1982), and conditioning was assessed at different periods of time after antigenic stimulation (Ader et al., 1982). In all these cases the conditioned immunosuppressive effect, although not always large, has been consistently seen (Ader, 1980, 1981a). This robustness of the conditioned immunosuppressive effect is summarized by Ader (1981a) who states:

> ...we have changed the CS and the US, varied the dose of immunosuppressive drug, increased the number of conditioning trials, increased the number of times conditioned animals were reexposed to the CS, decreased the possibility that control groups were experiencing some of the stimuli that comprised the complex CS, lengthened the interval between the conditioning and subsequent antigenic stimulation in order to reduce the residual immunosuppressive effects of CY, equated

> fluid consumption, varied sample time, and used mice as well as different strains of rats. The basic phenomenon could be observed under a variety of circumstances but, despite the methodologic refinements, we have not magnified the effects or conditioning. The results have been consistent and independently verifiable, but the effect has remained small. (p. 433)

In addition to demonstrating the generality of conditioned immunosuppression in their model, Ader and colleagues have also sought to quell arguments of the conditioning phenomenon being due to a stress induced increase in steroid levels leading to immunosuppression. In Ader's conditioning protocol cyclophosphamide (CY) has served as the UCS for suppression of antibody activity. It is known that both lithium chloride (LiCl) and CY are effective UCSs for producing taste aversions, elevations in corticosterone levels and conditioned adrenocortical responses (Ader, 1976). The two stimuli differ in that LiCl does not suppress the antibody immune response to SRBC, and as such, LiCl is a useful tool for assessing whether increased corticosterone levels superimposed upon residual immunosuppressive effects of cyclophosphamide mediate conditioned immunosuppression. Two experiments (Ader & Cohen, 1975; Ader, Cohen & Grota, 1979) assessed this

question. Ader and Cohen (1975) used their standard protocol but substituted LiCl for CY. The rats developed a taste aversion when subsequently tested but exhibited no conditioned immunosuppressive effects. Ader et al. (1979) also failed to find conditioned immunosuppression in animals that received LiCl as the UCS. In addition animals receiving injections of corticosterone at the time they were to be reexposed to the CS (saccharin) also did not show significant reduction in antibody activity compared to controls. These experiments then, lent no support to the hypothesis that conditioned immunosuppression is the result of a non-specific stress reaction mediated by glucocorticoids.[6]

Other researchers have also attempted to extend the generality of conditioned immunosuppression by using measures of immune functioning different from Ader and colleagues. For instance, Gorczynski, Macrae and Kennedy (1983) (c.f. Gorczynski & Kennedy, 1984) using Balb/c mice paired saccharin (CS) and cyclophosphamide (UCS) three times with 21 day intertrial intervals. Mice were then injected with sheep red blood cells (antigen) and a plaque forming cell response (PFC)[7] was measured 6 days later. Gorczynski

[6] The reader should be aware that Gorczynski, Macrae and Kennedy (1983) were unable to obtain conditioned immunosuppressive responses in adrenalectomized mice, but according to Ader & Cohen (1985) pp. 395 footnote 13, Gorczynski has subsequently been able to obtain taste aversions based on the experimental paradigm used in his laboratory.

et al. (1983) found conditioned immunosuppression in this experiment but only in animals that received CS-UCS pairings earlier during the day. Additional evidence of conditioned immunosuppression was also found in animals reexposed to immunologically inert cues after pairings of these cues with rotational stress (UCS) which unconditionally produced immunosuppression (Gorczynski et al. 1983).

Another group of researchers has also used the PFC response as a dependent measure to assess effects of taste aversion conditioning on immune system activity. Similar to Ader and Cohen (1975), McCoy, Roszman, Miller, Kelly and Titus (1986) began by adapting female Fischer 344 rats to water deprivation for five days. On the 6th day animals were randomly assigned to one of four conditioning groups: 1) Group C which received 15 minutes exposure to 0.15% saccharin solution followed 30 minutes later by ip injections of 50 mg/kg CY; 2) Group U received the same treatment as Group C; 3) Group P which received Sac followed 30 minutes later by saline and 4) Group R which received 15 minutes exposure to water followed by CY. Water deprivation

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[7] Rather than measuring serum antibody titers (as has been done by Ader and colleagues) Gorczynski and coworkers enumerate individual antibody-forming lymphocytes in a plaque assay. Immune lymphocytes from SRBC-immunized animals are incubated with the antigen and complement in a semisolid supporting medium (e.g., agar). A clear zone of hemolysis (i.e., a plaque) occurs around each antibody releasing cell and these plaques can be counted. Peak PFC responses occur before peak serum antibody titers can be detected, and as such PFC responses are a more sensitive measure of antibody activity. (Footnote adapted from Ader & Cohen, 1985, pp. 395).

resumed for days 4-6. On day 7 all rats were injected ip with antigen (SRBC) and exposed 30 minutes later to Sac (Groups C and P) or water (Groups U and R) drinking solutions. Thirty minutes later Group U was administered a further injection of CY to determine its unconditional effects while groups C, P and R received saline. Six days later (Day 13 - 9 days after conditioning) animals were sacrificed and assayed for PFC responses. Data on fluid intake by the rats showed Group C animals who received Sac + CY pairings and were reexposed to Sac to significantly reduce fluid intake relative to Group U and P animals and relative to their own fluid intake on the day of conditioning. Immunological data on PFC responses were consistent with data on fluid consumption. Group C exhibited significantly lower PFC responses than Group P or Group R replicating conditioned immunosuppression observed by Ader and Cohen (1975) using serum antibody titers. In a second experiment McCoy et al. (1986) utilized the same protocol with minor modifications using Balb/c mice as subjects. They obtained essentially the same results in fluid consumption and immunological data. In addition, in a third experiment with Balb/c mice these results were also replicated and extended to a group with a 3 hour delay between Sac and CY administration.

Conditioned immunosuppression has also been verified by Klosterhalfen and Klosterhalfen (1983b) who studied adjuvant

induced arthritis in female Han Wistar rats.[8] Conditioned animals received a saccharin-vanilla drinking solution (CS) paired with cyclophosphamide (UCS - 100mg/kg in experiment 1; 80 mg/kg in experiment 2). Nonconditioned animals received the CS and UCS in a noncontingent manner. Ten days later animals were reexposed to the CS and received a subplantar[9] injection of Freund's complete adjuvant (CFA) to induce arthritis. Hind paw swelling was assessed by two raters at various periods after CFA injection and subsequent CS reexposures to quantify immunosuppression. Dramatic swelling occurred in injected paws 24 hours later, but, there were no group differences. In contrast, re-exposure of conditioned animals to the CS at the time of injection of CFA, and, 2 and 4 days later significantly attenuated swelling seen in the uninjected paw relative to control animals when measured 12-20 days after CFA injection.

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[8] Adjuvant induced arthritis is a widely used animal analog of human rheumatoid arthritis. Adjuvant arthritis can be established by injecting Freund's complete adjuvant (CFA - a mixture of killed human mycobacterium tuberculosis incorporated in a water-oil emulsion) into the hind paw of a rat. Within 24 hours this paw swells considerably. On about the 12th postinjection day, the uninjected paw also starts to show signs of inflammation. The volumes of both paws increase during the following week and slowly decrease thereafter. In most animals the injected hind paw gets much thicker than the contralateral one. (Footnote adapted from Klosterhalfen & Klosterhalfen, 1983b, pp. 463).

[9] A subplantar injection is one given beneath the sole of the foot.

Another recent study (Sato, Flood & Makinodan, 1984) investigated the effect of a stimulus paired with shock on immunologic recovery in mice exposed to X-irradiation. Balb-c mice were subjected to 25 pairings of a buzzer (CS) and 3 seconds of .35mA inescapable electric footshock (UCS) for five conditioning sessions. Two days after the last session the mice were exposed to low dose X-irradiation (200 Rads) to suppress immunological reactivity. Fourteen days later mice received six sessions of five CS reexposures. Antigen (SRBC) was injected iv after the 4th CS reexposure trial. Spleens were removed and assessed for plaque forming cell responses 4 days after antigen injection. Animals unirradiated and stressed showed no suppression of antibody activity. In contrast, animals irradiated and reexposed to the CS associated with inescapable shock were immunocompromised relative to similarly trained groups not reexposed to the CS at test. This experiment then further supports the existence and generalizability of conditioned immunosuppression of humoral immune responses.

Finally, in contrast to the majority of studies finding conditioned suppression of a humoral immune response, Jenkins, Chadwick and Nevin (1983) have demonstrated conditioned <u>enhancement</u> of antibody production. These researchers used a variation of Ader's taste aversion paradigm. After animals were adapted to drinking their daily water intake during a 30 minute interval the

experiments began. In a first experiment there were 3 groups: 2 conditioned groups, Group CS2 and Group CSo; a nonconditioned (NC) group. On the day of conditioning (Day 0) Groups CS2 and CSo received 0.1% saccharin drinking solution (SAC) and ip injections of LiCl (128 mg/kg in a volume of 20 ml/kg). This treatment constituted the CS. These animals then received the US - an ip injection of antigen (2 ml/kg of 1% thrice washed suspension of sheep red blood cells - SRBC). Group NC received normal drinking water, ip injections of water and ip injections of SRBC. On days 7 and 9 Groups CS2 and NC were exposed to SAC + LiCl (CS) while Group CSo received normal drinking water and ip injections of water. On day 13 blood was drawn from all rats for hemagglutinating antibody titer analysis. On day 16 all three groups received treatment identical to that on days 7 and 9, and, on day 20 blood was again removed from all rats to assess antibody titers. Experiment 2 was a replication of experiment 1 with 2 differences. Firstly, the experiment was terminated on day 13 after removal of blood for antibody analysis. Secondly, an additional group, US, received SAC + LiCl (CS) plus SRBC (US) on the conditioning day (Day 0); received normal drinking water + injections of water plus SRBC (US) on days 7 and 9. Blood was also taken from this group for antibody analysis on day 13. No differences were found between NC and CSo groups in experiment 1 at day 13 or 20, nor in experiment 2 at day 13, therefore these 2 groups were pooled for comparison to CS2,

within each experiment. The results showed that group CS2 had significantly higher hemagglutinating antibody titers compared to the pooled control group for both experiments 1 and 2 on day 13. Although higher titers were also observed in Group CS2 on day 20 in experiment 1, this difference was not significant. These results then show conditioned enhancement of a humoral immune response (hemagglutinating antibody titers) can also be obtained using Ader's taste aversion paradigm.

Conditioning and Cell Mediated Immunity

In addition to demonstrations of the conditionability of humoral immune responses (antibody mediated immunity) other evidence suggests that conditioning can influence various types of cells involved in immunity collectively called cell mediated immunity.[10]

[10] In contrast to the previous section which dealt with humoral immunity, this section deals with research in cell mediated immunity. Cell mediated immunity is a bit of an archaic term which used to be synonymous with T-cell (thymus cell) mediated immunity. T-cell mediated immunity involves the production of 'sensitized' lymphocytes which have antibody-like molecules on their surface ('cell-bound antibody'). These resultant cells are involved in rejection of skin transplants and delayed hypersensitivity reactions. The current definition of cell mediated immunity is much broader, including any type of immune functioning delivered by a whole cell. Rather than talk about immune functioning per se, current immunologists talk about immune functioning regulated by classes of cells. Some examples of cell classes are beta cells which produce antibodies, T-cells which kill or assist in killing other cells (helper T cells, suppressor T cells, Killer T cells, allo-responsive T cells), macrophages, PMN's (polymorphonuclears) and NK (Natural Killer) cells. (Footnote adapted from Roitt, I. M.,

Bovbjerg, Ader and Cohen (1982) were able to modify their standard taste aversion conditioning protocol to investigate the possibility of conditioned immunosuppression of a local graft-vs-host response (GvHR).[11] Female (Lewis x Brown Norway) F1 rats were subjected to different treatments 48 days before induction of a local GvHR. Conditioned animals were exposed to a 0.15% sodium saccharin drinking solution (Sac) followed by ip injections of 50mg/kg CY. Nonconditioned (NCr) animals received CY followed by saccharin 28 days later. Placebo (P) animals received saccharin and ip injections of saline. On the day of grafting 48 days later (day 0), all animals received an injection in the right hind footpad of splenic leucocytes obtained from female Lewis donors. Previously treated conditioned animals were then divided into three subgroups. The experimental group (CS reexposure - CSr) was reexposed to Sac and injected with saline on day 0, injected with 10mg/kg CY 1 day after the graft (day 1) and again reexposed to saccharin and saline on the second day after grafting

1977, pp. 47).

[11] In a GvHR, grafted T lymphocytes recognize histoincompatibility alloantigens on cells of the host (but not vice versa). In the local GvHR reaction parental strain lymphoid cells are injected into the hind foot footpads of F1 hybrid offspring. The recognition of nonself by the injected donor cells results in the proliferation and recruitment of donor and host cells in the regional draining lymph node (popliteal node). This proliferation of cells is quantified by comparing the weights of lymph node that drains the site of injection and the contralateral node. (Adapted from Ader and Cohen, 1985, pp. 395; Bovbjerg, Ader & Cohen, 1982, pp. 583).

(day 2). Another conditioned subgroup (NO CS - CSo) was injected with CY (10mg/kg) on day 1 as a control for group CSr. The third conditioned subgroup (Group US) experienced no reexposure to saccharin but received CY (10mg/kg) on days 0, 1, and 2 to define the unconditional effects of the drug. Nonconditioned animals received Sac on days 0, 1, and 2 and CY (10mg/kg) on day 1 as did group CSr. Finally, P animals received only Sac on days 0, 1, and 2 to control for any of its unconditional effects. Five days after grafting popliteal lymph nodes were removed, dried, and weighed to quantify the GvHR. As expected group US showed the lowest GvHR (lightest ipsilateral node weights) consistent with the immunosuppressive effects of CY. In contrast, P animals showed the greatest GvHR (largest ipsilateral node weights). Group NCr and CSo showed lower GvHR than the P animals but only the latter difference was significant; both groups showed significantly greater GvHRs relative to group US. Finally, the critical experimental group, CSr, which received two CS reexposures had significantly lower ipsilateral node weights than P, NCr and CSo groups, and, in addition, it did not differ from group US which received two more 10mg/kg CY injections. These results extended conditioned immunosuppression to a GvHR 7 weeks after initial conditioning.

In a subsequent study, Bovbjerg, Ader and Cohen, (1984) again used a local GvHR in female Lewis x Brown Norway F1

rats to attempt to replicate and extend their previous results. Adding to the design of the earlier study, Bovbjerg et al. (1984) interspersed extinction trials (saccharin exposure + saline injection) during the seven week period between conditioning and induction of the GvHR. Three extinction groups were included: CS-4 (a group receiving four extinction trials - one trial every 8 days), CS-9 (a group receiving nine extinction trials - one trial every 4 days) and CS-18 (a group receiving eighteen trials - one trial every 2 days). Consistent with results of the previous study, animals receiving three exposures to Sac (CS) and CY (UCS) showed lowest GvHRs and P animals (Sac + Saline) exhibited greatest GvHRs. Conditioned animals reexposed to Sac (CS-0) after graft induction also again showed significant suppression of the GvHR relative to conditioned animals not reexposed to the CS (CSo). Conditioned animals receiving four extinction trials (CS-4) also exhibited significant suppression of the GvHR relative to CSo. However, groups CS-9 and CS-18 were not significantly suppressed relative to CSo indicating further extinction trials were successful in attenuating the conditioned suppression of the GvHR. This information thus further extends the role of conditioning parameters in modulation of cell mediated immune processes.

Much of the prior research in conditioning of immunobiologic responses has used CY (which has noxious

gastrointestinal effects) as a UCS for taste aversion and immunosuppression. In an attempt to determine whether behaviorally conditioned immunosuppression of a cell mediated immune response was possible with UCSs other than CY in a taste aversion paradigm, Kusnecov, Sivyer, King, Husband, Cripps, and Clancy (1983) used a biologic immunosuppressant (rabbit antirat lymphocyte serum - ALS) which selectively destroys lymphocytes in rats without other side effects. Male Wistar rats were adapted to a water deprivation schedule similar to that of Ader and Cohen (1975). On the conditioning day (day 0) animals were randomly assigned to one of three treatment groups. One group (saccharin/rabbit anti-rat lymphocyte serum - Sac/ALS) received exposure to 0.3% saccharin in tap water (CS) followed by a 0.2 ml ip injection of ALS (UCS). A second group (saccharin/normal rabbit serum - Sac/NRS) received similar treatment receiving normal rabbit serum as the UCS. A third group (water/rabbit antirat lymphocyte serum - Water/ALS) received tap water as the CS followed by ALS. All animals were given ad lib access to food and water for the next 8 days. On days 9-13 water deprivation was reinstuted. On the fourteenth day after conditioning animals were reexposed to the CS (either saccharin or water) and subsequently again given ad lib access to food and water. On day 21 all animals were sacrificed, mesenteric lymph nodes removed and immunological reactivity of dissociated cells were assessed via a mixed lymphocyte

culture.[12] Results indicated that group Sac/ALS exhibited a taste aversion to saccharin on the 14th day after conditioning relative to Sac/NRS and Water/ALS control groups. Reactivity of mesenteric lymph node cells showed that the Sac/ALS group was significantly less reactive compared to Water/ALS and Sac/NRS groups, while the Sac/NRS and Water/ALS groups did not differ. The data therefore illustrates that saccharin paired with a biological immunosuppressant (ALS) produces a greater suppression of mesenteric lymph node cell reactivity upon reexposure to the CS than animals receiving only ALS (Water/ALS) and demonstrates conditioned immunosuppression of a cell mediated response with UCSs other than CY are possible.

Some evidence suggesting the influence of conditioning manipulations in modulating cell mediated immune responses has also been found in humans. Smith and McDaniels (1983)

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[12] In the mixed lymphocyte culture reaction, lymphocytes from two histoincompatible animals are cocultured for several days. In this case spleen cells from a different strain of rat (Inbred male and female rats of the DA strain) were cocultured with cells of the mesenteric lymph nodes of male Wistar rats. The ensuing proliferation of T-cells is quantified by scintillation spectrometry of the cultures that were pulsed with tritiated thymidine for several hours prior to the termination of the culture period. Proliferation reflects the recognition of foreign histocompatibility alloantigens by T-cells that do not themselves display the same antigens. If lymphocytes from one of the animals are prevented from proliferating, then the thymidine incorporation reflects proliferation of cells from the animal that provided the responder cells (i.e., the animal that was treated with ALS - in this case the Wistar rats) (Footnote adapted from Ader and Cohen, 1985, pp. 395).

were interested in determining whether the delayed type hypersensitivity reaction (DTH)[13] to tuberculin in humans could be reduced by conditioning manipulations. Seven volunteer subjects participated in an experiment in which they were subjected to 6 monthly tuberculin skin testing sessions. A nurse blinded to the experimental protocol administered treatments. For 5 monthly sessions one arm of each subject was consistently administered a substance from a green vial (tuberculin), while the other arm received a substance from a red vial (saline). On the test trial (month 6) the contents of the vials were reversed and each subject now received tuberculin in the arm that previously received saline and vice versa. The UCS in this situation was the tuberculin injection which produced erythema and induration (UCRs) while the CS consisted of the multitude of cues in the drug administration situation (e.g., the vials the drug was in, the room and day of the week treatment took place, the nurse etc.). Each subject was monitored for the amount of erythema and induration present in each arm 24 and 48 hours after each of the 6 monthly treatments. Results indicated no erythema or induration after any of the saline trials. However, the arms that received tuberculin after

[13] Delayed type hypersensitivity (DTH) reactions are inflammatory responses that are initially mediated by T lymphocytes. They are measured by a local skin reaction (erythema - redness of skin due to congestion of capillaries; induration - hardening of the skin) that occurs 24-48 hours after the cutaneous challenge with an antigen to which an individual has previously been immunized (sensitized). (Footnote adapted from Ader and Cohen, (1985), pp. 395)

five repeated saline trials showed significant diminution in erythema and induration relative to the stable level of responding observed in the same arms during the five previous saline trials. These results then are consistent with a conditioned suppression (diminution) of a delayed type hypersensitivity response perhaps as a result of CS preexposure (latent inhibition - Lubow & Moore, (1959).

Additional evidence consistent with the ability of conditioned immunopharmacologic responses to modulate cell-mediated immune functioning has been provided by Gorczynski, Kennedy and Ciampi (1985). Using a taste aversion protocol similar to Ader and Cohen (1975), Gorczynski et al. (1985) exposed Balb/c female mice to three pairings of 1% saccharin drinking solution and ip injections of 125mg/kg CY at 21 day intervals. Three weeks after the last trial animals received iv tail vein injections of a Balb/c positive plasmacytoma tumor. Animals were then either reexposed to saccharin or plain drinking water. Results indicated that animals receiving Sac and CY conditioning trials and reexposed to Sac had significantly higher mortality rates and significantly increased levels of plasmacytoma tumors. Further experimentation revealed increased levels of histamine type II receptor bearing T suppressor cells in spleen cells of these animals, and it was speculated that conditioned increases in levels of these suppressor cells may have been responsible for increased plasmacytoma tumor

susceptibility as the administration of cimetidine (a histamine type II receptor antagonist) reversed the mortality previously seen.

A recent study has also provided evidence for conditioned suppresssion of a cell mediated immune response. Using Ader's taste aversion paradigm O'Reilly and Exon (1986) investigated whether several immune responses could be concomitantly conditioned in individual Sprague-Dawley rats. The immune responses measured included: (a) antibody production to T-dependent keyhole limpet hemocyanin (KLH) - a measure of serum immunoglobulin G (IgG) antibodies, (b) delayed type hypersensitivity reactions (foot pad swelling) to bovine serum albumin (BSA), (c) natural killer cell (NKC) cytotoxicity to tumour cells, (d) 2 immunoregulatory cytokines - lymphocyte derived interleukin 2 (IL2) and macrophage-derived prostaglandin E (PGE), (e) spleen weights, (f) number of splenocytes, and, (g) number of resident peritoneal cells. After animals had been adapted to water intake, conditioning treatments began. On the day of conditioning (Day 0) conditioned animals received 0.15% sodium saccharin solution (SAC) during their 30 minute drinking periods followed by subcutaneous (sc) injections of 50 mg/kg cyclophosphamide (CY). Nonconditioned (NC) animals received plain drinking water and CY. Placebo (P) animals received plain water and sc injections of vehicle. On day 15 each rat was injected sc with BSA to induce a delayed

type hypersensitivity reaction (footpad swelling) and KLH to induce a humoral (antibody) immune response. On day 22 conditioned animals were divided into 3 subgroups. Group CS2 received SAC + sc injections of vehicle, group CSo received water + vehicle, while group US received water + CY. NC animals received SAC + vehicle while P animals received water + vehicle. All rats also received a footpad injection of BSA to assess suppression of delayed type hypersensitivity (footpad swelling) on day 23. On day 23 footpads were measured to assess delayed type hypersensitivity and received an additional KLH injection to induce IgG antibody production. On day 26 exposure to SAC, water, CY or vehicle treatment combinations were exactly as on day 22. Finally, on day 29 all animals were sacrificed and all other immune measures were assessed. Results showed that group CS2 significantly reduced fluid intake on days 22 and 26 compared to group NC, indicating that a taste aversion had developed. Results of the immune measures, however, showed that only the cellular immunity response - NKC cytotoxicity was significantly suppressed in group CS2 relative to group CSo. Thus, this experiment further extends conditioned suppression of cellular immunity to NKC[14] cytotoxicity.

[14] Natural Killer Cells are a subpopulation of granular lymphocytes believed to be the putative efffector cells for surveillance against incipient neoplasia (see subsequent section on NK cells for further information). NKC cytotoxicity is measured in a standard in vitro ^{51}Cr release assay. Essentially, spleens are disaggregated to single cell suspensions and red blood cells are lysed by

Finally, in contrast to the majority of research demonstrating conditioned immunosuppression of various types of immune responses, Gorczynski, Macrae and Kennedy (1982) have provided evidence for conditioned enhancement of a cell mediated immune response. They studied the in vivo priming of the cytotoxic T lymphocyte responses between individuals of different inbred strains of mice. In this paradigm introduction of a foreign alloantigen[15] (UCS - in this case a skin graft from a different strain of mouse) induces an immune response (UCR - increase in cytotoxic T lymphocyte precursor - CTLp) which can be assessed in tissue culture. The CS in this paradigm consists of all the environmental cues involved in preparation of the mouse for skin grafting e.g., shaving of the area to be grafted, handling for ip administration of pentobarbital anesthetic, excision of the dermis in the area to be grafted, and encasement of the grafted area in gauze and plaster of paris for 9 1/2 days all contribute to the CS complex. The administration of the

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hypotonic shock. The remaining white blood cells are incubated in vitro with an NK cell sensitive target, YAC-1 lymphoma tumour cells. The tumour cells are labelled with ^{51}Cr and NK cell cytotoxicity is assessed by the amount of specific ^{51}Cr released from lysed YAC-1 tumour cells. NK cell cytotoxicity is directly proportional to the amount of ^{51}Cr release.

[15] Alloantigens are antigens obtained from one individual (or inbred line) that will incite a specific immune reaction when they are introduced into another individual (or inbred line) of that same species. In this case the tailskin grafts from C57BL/6J mice are the alloantigens that produce increases in cytotoxic T lymphocyte precursors in CBA mice recipients. (Footnote adapted from Ader and Cohen, (1985), pp. 395).

UCS in this model is the actual grafting of allogeneic tissue (tailskin grafts from C57BL/6J mice) to the graft recipient (male CBA mice) which produces increases in cytotoxic T lymphocyte precursors (UCR). Conditioning involved repeated pairings of the graft preparation and grafting procedure (CS + UCS) over 40 day intervals (the amount of time required for healing of all wounds in the manipulations used and the recovery of CTLp to baseline levels). Three CS-UCS pairings were used in these experiments followed by presentation of the CS alone (sham graft). The results in two different experiments showed that more than 50% of animals conditioned and exposed to sham grafting exhibited increases in CTLp (responders) while the remainder showed no response. In a second phase of the experiment these responders were then divided into two groups. One group received two additional conditioning trials and the other received two extinction trials (CS exposures - sham grafts). When both of these subgroups were subsequently reexposed to the CS, those animals receiving additional CS-UCS pairings showed an increase (enhancement) in CTLp over their previous levels while animals in the extinction condition displayed a significant decrease in CTLp from their previous responses. These results are provocative in the demonstration of conditioned enhancement of an immune response and might be useful therapeutically if a less "aversive" method of producing the enhancement could be found.

Other Evidence of Conditioned Immune Responses

Most of the contemporary research in conditioning of immunobiologic responses has demonstrated suppression of various immune mechanisms. One might ask, "What is the adaptive significance of an individual lowering its immunity?". Although it provides a possible explanation for disease onset, it seems almost paradoxical for an organism to learn how to increase its susceptibility to disease! For this reason, Ader and Cohen (1982) searched for a paradigm in which conditioned suppression of an immune response would be in the survival interests of the organism. The paradigm chosen was an animal model of autoimmune[16] disease called systemic lupus erythematosus (SLE). In SLE, female New Zealand (NZF1) hybrid mice develop a lethal glomerulonephritis (inflammation in the kidney with specific inflammation of the capillary loops in the renal glomeruli) and progress of the disease can be monitored by the rate of development of proteinuria (excess of serum proteins in the urine). Progress of the disease can be retarded by repeated administration of CY. Therefore the question was whether

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[16] Immune mechanisms of the body allow for differentiation of self components from non-self components. When there is a breakdown of these mechanisms the body can no longer separate self from non-self and a condition called autoimmunity (immunity against self) results. The whole spectrum of diseases and disorders involving attack of the body by its own defences are referred to as autoimmune disease. These disorders can be the consequence of cellular and/or antibody-mediated immune reactions. (Footnote adapted from Roitt, I. M., 1977, pp. 265; Ader & Cohen, 1985, pp. 395).

conditioning manipulations would retard proteinuria and mortality associated with onset of SLE. Four month old, female (NZF1) mice received repeated weekly pipette administrations of 0.15% sodium saccharin solution (SAC - CS) with ip injections of either 30mg/kg CY (UCS) or saline (placebo) for 8 weeks. Group C100 received Sac and CY weekly for all 8 weeks. Another conditioning group (C50) received 50% partial reinforcement of CY for 4 weeks (four Sac + CY pairings and four Sac + placebo pairings). A third nonconditioned group (NC50 - a control for C50) received the same number of Sac and CY exposures but in noncontingent fashion (i.e., on different days of the same week). A final untreated control group received eight weekly noncontingent Sac and Saline pairings. As expected group C100 developed proteinuria significantly more slowly than all other groups. Group C50 (50% partial reinforcement) developed proteinuria significantly more slowly than untreated controls and group NC50 which received equal amounts of CY. Similar results were seen in mortality data. Group C50 survived significantly longer than untreated controls and significantly longer than group NC50 which received equal amounts of CY. In addition, group C50 did not differ in rate of mortality from Group C100 which received twice as much CY.

A subsequent study investigated the effects of extinction on modulating the development of SLE.

Essentially the same design was used as in Ader and Cohen (1982) except that the partially reinforced group (C33) received Sac and CY pairings only 1/3 of the time (c.f., Ader & Cohen, 1985). After initial conditioning training the 3 groups C100, C33, and NC33 were subdivided into groups which received: (a) additional Sac and CY pairings, (b) extinction (Sac + placebo) trials or, (c) no treatment. Results indicated that C100 animals receiving additional pairings lived longer than those deprived of such pairings. Furthermore, animals receiving extinction and partial reinforcement (C33) respectively, did not differ in mortality from each other or the C100 condition.

These results provide evidence that conditioned immunosuppression as assessed by the delayed onset of SLE and decreased mortality is a reliable effect. In addition the procedures of partial reinforcement and extinction do not eliminate the observed effects.

As can be seen from the review of current data on conditioning of immunological responses, evidence has accrued demonstrating conditionability of humoral, cell, or possible combinations of humoral and cell mediated immuniy (autoimmune disorders).

Conditioned Tolerance and Immune Responses

Recent evidence (Siegel, 1979, 1983) suggests that development of tolerance to drug effects can be conceptualized in a Pavlovian conditioning model. Since much of contemporary research in conditioning of immunobiologic responses uses drug UCSs as part of their methodology (e.g., cyclophosphamide), development of tolerance to these drugs could potentially influence the immune responses observed in these experiments. Therefore a brief review of evidence implicating the role of associative processes in development of drug tolerance is provided.

Definitions of Tolerance

The phenomenon of drug tolerance refers to the decreasing systemic effects of a drug over the course of its repeated administrations, or, the necessity of increasing the amount of the drug over repeated administrations to maintain the initial effects of the drug (Siegel, 1979).

Earlier theories attempting to explain the development of drug tolerance were systemic theories (Cochin, 1970; Collier, 1965) emphasizing physiological changes induced by earlier drug administrations that functionally reduced effects of the drug on subsequent administrations (c.f. Siegel, Hinson & Krank, 1978). These theories attribute

tolerance only to pharmacological/physiological changes and make no provisions for the role of associative effects in tolerance development.

More recently Siegel (1979, 1983) has proposed a model of drug tolerance incorporating Pavlovian conditioning principles. This "conditioned" drug tolerance model is an outgrowth of work by Wikler (1973) who acknowledged the role of pharmacological learning in tolerance development, and of conditioned opponent process theories of conditioning (Schull, 1979). (A review of different interpretations of the influence of conditioning on drug tolerance can be found in the Appendix at the end of this thesis.)

A learning analysis of tolerance is built upon the work of Pavlov (1927, p. 35-37) who suggested that the routine administration of a drug constitutes a conditioning trial. In this model the pharmacological effects of the drug (the Unconditional Stimulus, UCS) is frequently preceded/accompanied by many cues (Conditional Stimuli, CSs) unique to the drug administration context. These cues consist of environmental stimuli, rituals and procedures which reliably precede the drug effect. Development of any associations between predrug cues (CS) and the effect of the drug (UCS) may be revealed by replacing the drug with a placebo in the usual drug administration situation and then monitoring the appropriate response system affected by the UCS (See Siegel 1979, 1983 for a review). When this

procedure is executed, the observed CR sometimes mimics the UCR, while in other situations it opposes the UCR (Siegel, 1975, 1977, 1978, 1979, 1983; Siegel, Hinson & Krank, 1978). This "opponent" CR has been observed with a variety of drugs, in numerous physiological systems employing different conditioning paradigms (See Siegel 1979, 1983 for a review). For example, morphine may produce bradycardia, analgesia and hyperthermia as UCRs with accompanying opponent CRs of tachycardia, hyperalgesia and hypothermia (Siegel, 1979, 1983).

The presence of the aforementioned conditioned opponent drug responses form the basis of a conditioning analysis of drug tolerance (Siegel, 1979, 1983). If a conditioned compensatory (opponent) response develops to any of the cues in the predrug administration ritual/context, its summation with the unconditional effects of the drug will contribute to the reduction of the net drug effect, i.e., tolerance will develop to the drug. Thus, a conditioning model of tolerance emphasizes the gradual development of compensatory (opponent) CRs as being responsible for the diminishing effects of the drug.

Support for a Conditioning Analysis of Tolerance

The most powerful evidence corroborating the contribution of conditioning factors in development of tolerance is provided by conditioning manipulations known to affect CRs, and hence tolerance. This approach has been employed in studying tolerance to the effects of ethanol and barbiturates but most extensively in the study of morphine tolerance (See Siegel, 1979, 1983 for a review). Therefore, data from the morphine tolerance literature will be briefly reviewed.

Situational Specificity. One series of studies may be referred to as environmental or situational specificity designs. All of these experiments incorporated two groups receiving morphine tolerance training in the presence of cues that reliably signaled the drug administration, e.g. the situation or context of the drug administration ritual. In all cases the effects of the drug were assessed by a subsequent tolerance test phase. One group was usually tested by receiving the drug in the presence of cues it was exposed to during tolerance training (same tested). The other group was generally tested by receiving the drug in the presence of cues different from those received during tolerance training (different tested). A conditioning analysis of tolerance would predict greater tolerance in groups that were same-tested than different-tested as cues

associated with the the drug should produce CRs which reduce the net drug effect. Considerable evidence has accrued to substantiate this analysis of morphine analgesic tolerance in rats (Adams, Yeh, Woods & Mitchell, 1969; Ferguson, Adams & Mitchell, 1969; Kayan & Mitchell, 1972; Kayan, Woods & Mitchell, 1969) using a hot plate (Siegel, 1975; Krank, Hinson & Siegel, 1981), a paw pressure analgesiometer (Siegel, 1976), a tail flick (Advokat, 1980) or a flinch jump apparatus (Tiffany & Baker, 1981) to measure analgesia. Some evidence also suggests the influence of situational cues in modulation of heroin overdose death in humans (Siegel, Hinson, Krank & McCully, 1982).

Other Manipulations that Attenuate Tolerance Acquisition. Another series of experiments demonstrating the influence of conditioning in development of morphine tolerance are those using manipulations of the putative CS. These manipulations consist of presenting only the CS before (CS preexposure - latent inhibition - Lubow, 1973; Lubow & Moore, 1959), during (partial reinforcement - PRF - Mackintosh, 1974, p. 72-75; Marx, 1971, p. 163-165), or after (extinction - Schwartz, 1978, p. 70) conditioning trials, and, all attenuate CRs and hence tolerance development.

In the CS preexposure experiments (Siegel, 1977, Experiment 3; Tiffany & Baker, 1981) rats were repeatedly

exposed to all cues in the morphine administration procedure that would later signal the effects of the drug. Assessment of CS preexposure effects was executed by monitoring the development of morphine analgesic tolerance in a subsequent tolerance acquisition test. Evidence from the two experiments consistently shows that relative to groups receiving no prior training, CS preexposure groups were slower to acquire tolerance to the analgesic effects of morphine, i.e., morphine analgesic tolerance was latently inhibited.

Experiments using partial reinforcement (PRF) (Siegel, 1977, Experiment 4; Siegel, 1978, Experiment 3) intersperse placebo treatments (conditioning procedure with physiological saline injection) with morphine injections during tolerance training. Therefore the cues of the conditioning situation are not always followed by the pharmacological effects of the drug. The effect of partial reinforcement is then assessed by observing the tolerance which occurs when both predrug cues and morphine are administered on a test session. Partial reinforcement groups treated in this manner show significantly reduced tolerance to the analgesic (Siegel, 1977, Experiment 4) and pyretic (Siegel, 1978, Experiment 3) effects of morphine relative to continuously reinforced control groups.

Extinction is the process of reducing established CRs by presenting the CS without the UCS. Experiments in the

morphine tolerance literature utilizing this procedure first repeatedly pair predrug cues with morphine until tolerance develops. Extinction groups then receive exposure to only the CS (predrug cues). Finally tolerance is assessed in all groups by reexposure to the drug in the presence of predrug cues. A conditioning theory of tolerance would predict that extinction should attenuate or reverse tolerance if tolerance development is due to a conditioned compensatory response opposing the effects of the drug. This is exactly what has been found. Tolerance to the analgesic (Siegel, 1975, Experiment 3; Siegel, 1977, Experiments 1 and 2; Siegel, Sherman & Mitchell, 1980) and pyretic effects of morphine (Siegel, 1978, Experiment 2) was reversed suggesting tolerance development is a conditioning phenomenon.

A final manipulation shown to moderate morphine tolerance development is the use of an explicitly unpaired procedure where the CS consistently predicts the absence of the UCS. The CS is subsequently paired with the UCS and acquisition of CRs is monitored. This procedure typically retards or inhibits development of CRs relative to groups receiving CS - UCS pairings. Siegel, Hinson and Krank (1981) used this procedure to examine development of morphine tolerance. Consistent with a conditioning analysis, groups which received explicit unpairings of predrug cues and morphine were significantly less tolerant

to the analgesic effects of morphine relative to a group receiving predrug cues and morphine pairings.

A Departure from Contemporary Immune Conditioning Research

A departure from contemporary research in conditioning of immunobiologic responses concerns the use of a conditioned drug tolerance paradigm to study Natural Killer (NK) cell activation (Dyck, Greenberg & Osachuk, 1986). Before delving into the specifics of the departure, it is necessary to provide a description of NK cells, including their role in immune system functioning and resistance to disease.

Natural Killer (NK) Cells and Immune Resistance to Tumours

Natural Resistance. During the 1970's the dominant theory of immune surveillance against tumors postulated that T-cells or thymus dependent lymphocytes were the major mediators of anti-tumor immunity (c.f. Greenberg, Dyck & Sandler, 1984). However, tests of tumor immunity in the congenitally athymic nude mouse which is devoid of T-cell activity still showed resistance to tumors (Rygaard & Povlsson, 1976), and this led to a reevaluation of tumor immunology (Moller & Moller, 1976) and a search for other

effector cells involved in tumor surveillance (Greenberg & Greene, 1976). Research since this time has focused on non T-cell mediated immunity referred to as 'Natural Resistance'. Natural resistance defences include humoral (macrophages), cellular (Natural Killer - NK Cells), and T-independent natural antibody mechanisms (c.f. Greenberg, Dyck & Sandler, 1984), however, increasing numbers of studies point to NK cells as being the putative effector cells for surveillance, control of tumor cells, and metastasis (Karre, Klein, Kiessling, Klein & Roder, 1980).

Natural Killer (NK) Cells. Natural Killer[17] Cells are a subpopulation of lymphocytes found in a wide range of mammalian and avian species (Herberman & Ortaldo, 1981). They compose only about 5% of the peripheral blood or splenic leucocytes in man and other species and are only identifiable morphologically, i.e., NK cells are large granular lymphocytes (Herberman & Ortaldo, 1981). Natural Killer cells destroy other cells by cell lysis[18] and are intermediate in specificity and speed of reaction between T-cells (which are relatively slow and highly specific in the targets which they attack) and macrophages and PMN's (which act rapidly and are regarded as non-specific for targets)

[17] See Herberman & Ortaldo, 1981 for an excellent review of major effector cells in the immune system (T-cells, macrophages, monocytes, and PMN's) and their relationships to NK cells.

[18] Cell lysis is the destruction or decomposition of cells. The mechanism by which NK cells lyse other cells is not completely understood (Herberman & Ortaldo, 1981).

(Herberman & Ortaldo, 1981).

Some additional characteristics of NK cells and other effector cells can be found in Table 1.

Insert Table 1 about here

Natural Killer cells are important in that they in combination with macrophages and PMN's are thought to be part of a broader range primary defense system that can immediately respond to foreign materials entering the body until more potent long term forms of immunity can intervene (Herberman & Ortaldo, 1981).

The evidence supporting the effects of NK cells in natural resistance to tumors comes largely from studies correlating NK levels and tumor resistance. For example, Kiessling, Petranyi, Klein and Wigzell (1975) found that NK sensitive tumors grew less well in genetic hybrids with higher in vivo NK levels. In contrast, homozygous recessive bg/bg mutant mice that have low NK levels showed less resistance to NK sensitive tumors than their heterozygous (bg/+) counterparts (Karre et al., 1980). Similarly, in a colony of beige mice with a selective deficit of NK activity (Roder & Duwe, 1979) a high incidence of lymphomas was noted (Loutit, Townsend & Knowles, 1980).

Human analogs to these animal studies also show similar results. For example, patients who have a severe deficit in NK activity (Chediak-Higashi syndrome) (Roder, Haliotis, Klein, Korec, Jett, Ortaldo, Herberman, Katz, & Fauci, 1980) also exhibit greater incidence of lymphoproliferative diseases (Dent, Fish, White & Good, 1966). In addition, kidney allograft recipients who have received immunosuppressive drugs to prevent tissue rejection have higher risks of developing lymphoproliferative and other tumors and also show severely depressed NK levels (Lipinski, Turz, Kreis, Finale & Amiel, 1980).

Direct manipulation of NK activity can also be used to study resistance to tumors. Stimulation of mice with Polyinosinic Polycytidylic Acid (Poly I:C) which induces interferon and NK activity shows a decrease in tumor load relative to untreated controls (Greenberg, Dyck & Sandler, 1984; Riccardi, Santoni, Barlozzari, Puccetti & Herberman, 1980). Conversely, one can reduce NK activity in vivo by injecting anti-asialo GM1 antiserum intravenously (Greenberg, Dyck & Sandler, 1984; Gorelik, Wiltrout, Okumara, Habu & Herberman, 1982) which has the result of increasing susceptibility to tumors.

Recent research of NK cell activity in humans also shows promise. In one experiment (Kiecolt-Glaser, Garner, Speicher, Penn, Holliday & Glaser, 1984) various questionnaires and measures of immunology were collected

from medical students after one exam and one month later during final examinations. These students exhibited significantly reduced NK cell activity from the first to second set of exams and this reduction was particularly pronounced in individuals scoring high on the UCLA loneliness, and Social Readjustment Rating Scales. Similarly in a sample of newly admitted psychiatric inpatients (Kiecolt-Glaser, Ricker, George, Messick, Speicher, Garner & Glaser, 1984) high UCLA loneliness scale scorers displayed significantly lower NK cell activity; a multiple regression equation selected loneliness as the best predictor of NK cell activity in this same group of individuals. Further research (Kiecolt-Glaser, Glaser, Williger, Stout, Messick, Sheppard, Ricker, Romisher, Brimer, Bonnell & Donnerberg, 1985) has shown that NK cell activity was significantly increased in geriatric residents after engaging in one month of progressive relaxation training. Another group of researchers (Locke, Kraus, Leserman, Heisel & Williams, 1984) examined correlations between reported life change stress (LCS) and psychiatric symptoms with natural killer cell activity in undergraduate college students. Students exhibiting high scores on the anxiety, depression, obsessive-compulsive and interpersonal sensitivity subscales of the Hopkins Symptom Checklist had significantly lower levels of NK activity. In addition an interaction was found between LCS and psychiatric symptoms in prediction of NK activity, i.e., Students defined as good

copers (reporting low psychiatric symptom distress in presence of high LCS) displayed significantly higher NK activity (3 times higher) compared to individuals defined as poor copers (reporting high psychiatric symptom distress and high LCS). Finally, Levy, Herberman, Maluish, Schlien and Lippman (1985) found that female breast cancer patients exhibiting poor coping styles and fatigue and depressive affect had lower NK cell activity. Perhaps more important however was the finding that NK cell activity was the only significant predictor of breast cancer (axillary lymph node status) in a stepwise multiple regression equation with other variables.

The aforementioned studies are a selected sample of the research that is currently being conducted concerning the functional significance of NK activity. The data however are consistent in suggesting the importance of NK activity as a primary effector cell in defense against incipient neoplasia as well as other foreign substances (e.g., viruses - Herberman & Ortaldo, 1981) entering the body. Hence, the identification of factors capable of influencing the regulation of NK cell activity, including factors associative in nature, are worthy of study theoretically as well as practically in potential treatment of diseases/disorders that may be related to NK cell surveillance functions.

The Departure - Conditioned Tolerance of NK Cell Activity

The departure from contemporary research in conditioning of immune responses is the use of a conditioned tolerance model to investigate NK cell activity (Dyck, Greenberg, & Osachuk, 1986). This approach was taken in an attempt to demonstrate further generality in the conditioning of immune responses, and, to circumvent what were seen as several potential shortcomings of contemporary immune conditioning research.

Firstly, although the existing literature supports the idea of a direct interaction between the CNS and the immune system, the previous sections of this literature review indicate that most of the evidence is based on a highly restricted range of conditioning methodologies, relying almost exclusively on the taste aversion paradigm. (e.g., See Tables 2 and 3)

Insert Tables 2 and 3 about here

It is well known that taste aversion learning is characterized by features which distinguish it from other conditioning phenomena (e.g. selectivity, rapid acquisition, slow extinction). Procedural pitfalls of this paradigm have also been identified (e.g. differences in handling, exposure to the CS, and injections in conditioning and control groups; Klosterhalfen & Klosterhalfen, 1983a). Together

this information questions whether the conditioning of immunobiologic responses is a general phenomenon permitting associations between a broad range of environmental signals and immunological consequences, or, whether it is restricted only to highly "prepared" systems.

Secondly, much of the contemporary immune conditioning research has used highly aversive stimuli. For instance, in the taste aversion studies, a common UCS is the highly aversive and toxic immunosuppressive drug cyclophosphamide, (e.g., See tables 2 and 3). When researchers have attempted to use less aversive UCSs, the CSs have involved highly aversive and painful procedures (e.g. skin grafts and immobilization by plaster of paris, Gorczynski, McCrae & Kennedy 1982). Therefore it is uncertain whether conditioning of immune responses is idiosyncratic to only highly aversive stimuli. If stimuli other than highly "prepared" aversive stimuli are capable of producing conditioning effects it becomes more likely that associative processes play an important adaptive role in immune functioning in the natural environment.

Thirdly, an assumption in use of the taste aversion paradigm is that the conditioned immune responses mimic the unconditioned responses upon which they are based (in all of the published reports, conditioned immune responses have resembled the unconditioned responses). By the brief review of the conditioning of morphine tolerance literature (where

CRs often oppose the effect of the UCR and tolerance develops) it should be apparent that conditioned immune responses opposite in effect to their unconditioned responses should be possible, especially since most UCSs used in conditioning of immune responses are drugs.

Finally, as one may again see in Tables 2 and 3, the majority of the research investigating conditioning and immune responses utilizes Pavlovian conditioning paradigms. However, these paradigms, the majority being taste aversion experiments, do not allow the experimenter strict control over delivery of stimuli in the conditioning protocol. For example, in taste aversion experiments animals control CS exposure by how much fluid they drink and whether or not they choose to drink. Therefore conditioning protocols in which the experimenter does have control over stimulus delivery are closer to Classical Conditioning designs and are superior methodologically. Tolerance training protocols are an improvement in this direction as the delivery of all stimuli are under the control of the experimenter, and they differ from Classical Conditioning only because the CS and UCS (drug) are presented at final test injection rather than the CS alone. For this reason as well as the previously mentioned points, Dyck et al. (1986) adopted a tolerance training protocol to study conditioning of immunobiologic responses.

Using Siegel's work on morphine tolerance as a guide, Dyck et al. (1986) investigated the development of tolerance to polyinosinic polycytidylic acid (Poly I:C) a known NK cell stimulator. They examined whether the observed tolerance effects were reversible by two decremental conditioning procedures used in the morphine tolerance literature (Siegel, 1977, 1978, 1983) - extinction and CS preexposure.

The tolerance protocol consisted of repeated weekly pairings of a complex environmental CS (exposure to olfactory and light cues) with intraperitoneal injections of Poly I:C (A UCS for NK cell activation). Groups receiving 4 weekly exposures to environmental cues and Poly I:C (tolerance trained groups) were compared to unhandled controls or groups receiving equal exposure to cues paired with saline injections (placebos) to determine whether the immunostimulatory effect of Poly I:C would become attenuated over repeated administrations. This was evaluated by a final test injection which preceded the measurement of NK cell activity.

In the extinction experiment (Experiment 1) two groups received the same training as the tolerance trained group followed by either 4 or 8 extinction trials (exposure to complex environmental cues and saline) prior to final test injection. Tolerance developed in the tolerance trained group such that NK cell activity was not different from

unstimulated controls receiving exposure to cues and saline. Tolerance was also reversed in the two extinction groups. Although NK activity did not differ between the two groups both had significantly higher NK activity relative to the tolerance trained group.

The CS preexposure experiment (Experiment 2) was essentially similar in design to the extinction experiment. It differed in that one group (CS preexposure group) received 6 weekly preexposures to odor cues paired with saline prior to 4 weekly tolerance training sessions and a final test injection. Reduced NK cell activity was again seen in the tolerance trained group and this tolerance was latently inhibited in the CS preexposure group.

These initial results imply that the CNS affects NK cell activity in a direct manner and support a conditioning analysis in the development of tolerance to the immunostimulatory effects of Poly I:C.

THE PURPOSE OF THE PRESENT STUDY

It has been demonstrated that the tolerance which develops to drug-induced (Poly I:C) Natural Killer (NK) cell activation is attenuated by two Pavlovian decremental conditioning procedures: Extinction, and CS pre-exposure (Dyck et al., 1986). To further evaluate the role of associative processes in the development of tolerance to NK cell stimulation the present study examined the effects of a known decremental Pavlovian conditioning training parameter - partial reinforcement (PRF).

In a Pavlovian conditioning paradigm, partial reinforcement refers to the procedure of pairing a CS and UCS on some trials (reinforced trials) and presenting only the CS on other trials (non-reinforced trials). The ratio of reinforced to total number of trials defines the partial reinforcement schedule. This procedure leads to poorer acquisition of a CR relative to a procedure where the CS is consistently followed by the UCS (continuous reinforcement, CRF) (Mackintosh, 1972, p. 72-75; Marx, 1971, p. 163-165), and generally, the lower the ratio of reinforced to total number of trials (i.e. the leaner the PRF schedule used), the poorer is the development of the CR.

Within the context of a drug conditioning model the CS consists of those cues which accompany the drug (drug administration ritual) with the systemic effect of the drug constituting the UCS: Partial reinforcement then refers to the presentation of drug cues with placebo on some proportion of trials. In this model, Siegel (1983) has shown that PRF retards the development of tolerance to both the analgesic and pyretic effects of morphine. Since Dyck et al. (1986) have used the model to study tolerance of drug-induced NK cell activity by Poly I:C (UCS), it was expected that PRF of the CS (peppermint odour cues and injection ritual) with the UCS (Poly I:C) would retard the subsequent development of tolerance to the systemic effects of the drug. Furthermore, relatively leaner PRF schedules were expected to produce correspondingly greater reductions of tolerance.

Two hypotheses followed directly from these assumptions:

1. It was hypothesized that relative to CRF, PRF would attenuate the subsequent development of tolerance to the stimulatory effect of Poly I:C upon NK cell activity.
2. It was hypothesized that PRF schedules with lower ratios of reinforced to total number of trials would produce greater attenuation of tolerance to the stimulatory effect of Poly I:C upon NK cell activity.

A 50% PRF schedule has been used in many experiments (Marx, 1971, p. 163-165; Siegel, 1983) and would have been sufficient to test hypothesis one. However, at least two PRF schedules sufficiently different in number of reinforced to total number of trials were necessary to test hypothesis two. To this end, three PRF schedules were generated (55% PRF, 38% PRF and 29% PRF) to test experimental hypotheses in this thesis.[19]

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[19] See section on experimental design and Table 4 for details.

METHOD

Subjects

The subjects were 56 experimentally naive, female, 5 wk. old DBA/2J mice obtained from Jackson Laboratories, Bar Harbor Maine. This strain of mice was initially selected for previous research (Greenberg, Dyck & Sandler, 1984; Greenberg, Dyck, Sandler, Pohajdak, Dresel & Grant, 1984) and is currently being used to study drug-induced tolerance of NK cell activity (Dyck et al. 1986). The DBA/2J strain was chosen as they have low to medium basal NK cell activity and provide an appropriate model to stimulate NK activity and study its reduction when tolerance develops.[20] The mice were housed in groups of 4 in standard polypropylene cages with filter bonnets and maintained on a 12 hour light cycle with food and water ad libitum throughout the experiment. Cage cleaning and replacement of food and water were co-ordinated with the injection ritual. The mice otherwise remained undisturbed between injections.

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[20] This information was obtained from a personal communication with Dr. A. H. Greenberg.

Materials

The Putative CS (Conditional Stimulus) - Peppermint Extract. The CS used in this experiment (peppermint extract) has previously been found to be an effective CS for conditioning (Dyck et al., 1986). Using a pasteur pipette, approximately 2 mls. of peppermint extract were spread liberally over 300 cc of absorbent bedding in a standard polypropylene cage (28 cm long x 17 cm wide x 12 cm high) which was immediately covered by a sheet of plexiglass. This "peppermint box" when covered by the plexiglass lid served as the conditioning apparatus into which mice were placed. As a precaution to minimize other potential odour cues (urine, defecant, pheromones) from influencing the conditioning procedure, a fresh "peppermint box" was prepared for each squad of mice.

The UCS (Unconditional Stimulus) - Poly I:C. Poly I:C (polyinosinic polycytidylic acid) is a synthetic polynucleotide which reliably stimulates NK cell activity. The mechanisms by which activation occurs are not completely understood although it is known that Poly I:C stimulates macrophages which produce a variety of products including interferon (Lucas & Epstein, 1985) a known NK cell modulator (Gidlund, Orn, Wigzell, Senik & Gresser, 1978; Trinchieri & Santoli, 1978). In preliminary experiments (Dyck et al.,

1986), the dose response curve of Poly I:C induction of splenic NK cell activity was analyzed, sacrificing mice 18-20 hrs. after drug administration. A dose of Poly I:C (20 ug) on the linear portion of the response curve was selected so that deviations from control responses would be more easily detectable. A second aspect of the NK response that was determined was the time at which NK cell levels returned to baseline. This was found to be 6 days after Poly I:C injection, consequently, previous experiments used a 7 day intertrial interval (Dyck et al., 1986). Tolerance to Poly I:C was also found to develop after 4 weekly injections in the presence of olfactory (peppermint extract) and drug injection cues (Dyck et al., 1986). This experiment also used the same parameters of a 20ug/mouse injection of Poly I:C with a 7 day intertrial interval.

Preparation of Poly I:C proceeded in the following manner. All stock was prepared in a laminar flowhood with sterile glass pipettes and containers to ensure sterility of the stock solution. Poly I:C was dissolved in sterile Hanks Balanced Salt Solution (HBSS) containing phenol red indicator to produce a 20 ug/mouse (.2 mg/ml) stock solution. This stock solution was then sterilized by filtering through a 22 micron millipore filter. Volumes of stock required for a particular treatment day were then aliquotted into sterile vials, capped, sealed with parafilm and frozen at -20 degrees celsius. A vial of Poly I:C for

use on a particular treatment day would then be removed from the freezer, thawed in a 37 degrees celsius water bath and vortexed to ensure mixing of the thawed stock solution. This procedure ensured all animals received an identical Poly I:C stock over the duration of the experiment.

Placebo. The placebo or vehicle used for injection on non-reinforced or partially reinforced trials was sterile Hanks Balanced Salt Solution containing phenol red indicator. The same stock solution of HBSS used to prepare the Poly I:C was retained for this purpose. The HBSS was refrigerated until needed on a treatment day. Again working in a laminar flowhood, an appropriate volume of sterile HBSS would be aliquotted into a sterile vile, capped, and then warmed in a 37 degrees celsius water bath before use on a particular treatment day.

Experimental Design

The design used to test experimental hypotheses in this thesis may be seen in Table 4.

Insert Table 4 about here

Group A was an unstimulated control group that received a single saline injection with conditioning cues (Sc) on the day of the test. Group B was a stimulated control group

(Handled Stimulated Control) and received a single injection of Poly I:C with conditioning cues (Pc) on the day of the test. The comparison of Groups A and B was to indicate the effect of a single drug injection on NK cell activity. Group C was the CRF tolerance trained condition and consisted of 4 weekly Poly I:C injections with conditioning cues (Pc) prior to the final test injection. Comparison of Groups B and C was to demonstrate the development of tolerance to the repeated immunostimulatory effects of Poly I:C. Groups D, E and F were the PRF conditions. These groups were exposed to conditioning cues and Poly I:C (Pc) at the same time as group C and received the same number of CS-UCS pairings. Partial reinforcement (PRF) was accomplished in these groups by interspersing different numbers of unreinforced trials (conditioning cues + saline injections - Sc) between reinforced trials (Pc). Therefore group D was a 55% PRF schedule as 5 of 9 trials were reinforced. Similarly, groups E and F were 38% and 29% PRF schedules as 5 of 13 and 5 of 17 trials were reinforced, respectively. Comparisons of groups D, E and F to C were to reveal any effects of PRF on tolerance development thereby testing Hypothesis 1. Comparisons among groups D, E and F were to unveil any relative differences in tolerance due to different PRF schedules thereby testing Hypothesis 2. Since the partial reinforcement groups were to receive more handling and injections than the CRF group, group G (Handled-Injected Stimulated Control) was included as a

control for handling and injection effects. Finally to equilibrate other groups on handling when the tolerance or PRF groups were receiving conditioning treatments they were handled only (H) i.e., they were removed from the colony room, received a single cage cleaning with food and water replacement and were then returned to the colony room.

Conditioning Procedure

Two weeks after the mice arrived and adapted to the laboratory, the experiment began. Treatments were conducted during the light portion of the animals' light(7 A.M. - 7 P.M. light)-dark(7 P.M.- 7 A.M. dark) cycle on the same days at the same times over successive weeks. On each treatment day the mice were either exposed to a distinctive environmental stimulus (peppermint extract odour) paired with an injection or received only a single cage change with food and water replacement to equilibrate groups for handling effects. (See Table 4). Treatments commenced at 12:30 P.M.. Squads were always run in the order presented in Table 4 i.e. A, B, C, D, E, F and G. This was to ensure that mice not to be exposed to odor cues on a particular day e.g. A, B were not inadvertently exposed to lingering odor cues in the experimental room. All animals receiving conditioning treatments were then run. Conditioning sessions began by removing individual cages of mice from the

colony room. They were taken to an adjacent room and individually tail transferred from their home cages to the "peppermint box". After all mice had been placed in the "peppermint box" and the acrylic lid placed on top, a stopwatch was started. After 5 minutes in this distinctive environment, individual mice were removed from the box at random, swabbed with 70% ethanol, and using a 1cc tuberculin syringe and 26 gauge needle 3/8 of an inch in length, they were given an ip injection of either 100 ul. of sterile Hanks Balanced Salt Solution (S) or 20ug/mouse of Poly I:C (P). Following injection, the mice were placed in a homecage with fresh food, water and bedding, returned to the colony room, and left undisturbed until the next treatment day. On days in which animals were not exposed to cues and injections or handled, they were left undisturbed in their cages in the colony room.

Dependent Measures - Measurement of NK Cell Activity

Eighteen hours after the last drug injection all mice were sacrificed by cervical dislocation, their spleens removed, and assayed for NK cell activity.[21] Spleen cells were disaggregated through a nylon mesh, and red blood cells

[21] Due to the complexity of the Natural Killer Cell Assay it was conducted by technicians in the lab of Dr. A. H. Greenberg who regularly perform this task. This also served to keep the technician conducting the final assay from potentially influencing the results by being blind to group membership and the hypotheses of the study.

were lysed by 4 minutes exposure to .85% NH_4Cl solution. Cells were washed twice with Hanks Balanced Salt Solution (HBSS) and resuspended in complete RPMI 1640 medium with 10% fetal calf serum and 10 mM Hepes Buffer. Splenocytes were then counted on a hemacytometer and adjusted to their final cell concentrations. NK activity was measured in a standard 4 h chromium release assay using YAC-1 murine lymphoma cells. The lymphoma cells were labelled with sodium chromate (^{51}Cr) as target cells. Mixtures of 100 ul of spleen cell suspensions and 100 ul of labelled target cells (10^5/ml.) were co-cultured in microtiter plates in 150:1, 75:1, 37:1 and 18.5:1 effector to target ratios. Plates were then centrifuged at 200 g for 1 min. and placed in a humidified CO_2 incubator. Five-six hours later, plates were centrifuged at 1,000 g for 10 min., and 100 ul of the supernatant was removed from each well. The amount of radioactivity released from damaged cells (i.e., the amount of radioactivity released from YAC-1 murine lymphoma cells lysed by NK cells) was determined in a gamma counter and used to calculate percent specific cytolysis. Regression scores of cytotoxicity were calculated for each animal and transformed into lytic units/10^7 (LU/10^7) cells and LU/spleen (LU/10^7 cells X total spleen cells) where 1 LU = 30% cytolysis (Greenberg, Miller, Jablonski & Pohajdak, 1984). The expression of LU/10^7 cells is a measure of the proportion of NK cells to non NK cells in splenocytes i.e., the specific activity. The LU/spleen, on the other hand,

takes into account expansion or contraction of the spleen cell population since it modifies the LU/10^7 cells by the total splenocytes and is therefore a calculation of the total NK cells in the spleen.

RESULTS

The mean cytotoxicity scores (NK activity) of various treatment conditions expressed as LU/10^7 cells and LU/spleen are shown in Figures 1 and 2 respectively.

Insert Figures 1 and 2 about here

Corresponding descriptive statistics for Figures 1 and 2 can be found in Table 5.

Insert Table 5 about here

Inspection of Figures 1 and 2 show approximately the same trends in data across treatment groups for the two measures of cytotoxicity, although the variability within treatments is more pronounced with the LU/spleen measure. This is not surprising as the LU/spleen measure (an absolute measure) is a less sensitive measure of NK activity because it assesses cytotoxicity of all cells in a spleen compared to LU/10^7 cells (a relative measure) which estimates lysis only per 10^7 cells. The difference between the two measures may reflect migrations of spleen cells.

A Priori Tests - Tests of Experimental Hypotheses

Tests of experimental hypotheses were accomplished by planned pairwise comparisons among means of appropriate treatment conditions.[22]

Hypothesis 1

LU/10^7 Cells. As illustrated in Figure 1 and Table 5, two of the three PRF groups (Group D - 55% PRF; Group F - 29% PRF) exhibited visible increases in NK activity relative to the 100% CRF condition (Group C). However planned comparisons of Groups D (Mean = 7.76) and F (Mean = 8.27) with Group C (Mean = 5.15) revealed only Group F to have significantly higher NK activity, $t(49) = 1.71$, $p = .05$, one-tailed, while Group D was not significantly different, $t(49) = 1.43$, $p = .08$, one-tailed. In contrast to the other two PRF conditions, Group E the 38% PRF group (Mean = 5.20) did not exhibit substantially higher NK levels compared to Group C (Mean = 5.15) and was not different, $t(49) = .03$, $p = .49$, one-tailed.

LU/Spleen. Similar to the LU/10^7 cells data, two of the three PRF groups in Figure 2 and Table 5 (Group D - 55% PRF; Group F - 29% PRF) had higher NK levels relative to the 100% CRF group (Group C). However, in contrast to the

[22] Although not all of the contrasts were orthogonal to each other, they were the appropriate a priori comparisons to be made upon theoretical grounds and as such were implemented.

LU/10^7 cells data, neither Group D (Mean = 49.84), $t(49)$ = 1.18, p = .12, one-tailed, nor Group F (Mean = 50.39), $t(49)$ = 1.24, p = .11, one-tailed, showed significantly higher NK levels compared to Group C (Mean = 40.07). In addition, although Group E (38% PRF; Mean = 35.19) unexpectedly had lower NK levels than Group C (Mean = 40.07), this difference was not significant, $t(49) = -.59$, p = .28, one-tailed.

Hypothesis 2

LU/10^7 Cells. Planned pairwise comparisons among the three PRF conditions were also performed. The means of the 55% (Group D), 38% (Group E) and 29% (Group F) PRF conditions were 7.76, 5.20 and 8.27 respectively (See Figure 1 and Table 5). No differences in NK activity were found between Group E (38% PRF) and Group D (55% PRF), $t(49)$ = -1.39, p = .09, one-tailed, or between Group F (29% PRF) and Group D (55% PRF), $t(49)$ = .28, p = .39, one-tailed. However, Group F (29% PRF) exhibited significantly higher NK levels compared to Group E (38% PRF), $t(49)$ = 1.68, p = .05, one-tailed.

LU/Spleen. The means of the 55% (Group D), 38% (Group E) and 29% (Group F) PRF schedules on the LU/Spleen measure were 49.84, 35.19, and 50.39 respectively (See Figure 2 and and Table 5). Group E (38% PRF) was not greater than Group D (55% PRF), $t(49)$ = -1.77, p = .04, one-tailed; Group F (29% PRF) was not significantly greater than Group D (55%

PRF), $t(49) = .07$, $p = .48$, one-tailed. Similar to the $LU/10^7$ cells measure however, Group F (29% PRF) displayed significantly higher LU/Spleen levels compared to Group E (38% PRF), $t(49) = 1.83$, $p = .04$, one-tailed.

A Posteriori Tests - Comparisons Between Controls

As only a limited number of a priori comparisons were possible, data analyses in the following sections were done on an a posteriori basis. One-way ANOVA was computed for each dependent measure using all 7 treatment groups. Subsequent pairwise comparisons between groups were evaluated by Dunn's multiple comparison procedure (Kirk, 1968) with alpha set at .05.

Groups A, B and C

Comparison of Group A (Unstimulated Control) to B (Handled Stimulated Control) assesses the unconditional effects of Poly I:C in stimulating NK activity. In contrast comparison of Group B (Handled Stimulated Control) to Group C (100% CRF Tolerance Group) indexes tolerance to the immunostimulatory effects of Poly I:C. These comparisons were evaluated for each dependent measure.

$LU/10^7$ Cells. A 1-WAY ANOVA computed for all 7 treatment groups revealed a significant effect, $F(6, 49) = 16.80$, $p < .0001$. Subsequent post-hoc analysis by Dunn's

Procedure showed that the group given a single exposure to conditioning cues and Poly I:C (Handled Stimulated Controls - Group B) showed significantly increased NK cell activation relative to Unstimulated Controls (Group A), (See Figure 1 and Table 5). Mice receiving four repeated pairings of conditioning cues and Poly I:C injections before the final test injection - 100% CRF Tolerance Group (Group C) had lower NK levels (i.e., were tolerant) compared to Handled Stimulated Controls (Group B), although this was not significant by Dunn's Procedure,[23] (See Figure 1 and Table 5).

These results essentially replicate previous data (Dyck et al., 1986, Experiments 1 and 2) although the differences between groups in the current data are not as large. In addition, the Tolerance Group was not significantly lower than the Handled Stimulated Controls in this experiment, compared to the differences observed between these groups in Dyck et al., 1986, Experiment 1, $\underline{t}(38) = -2.65$, $\underline{p} = .006$, one-tailed, and, Experiment 2, $\underline{t}(52) = -8.30$, $\underline{p} < .0000$, one-tailed.

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[23] The use of Dunn's procedure does not allow calculation of exact probabilities of differences between groups. However if a t-statistic is calculated, $\underline{t}(49) = -1.33$, $\underline{p} = .09$, one-tailed, it becomes apparent that Group C (100% CRF Tolerance Group) is lower than Group B (Handled Stimulated Controls) but just falls short of being significant.

LU/Spleen. One-way ANOVA of the 7 treatment conditions on the LU/Spleen measure again showed significant differences between groups, $F(6, 49) = 11.71$, $p < .0001$. Similar to the LU/10^7 cells measure, post-hoc analysis by Dunn's Procedure revealed that Handled Stimulated Controls (Group B) had significantly higher NK levels than Unstimulated Controls (Group A), (See Figure 2 and Table 5). The 100% CRF Tolerance Group (Group C) again exhibited lower NK levels relative to the Handled Stimulated Controls (Group B) and this was also not significant by Dunn's Procedure,[24] (See Figure 2 and Table 5).

Group G - An Unexpected Result

Consistent with the graphical representation of NK data in Figures 1 and 2, means of treatment groups in Table 5 revealed an unexpected result - that Group G (Handled-Injected Stimulated Controls) displayed the highest NK activity of all treatment groups.

LU/10^7 Cells. The 1-WAY ANOVA for all 7 treatment groups showed a significant effect, $F(6, 49) = 16.80$, $p < .0001$. Subsequent post-hoc analysis of Group G to the other 6 Treatment conditions by Dunn's Procedure with alpha set at

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[24] If a t-statistic is calculated for the difference between Groups C and B on the LU/Spleen measure, $t(49) = -1.37$, $p = .09$, one-tailed, it again becomes apparent that Group C (100% CRF Tolerance Group) is lower than Group B (Handled Stimulated Controls) but just falls short of significance.

.05 verified that NK activity in Group G (Handled-Injected Stimulated Controls) was significantly greater than in all other treatment conditions, (See Figure 1 and Table 5).

LU/Spleen. Similar to the LU/10^7 cells measure, 1-WAY ANOVA of the LU/Spleen measure on the 7 treatment conditions showed significant differences between groups, $F(6, 49) = 11.71$, $p < .0001$. However in contrast to the LU/10^7 cells data, further exploration by Dunn's Procedure revealed Group G to be greater than only Groups A and E on the LU/Spleen measure, (See Figure 2 and Table 5).

DISCUSSION

For the sake of completeness, the data and analyses of both dependent measures (LU/10^7 Cells and LU/Spleen) were presented in this thesis. The reader should recognize that the similarity between the measures and analyses far outweigh the differences (See Figures 1 and 2). In addition, as an index of NK activity the LU/10^7 Cells measure is more sensitive and less variable thereby accentuating the differences between groups. For these reasons the interpretation of the data in the discussion will be based upon only the LU/10^7 Cells measure. It is hoped that this will facilitate the understanding of the data and avoid the confusion in discussing different interpretations based upon each dependent measure.

Tests of Experimental Hypotheses

Hypothesis 1 is Confirmed

The present investigation is consistent with previous evidence (Dyck et al., 1986) suggesting the influence of decremental Pavlovian conditioning training parameters in attenuation of tolerance to Poly I:C induced NK activation.

The data however provide only partial support for the experimental hypotheses. That is, while the leanest PRF schedule - 29% PRF (Group F) significantly increased NK activity relative to CRF (Group C), the three PRF conditions did not show a trend of increasing NK activity with leaner reinforcement schedules. Results showed that Group F (29% PRF) was significantly greater than Group E (38% PRF), (See Figure 1 and Table 5); Group E was lower than Group D (55% PRF) which did not differ from Group F (29% PRF). Therefore the NK activity in Group E compared to the other 2 PRF conditions is inconsistent with a conditioning analysis which would have predicted it to have NK activity intermediated to Group D and Group F.

Explanations of Observed PRF Effects

How can the results in the 3 PRF groups be explained? The simplest explanation is that conditioning occurs rapidly in this model and therefore only the leanest 29% PRF schedule will disrupt it sufficiently to significantly increase NK activity relative to the CRF Tolerance Group. This explanation is consistent with Siegel's demonstrations of reversal of tolerance to the analgesic and pyretic effects of morphine (Siegel, 1977, Experiment 4; Siegel, 1978, Experiment 3) in which relatively lean - 25% PRF schedules were utilized. The data in this experiment are also in agreement with PRF in classical (Brogden, 1939;

Fitzgerald, 1963; Froseth & Grant, 1961; Grant & Schipper, 1952; Hartman & Grant, 1960; Sadler, 1968) and in Pavlovian conditioning (Brimer & Dockrill, 1966; Willis, 1969; Willis & Lundin, 1966) in that significant decremental effects may only be observed with quite lean reinforcement schedules. In the comparative literature there is some uncertainty as to whether this effect is due to reduction in the rate of acquisition or final asymptotic levels of performance (Marx, 1971, pp. 163-164) while in the human eyelid conditioning literature lower asymptotes are generally the rule (Ross & Hartman, 1965, pp. 194). Thus the significant attenuation of tolerance in group F is consistent with previous conditioning work, unfortunately, the literature provides no explanation for the lower NK levels observed in group E.

A second potential explanation for the general lack of difference between the 3 PRF conditions may be the handling each of the 3 PRF groups received. Although the 3 groups ostensibly differed in number of unreinforced trials, if one considers the handling only trials (designated by H in Table 4) to be part of a complex CS in the conditioning protocol, it could be argued that the H trials may also be unreinforced trials. Thus if one considers the H trials as unreinforced trials, the 3 PRF conditions would effectively not differ and would all be 29% PRF schedules. This could explain the lack of difference between Groups D and E, and, D and F, but not the significant differences observed

between E and F. The above explanation is not totally satisfactory however if we also apply it to Group C. If the H trials in Group C are also considered unreinforced trials it would be a 29% PRF Group and we would not expect to see any differences between it and the PRF groups. But, as previously reported, the 29% PRF Group does have significantly higher NK activity than the 100% CRF Tolerance Trained Condition (Group C).

An additional point is noteworthy at this time. As can be seen in Table 4, even though the 3 PRF groups (D, E and F) received different amounts of handling only (H) and handling plus saline injections (Sc) there were no systematic increases in NK activity across groups with greater numbers of injections. This argues against a simple handling-induced increase in NK activity.

There is a third possibility for the observed results in the 3 PRF conditions. One could speculate that one of Groups D and E is anomalous. Since Group D is more similar to F than E (a leaner PRF schedule than D which would be expected to have higher NK activity) one might suspect that E is the anomalous group. Why it is lower than the other 2 PRF schedules is unknown at this time and the validity of this observation can only be ascertained by replication.

A Posteriori Results Requiring Further Explanation

Lack of Significant Tolerance in the Tolerance Group

Although NK levels observed in the CRF condition (Group C) were lower relative to the Handled Stimulated Controls (Group B) this difference was neither significant nor as pronounced as in previous observations[25] (Dyck et al., 1986). Similar to the explanation for the relative lack of differences between the PRF conditions, a possible reason for the less pronounced tolerance may be the additional handling (designated by H in Table 4) the animals in the present experiment received compared to that in previous research (Dyck et al., 1986). This handling was instituted as part of the design to equilibrate all groups on handling as much as possible. The effects of this handling may be interpreted in two ways. First, if we consider handling to be part of a complex CS controlling an opponent CR's development during tolerance training, this handling without drug administration on some days could constitute a CS only trial and would in effect be partially extinguishing the putative drug compensatory CR thus making the group less

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[25] As previously stated in the results, although the 100% CRF tolerance Trained Condition (Group C) was not significantly lower than the Stimulated Control Group (Group C) in this experiment, the differences between these groups $t(49) = -1.33$, $p = .09$, one-tailed, approaches significance and is therefore consistent with previous data.

tolerant. Effectively then the 100% CRF Tolerance Trained Condition (Group C) may inadvertently have become a PRF condition because of the handling only trials. Alternatively, repeated chronic handling may have been somewhat stressful thus leading to increased NK activation (Greenberg, Dyck & Sandler, 1984; Greenberg, Dyck, Sandler, Pohajdak, Dresel & Grant, 1984) and this handling-induced NK activation may have made the group appear less tolerant to the drug, however, this effect was not seen in Group A.

Differences between Handled Stimulated and Handled-Injected Stimulated Controls

The differences observed in experimental groups (purportedly due to PRF) is difficult to disentangle from the effects of numbers of injections since (a) this was allowed to vary across groups, and (b) this variable had a profound effect on the response of the control groups. Clearly the Handled-Injected Stimulated Controls (See Group G, Figure 1) had much higher NK levels (significantly higher) than the group that was merely handled (Group B).

Explanations for Differences. There are several potential reasons for the elevated NK levels observed in Handled-Injected Stimulated Controls (See Group G, Figure 1) relative to Handled Stimulated Controls (Group B).

The most immediate methodological difference between the two groups (See Table 4) is the number of exposures to

conditioning cues and saline injections each received. The Handled-Injected Stimulated Controls (Group G) received 16 more cue-injection pairings than the Handled Stimulated Controls (Group B) which were handled only (H) until the final test day when they received 1 cue + Poly I:C injection pairing. Similar results were also observed in Dyck et al., (1986) Experiment 1 where a Stress Control Group also showed NK levels above a Stimulated Control Group. The Stress Control Group in Dyck et al., (1986) Experiment 1 however received only 4 cue + Saline injections and 1 cue + Poly I:C injection, a total of 5 injections over all compared to 17 injections in the Handled-Injected Stimulated Control (See Group G, Table 4) in this study. Thus, some combination of this handling/cue-injection ritual may have been increasing NK activity in Handled-Injected Stimulated Controls.

A second explanation for elevated NK levels observed in Handled-Injected Stimulated Controls is the frequency of injections they received. This group received an injection every second day (See Table 4) as part of the cue + Saline injection ritual. In contrast the Handled Stimulated Controls received 1 cue + Poly I:C injection exposure. Perhaps the greater frequency of handling + cue-injection exposures in the Handled-Injected Stimulated Controls may also have influenced NK activity. Indeed, in all previous experiments using this paradigm (including Dyck et al., 1986, Experiment 1) the cue-injection ritual was always 7

days apart and no additional handling only (H) trials occurred in between, so, the effect of greater frequencies of handling + cue-injection pairings is unknown.

The foregoing procedural differences between Handled Stimulated and Handled-Injected Stimulated Controls may be interpreted in the following ways. First it is conceivable that the handling/cue-injection ritual may be more 'stressful' than simple handling per se.[26] Given the observation that repeated stress in the form of restraint or restraint plus inescapable tailshock increases the elimination of NK sensitive tumours (Greenberg, Dyck & Sandler, 1984; Greenberg, Dyck, Sandler, Pohajdak, Dresel & Grant, 1984), it is possible that stressful handling in the form of repeated injections could have amplified NK activity through similar stress-related neurohormonal and neurochemical alterations. However, to test this idea it would be necessary to independently assess these physiological responses. Furthermore, if the handling/cue-injection ritual has unconditional stress effects (i.e., increases NK activity) it is possible that these responses may be conditioned to cues in the handling protocol which

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[26] Observation of the behaviour of the mice over the duration of the experiment showed them to become more animated during conditioning treatments as the study progressed. The animals in addition engaged in stereotypical behaviours such as huddling together in one corner, tucking their tails underneath their bodies and squinting their eyes before being handled to be injected. In some cases animals tried to leap out of the peppermint box before they were to be injected.

may then be elicited on subsequent handling/cue-injection trials.

A second interpretation has to do with adaptation to stressful handling. Chronic stress has been shown to increase NK activity. If the handling/cue-injection ritual is stressful to the Handled-Injected Stimulated Controls, this procedure could be considered chronic stress which could explain the elevated NK levels observed in this group. The lower NK levels observed in the Handled Stimulated Controls could be explained by the single cue + Poly I:C injection acting like an acutely stressful episode leading to suppressed NK levels.

A final theoretical explanation of the differences between the two control groups is some combination/interaction of the aforementioned possibilities, i.e., some contribution of unconditional, conditional and/or chronic/acute stress responses in elevating NK activity.

Implications of Elevated NK Levels in Handled-Injected Stimulated Controls. Whether the explanation is empirical or theoretical and/or some combination of unconditional, conditional and chronic/acute stress, the superimposition of these hypothetical handling/cue-injection effects clearly enhanced the immunostimulatory properties of Poly I:C. The validity of each of these explanations is not known at this time, however, as a result, they do cast doubt on interpretation of the effect of PRF as solely a conditioned

drug effect. The alternative explanation is that some stress-induced alteration activated by the handling/cue-injection ritual is mediating reversal of the observed tolerance. This does not argue against a conditioning interpretation of the observed effects but suggests rather that conditoned stress effects as well as conditioned drug effects may contribute to the experimental outcome.

Regardless of what the actual explanation of the elevated NK levels in the Handled-Injected Stimulated Control is, it raises the issue of what is the appropriate control group for assessment of tolerance and its attenuation/reversal. If the Handled Stimulated Control (Group B) is used as the control group, the Tolerance Group (Group C) appears tolerant, and the 29% PRF conditon (Group F) appears to have reversed the tolerance, (See Figure 1). However if the Handled-Injected Stimulated Control is the control used, all groups appear tolerant. Thus both groups should be used as controls in the future.

General Conclusions and Future Directions

The aforementioned discussion suggests the following generalizations about the results of this experiment:

1. Tolerance to the immunostimulatory effects of Poly I:C seems to occur over four complex cue + Poly I:C exposure trials.

2. The observed tolerance may be reversed or attenuated by very lean levels of PRF, a Pavlovian decremental conditioning training parameter.
3. An alternative explanation of the increase in the 29% PRF condition may be combinations of unconditional, conditional, and chronic/acute stress responses associated with the handling/cue-injection ritual.
4. The attenuation of tolerance observed may reflect interactions of 2 and 3 above, i.e., the attenuation of tolerance may reflect effects of PRF, possible contributions of unconditional, conditional and chronic/acute stress effects, and the interaction of the two.
5. The interpretation of the tolerance phenomenon and its reversal/ attenuation will be affected by the choice of control group, i.e., When one compares the 29% PRF Group to the Tolerance and Handled Stimulated Control groups it appears as though tolerance has been reversed. However, when it is compared to the Handled-Injected Stimulated Control tolerance has not been attenuated by PRF.

To attempt to test each of the above generalizations it will be necessary to isolate conditioning effects from potential stress effects on attenuation of the observed tolerance. It is not possible at this time to delineate the contribution of each of the aforementioned effects, however several recommendations can be made.

In the future, when this paradigm is used, all groups should be compared to both Handled Stimulated Controls and Handled-Injected Stimulated Controls to assess the development and attenuation/reversal of the tolerance phenomenon.

If replications of this PRF experiment are undertaken it would be useful to try and reduce excessive handling as much as possible to minimize potential unconditional stress effects. In addition, the environment in the Handling Only Conditions (H) should be made as distinctive as possible from the CS in the Handled-Injected Conditions (Sc and Pc) to prevent the H conditions from acting as potential additional unreinforced (CS only) trials.

Subsequent research should use conditioning designs which equate amount of handling and injections in demonstration of conditioning effects in order to circumvent an alterate stress interpretation. This could be done for example using differential conditioning experiments in which all animals receive the same amount of handling, exposure to conditioning cues and injections. The procedure involves pairing one CS (CS_1) on reinforced trials and a different CS (CS_2) with the same animals on non-reinforced trials. Here the interpretation of the conditioning effect is made on the basis of stimulus control of the CR such that one would expect to see the CR when CS_1 is presented (e.g., tolerance) but not when CS_2 is presented.

Finally, attempts should be made to identify or determine the mechanisms/pathways for the observed tolerance and their attenuation. This may to some extent facilitate the separation of conditioned stress effects from conditional drug effects in this paradigm.

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Appendix A

APPENDIX - DIFFERENT INTERPRETATIONS OF CONDITIONING AND DRUG TOLERANCE

Within the last 15 years several conditioning models have been proposed which could explain the influence of conditioning factors upon the effects of drugs. What follows are the investigators who proposed the models, the models, and the interpretations, predictions and implications of the models with regard to conditioning influences on tolerance development.

Wikler (1973)

One of the first interpretations of conditioning influences upon drug effects has been provided by Wikler (1973). In his paper "Conditioning of Successive Adaptive Responses to the Initial Effects of Drugs", Wikler outlines 6 postulates to provide a framework to interpret drug effects, their direction, and the development of tolerance and/or sensitization to these drug effects. He then searches the drug conditioning literature of the time for evidence in support of his conceptualization. The postulates briefly are: (1) The nervous system consists of

an afferent, central processing and efferent arm ultimately innervating somatic and autonomic effectors, (2) Changes in an organism's external or internal environments act as UCSs which act on the afferent arm, producing central processing activities and UCRs (peripheral effector responses) which are judged to be adaptive, (3) Neutral stimuli (CSs) paired with these UCSs eventually evoke central processing activities identical or similar to those of the UCSs and produce CRs which are also considered adaptive, (4) Drugs may act on afferent, central processing or efferent portions of the nervous system, however, "only those drug effects are conditionable which are consequences of the unconditioned stimulus properties of those drugs" (Wikler, 1973, pp.194). Thus drugs (UCSs) acting on the afferent arm of the system will activate central processing and efferent UCRs; CRs will be in the same direction as the UCR. Conversely drugs (UCSs) acting directly on the efferent arm or effector sites will produce effects (UCRs) which will then produce unconditioned feedback activation or deactivation of afferent arms; CRs will resemble the feedback, i.e., the CRs will be adaptations or opposite in direction to the UCRs. Furthermore each of these CRs can be produced by pairing of CSs and UCSs in appropriate temporal contiguity., (5) Administration of a drug at neuronal receptor sites will produce unconditonal drug effects through central processing and efferent pathways; in addition will bring into play unconditioned feedback mechanisms which will reduce the

effect of the drug at this receptor site. Furthermore, with repeated drug administrations this feedback mechanism (counteradaptation) will become stronger, further reducing the drug effectiveness and producing tolerance, and, may even overshoot the unconditional effects of the drug; (6) Finally, these counteradaptation responses produce changes over time in : (a) the UCS processing activities of certain drugs; (b) the CRs which develop to CSs paired with the UCSs. As stated by Wikler (1973):

> ...when a CS is paired with such a drug repeatedly but at long intervals between drug administrations, the CR that is generated may resemble the initial UR evoked by the stimulus properties of that drug, but if the intervals between drug administrations are short, the CS may evoke a counteradaptive CR generally opposite in sign to the initial UR (unconditioned adaptive response) and the initial CR (conditioned adaptive response) (p.195)

In summary, according to Wikler's postulates, drugs act as UCSs at either the afferent or efferent level producing UCRs in each case. CSs paired with these UCSs can also produce CRs. The CRs to afferent UCSs mimmick the UCR, while, CRs to efferent UCSs are opposite in direction to the

UCR. Repeated drug administrations can produce drug tolerance and/or sensitization due to counteradaptive biological feedback mechanisms which are non-associative in nature. CRs can either mimmick or be opposite to UCRs and can change in direction over time. Thus although Wikler's postulates provide a framework to explain unconditioned and conditioned drug effects and their directions, it does not explicitly explain how these change over time, the mechanisms responsible for the changes, how the CRs and UCRs may interact, and how the CRs may change in direction over time.

Solomon and Corbit (1974)

A second theory appropriate to explanations of the effect of conditioning upon drug effects is the "Opponent Process Theory of Motivation" by Solomon and Corbitt (1974). The theory is more general than Wikler (1973), is a motivational theory, and, attempts to explain a variety of phenomena. It can also provide explanations of the influence of conditioning upon drug effects and development of drug tolerance.

According to the Opponent Process Theory of Motivation (Solomon & Corbit, 1974), affective phenomena are characterized by three stages. In the first stage the onset of an adequate stimulus arouses a hedonic state (A state) not occurring prior to stimulus onset, and coterminates with

stimulus offset. Subsequently, a second stage characterized by a hedonic state (B state) qualitatively different than the pre-stimulation state or the affective state of stage one appears. The affective state of stage two dies out slowly and is followed by a return to the pre-stimulation state (stage three). The quality and intensity of these A and B states change as a function of their repeated exercise i.e., the A state dissipates (A'), while the B state increments and lasts longer (B'). According to Solomon and Corbit (1974), these A and B states and their qualitative changes over time are explainable by 'a' and 'b' processes. Presentation of a US reliably triggers an 'a' process, which quickly reaches asymptotic levels and rapidly decays after US offset. The 'a' process activates a slave opponent 'b' process hedonically opposite in direction to that of the 'a' process. This 'b' process recruits less rapidly, has a longer latency and dies out more slowly than the 'a' process. The 'b' process is governed by a use/disuse principle i.e., greater strength accrues to the 'b' process with repeated exposures, and this is assumed to be non-associative in nature. The net hedonic state observed is assumed to be the result of the summation of the 'a' and 'b' processes. Solomon and Corbit (1974) contend that initial occurrence of 'a' and 'b' processes require no learning mechanisms, however, the 'a' and 'b' processes can be elicited by Pavlovian conditioning procedures when these unconditioned processes are present. Thus the elicitation

of 'a' and 'b' processes and their associated A and B states are determined by contiguity of the CS with each of the processes, i.e., if the CS occurs immediately before the UCS, the 'a' process will be conditioned; the CR will be biphasic as the 'b' process is a slave of the 'a' process. However if the CS appears after the UCS (backward arrangement) when the 'b' process is theoretically the strongest, the 'b' process will be conditioned and the CR will be monophasic.

Thus in a conditioned drug tolerance model, tolerance to the effect of a drug (UCS - 'a' process) develops as the 'b' process recruits. The model also predicts that conditioned tolerance should be maximized by backward pairings of the CS with UCS (drug) when the opponent 'b' process is greatest. According to the theory tolerance should also be possible if the 'a' process is conditioned as the 'b' slave process grows to 'a' and a well conditioned 'a' should produce a large biphasic response - mostly 'b' process, i.e., tolerance.

Schull (1979)

Schull (1979) has also developed a theory of motivation called "A Conditioned Opponent Theory of Pavlovian Conditioning and Habituation". The theory is essentially an outgrowth of Solomon and Corbit's (1974) theory.

In contrast to Solomon and Corbit (1974), Schull (1979) posits that the dynamic 'b' properties are not a function of 'b's slave role but under the control of Pavlovian conditioning procedures. Unlike Solomon and Corbit (1974), Schull (1979) argues that only the 'b' process is conditionable (conditioned opponent theory) and as such, in summation with the unconditioned 'a' process determines the net hedonic state observed.

Thus in explanation of conditioned tolerance, Schull's theory posits that development of tolerance to a drug occurs by conditioning of an opponent 'b' process and this occurs when the CS is in a forward temporal arrangement with the UCS ('a' process). As the conditioned 'b' process becomes larger over trials it summates with the unconditional effects of the drug ('a' process) and produces tolerance to the drug effects. Schull's explanation of tolerance would be strictly on the basis of conditioning factors - no mention of non-associative factors such as physiology or mechanisms of tolerance are described in Schull's theory. In addition the theory does not predict when and how the first CR develops to the CS.

Siegel (1979, 1983)

Siegel (1979, 1983) has proposed a theory of drug tolerance which is almost identical to Schull's (1979) conditioned opponent process theory, and has provided research in support of the theory (see Siegel, 1979, 1983).

According to Siegel, administration of a drug (UCS) produces a drug UCR that is initially quite large in magnitude. With repeated drug administrations however, the net response to the drug becomes diminished, i.e., tolerance develops to the drug. The development of tolerance is posited to be due to the development of a conditioned opponent drug CR which summates with the unconditional effects of the drug reducing the net drug effect. The CR develops to cues procedures and rituals in the drug administration context (CSs) which reliably precede the occurrence of the drug (UCS) a phenomenon that was first observed by Pavlov (1927, pp. 35-37). Evidence for this view of tolerance being due to a conditioned compensatory drug response has been provided by studies which demonstrate a response opposite in direction to the drug response occuring when a placebo is substituted for the drug in the usual drug administration context (e.g. hyperthermia to morphine administrations vs hypothermia which is elicited when saline is injected in the presence of drug signaling cues); by studies which show that tolerance to a variety of drugs is situation specific, and reversible by decremental conditioning procedures of extinction, CS pre-exposure, partial reinforcement and exteral inhibition (see section on Support for a Conditioning Analysis of Tolerance in this manuscript, and especially Siegel, 1979; 1983 for extensive reviews of evidence for a conditioning analysis of tolerance).

Like Schull (1979), Siegel's theory also does not predict when the conditioned compensatory CR develops or the mechanisms (if any) by which this occurs.

Eikelboom and Stewart (1982)

A final interpretation of the contribution of conditioning factors in modulating development of drug tolerance has been provided by Eikelboom and Stewart (1982). Their model (within a stimulus substitution framework) attempts to explain the finding in the drug conditioning literature that some CRs mimmick UCRs while others "paradoxically" oppose their UCRs. Their essential argument is that observation of "paradoxical" opponent CRs is due not to a special different type of conditioning that may be adaptive in nature, but rather, to the inappropriate identification of the unconditioned stimuli and unconditioned responses when conditioning drug-induced physiological responses. If the UCSs and UCRs are appropriately identified then all CRs resemble or mimmick their UCRs.

> Eikelboom and Stewart (1982) argue that:
> ...only when a drug acts on the input side, or afferent arm, of the central nervous system should its action be considered an unconditioned stimulus, and only those observed drug effects that are central-nervous-system mediated

> physiological reactions to such unconditioned stimuli should qualify as unconditioned responses (p. 510)

With regard to the interpretation of drugs acting on the efferent side of the CNS they state:

> ...drugs that act on the efferent arm will result in the activation, via the feedback system, of effectors that oppose or counteract the direct drug effect. It is thus argued that in the case of a drug that acts on an effector or on the efferent arm of a feedback system, the observed drug effect itself should be considered to be the unconditioned stimulus; the central-nervous-system mediated physiological reaction to such an effector produced unconditioned stimulus should be labeled the unconditioned response. Note that in this case the uconditioned response acts to oppose the direct drug effect, a consequence of the negative nature of the feedback (p. 512)

Thus to identify the unconditioned and conditioned effects of drugs within this model requires locating the site of drug action, after which predictions of directions

of UCRs and CRs can be made. The model adopts a stimulus substitution interpretation of conditioning, and, as such, CSs come to evoke properties identical or similar to the UCSs. Therefore, when a drug acts on the afferent arm of a system, its action on the CNS is the UCS and the CNS mediated response is the UCR; the CS when paired with the UCS produces a CR in the same direction as the UCR. Furthermore, when a drug acts on the efferent arm of the system the drug effect is the UCS and the response (opposite in direction to the drug effect) produced by negative feedback regulatory systems through the CNS is the UCR; the CR also mimmicks the UCR in this situation. Eikelboom and Stewart (1982) argue that the "paradoxical" opponent CRs other researchers have observed are a direct error in labelling the action of drugs which act on the efferent arm of the CNS as UCRs rather than the UCSs which via feedback through the CNS, produces a UCR opposite in direction to the observed drug effect.

The implications of this model to drug tolerance are that there are no conditioned counteradaptive or compensatory opponent CRs which develop producing tolerance. Tolerance is the result of non-associative regulatory feedback mechanisms which restore the organism to homeostasis; however, CRs which either resemble or oppose the drug <u>action</u> can be conditioned. In essence then, Eikelboom and Stewart (1982) argue about what the

appropriate definition of the UCS of a drug is. Their model is similar to Wikler's (1973) model in the use of a central processing notion between afferents and efferents to define a UCS.

The ramifications of Eikelboom and Stewart's (1982) theory are that it does away with the adaptive nature of the conditioned compensatory opponent process by replacing it with a non-associative feedback mechanism. As a result, for the model to work requires that all regulatory functions be controlled by feedback systems.

Summary

All of the aforementioned models have advantages and disadvantages and strengths and deficiencies in explaining a conditioning interpretation of tolerance.

The model selected to theoretically interpret the results of this thesis is the compensatory conditioning analysis put forth by Siegel (1979, 1983). Although the results could also be interpreted by Eikelboom and Stewart's (1982) conceptualization which places drug tolerance phenomena within a stimulus-substitution framework by focusing on the locus of action of particular drugs in relation to the CNS, it is perhaps premature to do so as it is not definitively known where Poly I:C acts to produce its immunostimulatory effects. In addition, empirical data

support a compensatory conditioning analysis of drug tolerace (Siegel, 1979, 1983), and, data from previous work (Dyck et al., 1986) fit most parsimoniously with a compensatory conditioning analysis. Therefore, this is the model that will be used in interpretation of data in this thesis.

Table 1

Characteristics of NK Cells and other Effector Cells

General characteristics of NK cells and other effector cells.

Morphology	T cells	Monocytes or macrophages	Polymorphonuclear leukocytes	NK cells
Size	Small (9 to 12 μm in diameter)	Large (16 to 20 μm)	Large (12 to 18 μm)	Medium (12 to 15 μm)
Ratio of cytoplasm to nucleus	Low	High	High	High
Nucleus	Round	Markedly indented	Multilobed	Slightly indented
General features				
Adherence to surfaces	–	+	+	–
Phagocytosis	–	+	+	–
Cell surface markers				
Receptors for sheep erythrocytes (human cells)	Have high-affinity receptors	–	–	+ on about 50 percent; have low-affinity receptors
Receptors for IgG	Less than 10 percent of cells have receptors	+	+	+
Antigens				
Human	Most or all cells react with 9.6, OKT3; subsets react with OKT4, OKT8	Most or all cells react with OKM1, anti-asialo GM1; subsets react with anti-Ia	Most or all cells react with OKM1, anti-asialo GM1	Most or all cells react with OKM1, anti-asialo GM1, OKT10; subsets react with 9.6 anti-Ia
Mouse	All cells express Thy 1, Lyt 1	Most or all cells express Mac 1, asialo GM1, Mph1		Most or all cells express asialo GM1, NK 1, NK 2, Ly11, Ly5, Qa5, ? Mph1

Table 1 (continued)

Some functional characteristics of NK cells and other effector cells.

Functional characteristics	T cells	Monocytes or macrophages	Polymorphonuclear leukocytes	NK cells
Spontaneous reactivity	–	+	+	+
Period for development or augmentation of cytotoxic reactivity	Primary response, > 5 to 7 days; memory response, 2 to 5 days	In vivo, 5 to 10 days; in vitro, 18 hours for most stimuli	In vitro, within minutes	In vivo, within 4 hours; in vitro, within 1 hour
Nature of target	Wide array of specific antigens and important role of major histocompatibility complex	Specificity not clearly defined; selectivity for tumor targets	Apparently nonspecific but some selectivity for tumor targets	At least several, widely distributed antigenic specificities
Cytotoxic reactivity against IgG antibody-coated targets	–	+	+	+
Activating factors	Specific antigens, lectins, lymphocyte activating factor (LAF), T cell growth factor (TCGF), interferon, T cell helper factors	Macrophage activating factor, interferon, wide variety of foreign materials (for example, bacterial endotoxin, phorbol esters)	Contact, lectins, cytochalasin E, phorbol esters	Interferon, lectins, antibodies, retinoic acid, TCGF, prostaglandin E (PGE)
Inhibition of reactivity	Specific and nonspecific T suppressor cells and factors, macrophage suppressor cells, interferon, PGE, cyclic AMP	PGE, phorbol esters	Inhibitors of serine esterases	PGE, nonspecific macrophage and other suppressor cells, phorbol esters, cyclic AMP
Factors promoting their growth	TCGF	CSF	CSF	TCGF
Possible mechanisms of cytotoxic effects	Protease, osmotic	Reactive oxygen species, protease, lysozyme, phagocytosis, PGE, interferon	Reactive oxygen species, protease, lysozyme, phagocytosis	Protease, lipase, cytotoxin
Production of soluble mediators	Wide array of lymphokines	LAF, colony stimulating factor (CSF), PGE, many enzymes, interferon	Many enzymes	Interferon, possibly TCGF

Note. From "Natural killer cells: Their role in defenses against disease" by R. B. Herberman and J. R. Ortaldo, 1981, Science, 214, p. 25;27

Table 2

Some Characteristics of Contemporary Studies in Conditioning of Humoral or Antibody Mediated Immunity

Author	Conditioning Paradigm + Pavlovian (P) or Classical (C)	Subjects	Antigen	CS	UCS	UCR	CR
Ader & Cohen, (1975)	Taste Aversion (P)	Male Charles River Rats	Sheep Red Blood Cells (SRBC)	Saccharin (SAC)	CY[a]	Suppressed Antibody Titers	Suppressed Antibody Titers
Rogers, Reich, Strom & Carpenter, (1976)	Taste Aversion (P)	Male Sprague Dawley Rats	SRBC	SAC	CY	Suppressed Antibody Titers	Suppressed Antibody Titers
Wayner, Flannery & Singer, (1978)	Taste Aversion (P)	Male Wistar Rats	SRBC and Brucella Abortus	SAC	CY	Suppressed Antibody Titers	Suppressed Antibody Titers
Ader & Cohen, (1981)	Taste Aversion (P)	Male Charles River Rats	SRBC	SAC	CY	Suppressed Antibody Titers	Suppressed Antibody Titers
"	"	"	"	"	Methotrexate	"	"
"	"	"	"	Sucrose Solution	CY	"	"

Table 2 (continued)

Ader & Cohen, (1985)	Taste Aversion (P)	Male Charles River Rats	SRBC	Chocolate Milk Solution	CY	Suppressed Antibody Titers	Suppressed Antibody Titers
Cohen, Ader, Green & Bovbjerg, (1979)	Taste Aversion (P)	Male BDF1 Mice	2,4,6 TNP-LPS[b]	SAC	CY	Suppressed Antibody Titers	Suppressed Antibody Titers
Ader, Cohen & Bovbjerg, (1982)	Taste Aversion (P)	Male Charles River Rats	SRBC	SAC	CY	Suppressed Antibody Titers	Suppressed Antibody Titers
Gorczynski, Macrae & Kennedy, (1983)	Taste Aversion (P)	Balb/c Mice	SRBC	SAC	CY	Suppressed Plaque Forming Cell (PFC) Response	Suppressed PFC Response
"	Conditioned Stress (P)	"	"	Inert Cues	Rotational Stress	"	"
McCoy, Roszman, Miller, Kelly & Titus, (1986)	Taste Aversion (P)	Female Fischer 344 Rats and Balb/c Mice	SRBC	SAC	CY	Suppressed PFC Response	Suppressed PFC Response
Klosterhalfen & Klosterhalfen, (1983)	Taste Aversion (P)	Female Han Wistar Rats	Complete Freund's Adjuvant	SAC/ Vanilla	CY	Suppressed Paw Swelling	Suppressed Paw Swelling

Table 2 (continued)

Sato, Flood & Makinodan, (1984)	Conditioned Stress (P)	Balb/c Mice	SRBC	Buzzer	Footshock	Suppressed PFC Response	Suppressed PFC Response
Jenkins, Chadwick & Nevin, (1983)	Taste Aversion (P)	Male Hooded Rats and Male Charles River Rats	-	SAC + Lithium Chloride	SRBC	Increased Antibody Titers	Increased Antibody Titers

[a]CY = Cyclophosphamide. [b]TNP-LPS = trinitrophenyl lipopolysaccharide.

Table 3

Some Characteristics of Contemporary Studies in Conditioning of Cell Mediated or Other Immunity

Author	Conditioning Paradigm + Pavlovian (P) or Classical (C)	Subjects	Cellular Immunity Stimulator	CS	UCS	UCR	CR
Bovbjerg, Ader & Cohen, (1982)	Taste Aversion (P)	Female Lewis x Brown Norway F1 Rats	Female Lewis Rat Splenic Leucocytes	Saccharin (SAC)	CY[a]	Reduced Popliteal Node Weights	Reduced Popliteal Node Weights
Bovbjerg, Ader & Cohen, (1984)	Taste Aversion (P)	Female Lewis x Brown Norway F1 Rats	Female Lewis Rat Splenic Leucocytes	SAC	CY	Reduced Popliteal Node Weights	Reduced Popliteal Node Weights
Kusnecov, Sivyer, King, Husband, Cripps & Clancy, (1983)	Taste Aversion (P)	Male Wistar Rats	Spleen Cells of Inbred Male and Female DA Rats	SAC	Rabbit Antirat Lymphocyte Serum	Suppressed Mixed Lymphocyte Culture Response	Suppressed Mixed Lymphocyte Culture Response
Smith & McDaniels, (1983)	CS Pre-exposure (P)	Humans	-	Contextual Cues	Tuberculin	Delayed Type Hyper-sensitivity Reaction	Suppressed Delayed Type Hyper-sensitivity Reaction

Table 3 (continued)

Gorczynski, Kennedy & Ciampi, (1985)	Taste Aversion (P)	Female Balb/c Mice	-	SAC	CY	Increased Plasma-cytoma Tumour Growth	Increased Plasma-cytoma Tumour Growth
O'Reilly & Exon, (1986)	Taste Aversion (P)	Male Sprague Dawley Rats	-	SAC	CY	Suppressed Natural Killer Cell (NKC) Activity	Suppressed NKC Activity
Gorczynski, Macrae & Kennedy, (1982)	Conditioning and Allogeneic Skin Grafts (C)	Male CBA/J Mice	-	Graft Preparation Procedure	C57BL/6 Mouse Skin Graft	Increase in Cytotoxic T Lymphocyte Precursor (CTLp)	Enhanced Increase in CTLp
Ader & Cohen, (1982)	Modified Taste Aversion[b] (P)	Female New Zealand F1 Mice	-	SAC	CY	Decreased Proteinuria and Mortality	Decreased Proteinuria and Mortality
Dyck, Greenberg & Osachuk, (1986)	Conditioned Tolerance (P)	Female DBA/2J Mice	-	Peppermint Odor + Light + Handling Cues	Poly I:C[c]	Increase in NKC Activity	Decrease in NKC Activity

[a]CY = Cyclophosphamide. [b]This study used an autoimmune model which can involve both humoral and cellular mediated immune responses. [c]Poly I:C = Polyinosinic Polycytidylic Acid.

Table 4

Design of Partial Reinforcement Experiment

Group	Label	n	Treatment (4 Weeks) Day 1	2	3	4	5	6	7	Test[a] (Week 5) Day 1
A	Unstimulated Control	8	H[b]	-[c]	H	-	H	-	H	Sc[d]
B	Handled Stimulated Control	8	H	-	H	-	H	-	H	Pc[e]
C	100% CRF Tolerance Group	8	Pc	-	H	-	H	-	H	Pc
D	55% PRF Group	8	Pc	-	H	-	Sc	-	H	Pc
E	38% PRF Group	8	Pc	-	Sc	-	Sc	-	H	Pc
F	29% PRF Group	8	Pc	-	Sc	-	Sc	-	Sc	Pc
G	Handled-Injected Stimulated Control	8	Sc	-	Sc	-	Sc	-	Sc	Pc

[a] Week 5 was the test day and mice were sacrificed 18-20 hours later for assay of splenic NK activity.

[b] Animals received a single cage cleaning and water replacement. This served to equilibrate handling of animals not receiving conditioning treatments with those receiving treatments.

[c] The slash denotes no treatment or handling for animals on a particular day. Mice were left undisturbed in their cages in the colony room.

[d] Mice received exposure to drug administration cues (peppermint extract odor + handling ritual - denoted by c) followed by a 0.1 ml. intraperitoneal injection of Hanks Balanced Salt Solution - Placebo (denoted by S) as described in methods. This treatment Sc effectively corresponds to a CS alone or unreinforced trial.

Table 4 (cont'd)

[e] Mice received exposure to drug administration cues (peppermint extract odour + handling ritual - denoted by c) followed by a 0.1 ml. intraperitoneal injection of Poly I:C (20ug/mouse of Polyinosinic Polycytidylic Acid - denoted by P) as described in methods. This treatment Pc effectively corresponds to a CS + UCS or reinforced trial.

Table 5

Descriptive Statistics of Two Measures of NK Activity by Treatment Condition

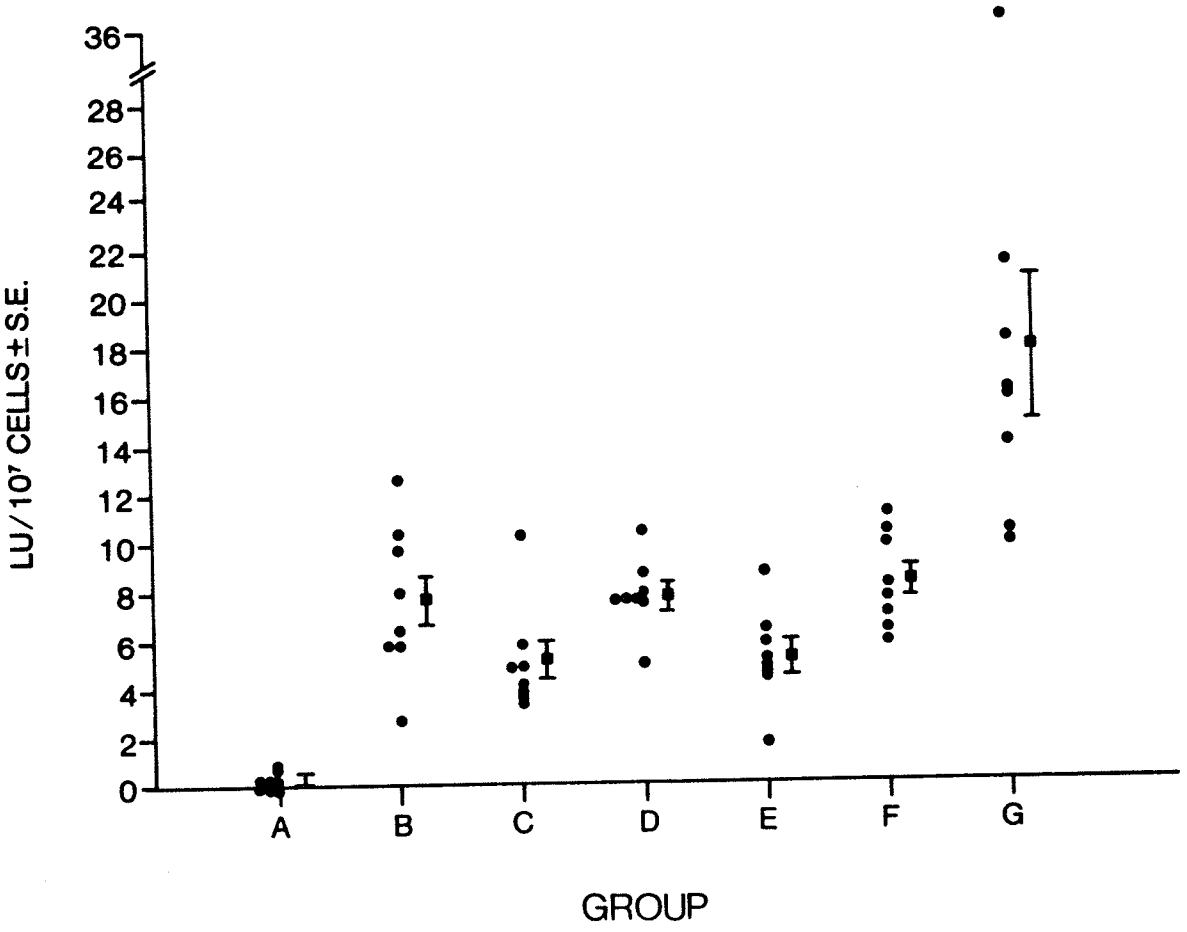
Group	Label	n	Measure of NK Activity					
			LU/10^7 Cells			LU/Spleen		
			$\bar{X}$.	S.D.	S.E.	$\bar{X}$.	S.D.	S.E.
A	Unstimulated Control	8	0.20	0.27	0.10	1.15	1.58	0.56
B	Handled Stimulated Control	8	7.60	3.14	1.11	51.41	18.20	6.43
C	100% CRF Tolerance Group	8	5.15	2.21	0.78	40.07	14.79	5.23
D	55% PRF Group	8	7.76	1.51	0.53	49.84	13.91	4.92
E	38% PRF Group	8	5.20	1.99	0.70	35.19	16.39	5.79
F	29% PRF Group	8	8.27	1.90	0.67	50.39	20.41	7.21
G	Handled-Injected Stimulated Control	8	17.78	8.37	2.96	63.90	22.30	7.88

Note. $\bar{X}$. = Group Mean; S.D. = Standard Deviation; S.E. = Standard Error of the Mean

Figure Captions

Figure 1. Effects of partial reinforcement upon tolerance to Poly I:C stimulation of Natural Killer (NK) cell activity expressed as lytic units/10^7 cells. (Cytotoxic activity of splenic NK cells is expressed as the mean [$\pm$ SE] lytic units/10^7 cells and for individual mice [black dots] within each treatment group. Experimental protocol for each group is described in Table 4 and descriptive statistics for each group can be found in Table 5).

Figure 2. Effects of partial reinforcement upon tolerance to Poly I:C stimulation of Natural Killer (NK) cell activity expressed as lytic units/spleen. (Cytotoxic activity of splenic NK cells is expressed as the mean [$\pm$ SE] lytic units/spleen and for individual mice [black dots] within each treatment group. Experimental protocol for each group is described in Table 4 and descriptive statistics for each group can be found in Table 5.)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LU/10⁷ CELLS±S.E.
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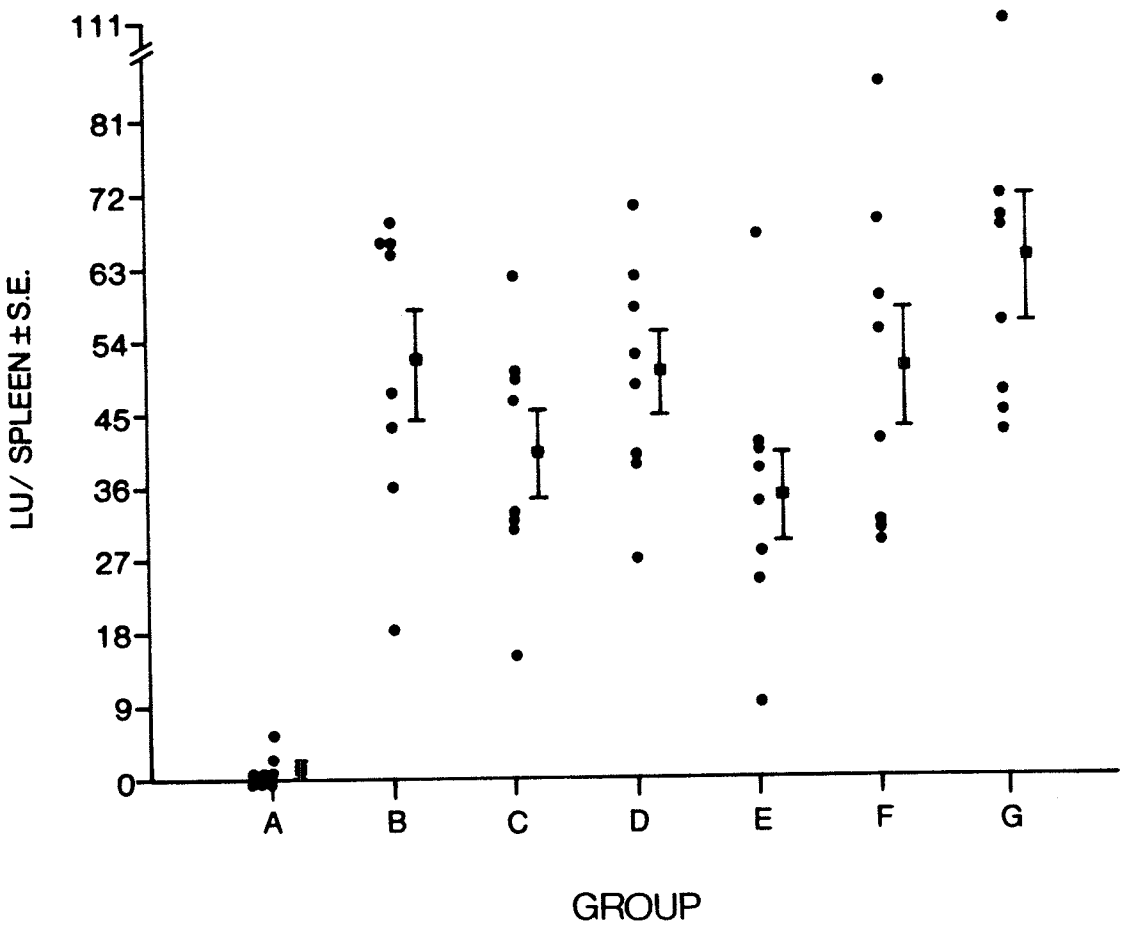
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