The Effects of Prenatal Nicotine Exposure on Behaviorally Conditioned Immunosuppression in Rats

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A thesis submitted to the Faculty of Arts and Science in partial fulfilment of the requirements for the Degree of Master of Arts.

February, 1993

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ISBN 0-315-86192-4

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ACKNOWLEDGEMENTS

I would like to thank Dr. K.Paul Satinder for his support and supervision of this research.

I would also like to thank Dr. L. Malek, of the Department of Biology, for the use of his laboratory space, for serological testing.

Also, thanks to Dr. Frederick R. Ball of the Thunder Bay Public Health Laboratory for the use of equipment, and last minute necessities for data collection.

Finally, sincerest appreciation to my loving and patient wife, Brenlee, and daughter Amber for putting up with my long hours in the laboratory, and for all her support and encouragement.

Abstract

Pregnant rats were administered nicotine (0.6 mg/kg/day) during different stages of pregnancy. Exposure occurred in either the first trimester (days 1-7), second trimester (days 8-14), third trimester (days 15-21), throughout gestation, or not at all (nonexposed controls). The effects of prenatal nicotine exposure on reproductive, developmental and reflex indices were studied. Prenatal nicotine exposure resulted in the birth of smaller sized litters for first trimester exposed dams, as compared to all other groups, except third trimester exposed females. Third trimester exposed females gave birth to smaller litters than second trimester and control females. Total weights of litters from third trimester females were lower than second trimester and control females. Furthermore, developmental age for eye opening was found to be earlier for first trimester animals as compared to third trimester or control rats. Also, animals exposed throughout gestation showed eye opening earlier than controls. Finally, first trimester exposed animals acquired the righting reflex sooner than all other groups except nonexposed control rats. Control animals demonstrated this reflex earlier than second or third trimester exposed rats.

Prenatal nicotine exposure, in this study, did not

appear to influence the acquisition or extinction of behaviorally conditioned immunosuppression in adult rats. The implications of this, regarding nicotine dose, the specific area of the immune system examined, and the developmental age of the subjects, are discussed.

The Effects of Prenatal Nicotine Exposure on Behaviorally Conditioned Immunosuppression in Rats

During the past twenty years, a large body of empirical research has focused on environmental factors which may influence immune system functioning. Psychosocial factors such as stress, including peer separation, marital discord, and anticipation of cancer chemotherapy have all been widely studied. In general, stress tends to produce immunosuppressive effects across most species. Furthermore, a plethora of research has explored the effects of conditioning and learning on immune system reactions. Taken together, the effects of stress and the data from conditioning studies lend strong support to a central nervous system (CNS) interaction and mediation of many immune system reactions. Furthermore, the field of teratology has not only explored prenatal drug effects on nervous system development and morphology, but also in utero drug effects on immune system development and function.

As such, the present study set out to explore the potential interactions between prenatal drug exposure on conditioned effects in the immune system. Specifically, since tobacco smoke has long been associated with many illnesses, in both the smoker and in the offspring of mothers who smoked while pregnant, the potential teratogenic effects of nicotine,

tobacco's primary psychoactive agent, were examined in conjunction with a conditioned immune system reaction.

The Immune System

The immune system is a division of the reticuloendothelial system (RES), and is responsible for the defence of the organism against foreign invasion into the internal biological environment (Golub, 1977). The primary role of the immune system is the detection of "self" from "non-self", and the subsequent destruction and elimination of any detected "non-self" intrusion.

The immune system possesses several functional organs, some central and others peripheral. The central organs of the immune system are the thymus and bone marrow, and the peripheral organs are the lymph nodes and spleen. The functional cellular units of the immune system are the lymphocytes, a specialized type of blood cell. Thymus derived lymphocytes or T-cells, mature within the thymus, while bone marrow derived lymphocytes or B-cells, mature within the bone marrow. Also, there are cells within the immune system known as macrophages. These are large scavenger cells whose primary function is the phagocytosis of larger invading antigens.

All lymphocytes and macrophages are derived from a

primordial blood forming cell which originates in the bone marrow. These cells are known as pluripotent hemopoietic stem cells (Golub, 1977). These stem cells in turn develop into either T-cell or B-cell precursors. T-cell precursors enter the blood stream and migrate to the thymus, where they develop into T-lymphocytes. B-cells do not require thymic processing for their development into functional cells, and as such mature within the bone marrow.

Immune Response

Internal exposure to an antigen (invading organism) sets into motion an immune response. The actual site of entry of the antigen stimulates macrophagic cell activity. More central immune functioning will involve one, or both, of two distinct central immune processes. These are cell mediated immunity, and/or humoral immunity.

Cell mediated immunity primarily involves T-cells, and is typically related to tissue rejection (eg; kidney transplant) and delayed hypersensitivity (eg; tuberculosis skin test). When an antigen makes contact with T-cell surface receptors, several chemical substances are produced which are cytotoxic in nature. Among these are the lymphokines, which are typically involved in inflammatory reactions (Golub, 1977), and communication with other immune system cells (Jaret,

1986).

Humoral immunity is distinct from cell mediated immunity in that humoral processes typically involve the production of antibody. Antibodies are large protein molecules, which possess receptor properties, distinct to each specific antigen to which an organism is exposed. Initial exposure to antigen, results in a pattern of antigen detection and initial antibody production referred to as the primary response. Subsequent exposure to the same antigen initiates a stronger and more robust proliferation of the same antibody referred to as the secondary response.

The biological environment of an organism, possesses the means for production of a vast amount of molecularly distinct antibodies, each with highly specific antigen binding capability. This occurs through antigen stimulation of B-cell surface receptors, called immunoglobulins.

There exist within the majority of mammals, five distinct classes of immunoglobulins- IgA, IgD, IgE, IgG, and IgM- each serving as a B-cell antigen receptor. When a specific antigen contacts an immunoglobulin receptor, the B-cell transforms into an immunoblast. This in turn develops into a plasma cell, whose functional purpose is production of antibody. This cell will produce the specific antibody which was

encoded on the surface immunoglobulin. In essence, the antibody is a modified and specific immunoglobulin molecule, able to bind exclusively to the invading antigen (Golub, 1977). Antibodies act in several ways: they combine with and neutralize antigens, as well as coating specific antigens thus aiding in their destruction by macrophages (Borysenko, 1984).

The typical immune response involves both cell mediated and humoral immune processes. In many cases, there exists a symbiotic functioning in which B-cell antibody production only occurs in the presence of specific T-cells known as "helper" T-cells. There is also similar functioning among T-cells. Many specific T-cell functions (effector T-cells) can only be completed with the assistance of "helper" T-cells (Golub, 1977). One antigen which typically initiates a B-cell/helper T-cell interaction is sheep red blood cells (SRBC). Finally, once invasion by the antigen is brought under biological control, immune system activity is slowed and terminated through the functioning of suppressor T-cells (Jaret, 1986).

Psychoneuroimmunology

Perhaps a major goal of scientific exploration is an attempt to rid humanity of sickness and disease. Until recently, it was widely believed that disease

states were exclusively biological in nature, and that the immune system functioned independently of the central nervous system (CNS). However, a large body of research now indicates that psychological and psychosocial factors can often contribute to immune system functions, and initiate the expression and progression of diseases traditionally believed to be caused by physical factors (Borysenko, 1984).

During the past fifteen years, new breakthroughs in the areas of Biology, Psychology and Immunology have given birth to a new and relevant field of study known as Psychoneuroimmunology. As indicated by Ader (1980) Psychoneuroimmunology is the study of the interactions between behavior, the CNS, and the immune system.

Attempts to demonstrate the interaction between psychological processes and immune system functioning were first documented by Soviet researchers, some 65 years ago (Metalnikov and Chorine, 1926; 1928). This early research reported that through traditional learning paradigms, classical condition of an immune response was possible. Specifically, guinea pigs were presented with a conditional stimulus (CS), such as a scratch or heating of the skin, followed by the presentation of the unconditional stimulus (US), an intraperitoneal (i.p.) injection of tapioca, Bacillus anthrax or a staphylococcus filtrate. When these

animals were presented with the CS, 12 to 15 days later, they demonstrated an elevation in immune system responsiveness in the absence of any antigen, with an increase in production of polynucleated peritoneal leukocytes (Metalnikov & Chorine, 1926).

In a later study, Metalnikov and Chorine (1928) examined the effects of classical conditioning on antibody formation. Rabbits received repeated pairings of a CS, similar to that of their previous study, with an injection of heat treated Vibrio cholera (US). Baseline blood samples were collected three weeks after the final CS-US pairing. On the following day some of the animals were reexposed to the CS alone and the remainder were undisturbed. Those animals reexposed to the CS showed rises in antibody titers, while those not reexposed showed no such elevations (Metalnikov & Chorine, 1928). This effect was further demonstrated in the same conditioned animals as long as two months after initial conditioning (Metalnikov & Chorine, 1928). Therefore, early research examining the interactions between classical conditioning and immune system functioning demonstrated the possible role of psychological processes in immune system functioning.

More recently, Ader and Cohen (1975) demonstrated conditioned immunosuppression, using one-trial taste aversion learning. In this study, an illness induced

taste aversion was produced in rats, by pairing a novel saccharin drinking solution, with cyclophosphamide (CY), an immunosuppressive, nausea producing drug. Three days after this initial conditioning, all animals were injected with sheep erythrocytes. Six days following inoculation, serum was collected and antibody titers were determined for all animals. Placebo treated animals (saccharin paired with saline injection) produced the highest antibody titers, followed by nonconditioned animals (CY injected but not paired with saccharin). The lowest antibody titers were produced by conditioned animals (Ader & Cohen, 1975). Furthermore, when taste aversion was induced using a nausea producing, nonimmunosuppressive drug, lithium chloride (LiCl), conditioned immunosuppression was not evident (Ader & Cohen, 1975). Ader and Cohen (1975) therefore conclude that conditioned immunosuppression is not necessarily mediated by acute elevations in adrenal corticosteroids, resulting from the nonspecific stress of taste aversion conditioning; since LiCl, which presumably induces equal levels of nonspecific stress as CY during conditioning, fails to produce conditioned immunosuppression (Ader & Cohen, 1975). The results of Ader and Cohen (1975) were further replicated by Rogers, Reich, Strom and Carpenter (1976). These authors indicate that further research examining the

effects of conditioned immunosuppression on separate classes of immunoglobulins, would be of interest for future study (Rogers et al., 1976).

Given that antigenic inoculation using SRBC involves both T-cell and B-cell interactions, several authors have attempted to extend the phenomenon of conditioned immunosuppression to thymus independent immune reactions. Wayner, Flannery and Singer (1978) examined the effects of conditioned immunosuppression using SRBC, as well as Brucella abortus (B. abortus), a T-cell independent antigen. The authors demonstrate a replication of Ader and Cohen's (1975) results using SRBC. However, Wayner et al. (1978) indicate that conditioned immunosuppression was not found with T-cell independent antigenic inoculation. Furthermore, the authors report that conditioned immunosuppression appears to be dependent upon some aspect of taste aversion, since conditioned immunosuppression was found to extinguish in conjunction with an extinction of taste aversion (Wayner et al., 1978).

In contrast to Wayner et al. (1978) who employed a rat model, Cohen, Ader, Green & Bovbjerg (1979) report the successful conditioned immunosuppression of a T-cell independent immune response, in the mouse, and using the antigen trinitrophenyl (TNP) coupled to the thymus independent carrier lipopolysaccharide. As such,

although contrasting evidence exists for the ability to condition a T-cell independent immune reaction, it is possible that reported effects may be both antigen specific and species specific.

Temporal factors have also been addressed regarding the CS-US pairing and CS reexposure, in the conditioned immunosuppression paradigm. Ader, Cohen and Bovbjerg (1982) indicate that reexposure to the CS may have long standing effects which alter the threshold of activation of cellular immune components, against SRBC. Specifically, these authors report that conditioned animals reexposed to the CS as long as 15 days after initial conditioning, show an attenuated antibody response as compared to controls (Ader et al., 1982).

The extent of conditioning effects on other immune system processes are also well documented. Several studies have attempted to demonstrate classically conditioned enhancement of immune system reactions. Jenkins, Chadwick and Nevin (1983) reported a conditioned enhancement of antibody production in rats exposed to the traditional taste aversion conditioning paradigm. These authors exposed rats to a novel saccharin drinking solution (CS), followed by an i.p. injection of LiCl. Within one hour of this pairing, animals were exposed to the US injection of SRBC. Using this paradigm, the authors reported enhanced production

of antibodies at 13 but not 20 days following CS-US pairing (Jenkins et al., 1983). However, methodological shortcomings preclude inference of this effect, since the authors excluded data from any animals producing titers below 4. Furthermore, all titers were performed in triplicate, and any sample with a range difference in titer of 2 or greater, between the three samples, was also excluded from the data. This procedure resulted in the exclusion of 15 animals from the original sample size of 66, almost one-fourth of the total sample, in essence guaranteeing significance. As such, these findings should be interpreted with caution, since animals not demonstrating elevations in antibody production were excluded from any statistical analyses.

However, other studies employing stricter methodological considerations have reported conditioned elevations in immunological reactivity. Ghanta, Hiramoto, Solvason and Spector (1985) demonstrated a conditioned increase in natural killer cell (NK) activity, when camphor, an olfactory CS, was repeatedly paired with poly I:C, a NK inducer. However, in a further attempt to demonstrate a conditioned increase of NK activity, Dyck, Greenberg and Osachuk (1986) demonstrated classically conditioned pharmacological tolerance using immunomodulatory drugs. Specifically,

repeated exposure, in mice, to poly I:C, produced a conditioned tolerance to the induction of NK activity, eight weeks after four treatments per week, with poly I:C (Dyck et al., 1986).

Other research has documented that cell mediated, graft-vs-host reactions could be suppressed using conditioning procedures (Bovbjerg, Ader & Cohen, 1982). Bovbjerg, Ader and Cohen (1984) replicated these earlier findings, indicating that conditioned rats produced milder graft-vs-host responses, to splenic leukocytes injected into the footpad. Furthermore, Bovbjerg et al. (1984) reported that this conditioned effect is subject to experimental extinction. Specifically, animals receiving either 9 or 18 extinction trials (i.e. exposure to saccharin without CY injections) did not differ significantly from controls, in graft-vs-host reactions. However, animals which received 0 or 4 extinction trials showed milder graft-vs-host reactions than did controls (Bovbjerg et al., 1984). Interestingly, extinction of conditioned immunological effects did not parallel the extinction of conditioned taste aversion effects, as was previously reported by Wayner et al. (1978). That is, while animals receiving 9 or 18 extinction trials exhibited extinction of conditioned immunosuppression, these animals still demonstrated marked taste aversion

to the novel saccharin solution. As such, these authors purport that since conditioned immunological responses are subject to extinction independently of the extinction of conditioned taste aversion, conditioned immunosuppression can be dissociated from conditioned taste aversion (Bovbjerg et al., 1984).

Ader and Cohen (1982) have reported that the use of conditioned immunosuppression/taste aversion was more successful than CY treatment alone, in delaying the onset of Autoimmune Systemic Lupus Erythematosus, in mice genetically predisposed to this condition. That is, mice receiving weekly presentations of saccharin and CY showed significantly delayed mortality, as compared to mice receiving no such treatment, or CY treatment alone (Ader & Cohen, 1982). As such, conditioned immunosuppression may have therapeutic value in the treatment of certain immunological conditions, such as autoimmune disorders.

Recent evidence indicates that women undergoing repeated infusions of cytotoxic agents for the treatment of ovarian cancer, experience both increased nausea and decreased immune function (conditioned immunosuppression), when they anticipate and return to the hospital (CS) for subsequent chemotherapy treatments (Bovbjerg, Redd, Maier, Holland, Lesko, Niedzwiecki, Rubin and Hakes, 1990). Specifically, *in*

vitro proliferation to the T-cell mitogens PHA and Con A were lower in blood samples obtained in hospital, as compared to those obtained on home visits, several days prior to admission for treatment (Bovbjerg et al., 1990). Although this study examined *in vitro* immune system reactivity, these results are consistent with much of the previously mentioned literature on conditioned immunosuppression using *in vivo* animal models.

The implications of such findings may be profound, since Gorczysnki, Kennedy and Ciampi (1985) have demonstrated that conditioned immunosuppression can modify host resistance to tumour growth. Gorczysnki et al. (1985) indicate that increased tumour growth and mortality was evident in mice exposed to conditioned taste aversion/immunosuppression, using CY, following transplantation of a syngeneic plasmacytoma. Therefore, this finding coupled with those of Bovbjerg et al. (1990), may support the theory that, generalized environmental cues in the therapeutic setting may come to serve as a CS for conditioned immunosuppression, and actually work to reduce the overall efficacy of chemotherapeutic treatment of neoplastic tumours.

Hence, early research in psychoneuroimmunology laid the foundation for exploration into CNS interactions and modulations of immune system

functioning. Later and ongoing research has attempted to apply these findings to the clinical milieu. One unexplored area of research in this experimental area is the role of drug exposure during pregnancy, and the immune system competency of the subsequent offspring.

Teratology and Immunity

Much research has been conducted examining numerous factors which are involved in immunological functioning, such as: psychosocial and biological interactions with the immune system. Among these are various chemical and pharmacological agents which may exert potent teratogenic effects.

In the field of teratology, perhaps the most widely and extensively researched pharmacological agent is alcohol. The effects of prenatal alcohol exposure on subsequent immune system functioning in offspring has been extensively researched.

A plethora of research has attributed prenatal alcohol exposure to immunological dysfunction, both *in vitro* and *in vivo.* Monjan and Mandell (1980) report that in utero exposure to alcohol was found to produce, in adulthood, suppression of *in vitro* mitogen induced lymphocyte reactivity. Furthermore, this depressed activity was found to be isolated to T-lymphocyte, but not B-lymphocyte reactivity. The effect was evident in

tissue cultures taken from rats at 7 and 11 months of age, but not 18 months of age, demonstrating a possible age dependent relationship (Monjan & Mandell, 1980).

Recent *in vivo* studies examining the effects of prenatal alcohol exposure on the immune system have supported earlier *in vitro* results. For example, Redei, Clark and McGivern (1989) examined immune responsiveness as measured by mitogen (Con A) induced T-lymphocyte proliferation, in spleen and thymus cells of 21 day old rats prenatally exposed to alcohol. The authors report a marked decrease in mitogen induced lymphocyte proliferation in alcohol exposed animals. This effect was found to be 8-fold less in spleen cells, and twofold less in thymus cells, for alcohol treated rats as compared to nonexposed controls (Redei et al., 1989).

Nair, Kronfol and Schwartz (1990) examined the effects of both alcohol and nicotine, *in vitro,* on NK, antibody-dependent cellular cytotoxic cell (ADCC), and lymphokine activated killer cell (LAK) activities. Alcohol alone was found to inhibit NK activity, as well as depress the lytic capacity of LAK cells. Also, nicotine alone was also found to inhibit NK activity. When alcohol and nicotine were added together in noninhibitory concentrations, a potentiated significant suppression of NK was evident (Nair, et al., 1990).

These authors speculate that the decreases in immune functioning evident in the presence of these common recreational substances, may contribute to the manifestation and occurrence of the diseases associated with smoking and drinking (Nair, et al., 1990).

In vivo studies of mothers who smoked during pregnancy report significantly lower levels of lymphocyte toxins in smokers than nonsmokers (Nymand, 1974). Furthermore, pregnant smokers were found to have a higher incidence of urinary tract infections, as well as febrile and non-febrile viral diseases (Nymand, 1974). It may be possible that such suppressions in immune system activity in the presence of nicotine, may depress the levels of passive immunity subsequently transferred from mother to newborn offspring.

As such, commonly available drugs, such as alcohol, are seen to exert teratogenic effects on immune system functioning. Furthermore, Ader (1983) indicates that prenatal exposure to many chemicals may result in no detectable defects at birth, but may still result in behavioral impairments later in life, as they emerge. One such commonly used product which has been found to exert minimal detectable defects at birth but several teratogenic behavioral impairments is tobacco smoke; and perhaps more specifically, nicotine (Abel, 1984).

Nicotine

Pharmacology of Nicotine

Nicotine, a central nervous system (CNS) stimulant, is the primary psychoactive agent in tobacco products, and that which attracts people to engage in tobacco use. Nicotine is primarily associated with the cholinergic system, in both the CNS and the peripheral nervous system (PNS). Nicotine readily crosses the blood-brain-barrier, as well as the placental barrier, thus rendering it a potential teratogen (Abel, 1984). However, the pharmacodynamic actions of the drug are dependent on dose, target organ and tolerance (Benowitz, 1986).

Nicotine has been found to activate the sympathetic nervous system, and in healthy human individuals increases blood pressure and heart rate by 10-20 BPM (Benowitz, 1986). Nicotine has also been found to increase levels of circulating norepinephrine (NE) and epinephrine (Cryer, Haymond, Santiago & Shah, 1976). Benowitz (1986) indicates that nicotine is associated with electroencephalographic (EEG) desynchronization, typical of autonomic stimulation and over-arousal. Furthermore, nicotine has been reported to increase endocrine levels of the above mentioned catecholamines, as well as vasopressin, growth hormone (GH), cortisol, adrenocorticotropic hormone (ACTH) and

beta-endorphin (Benowitz, 1986). It is this stimulation of the endogenous opiate system which may be implicated in the positive reinforcing properties of nicotine (Karras & Kane, 1980).

Nicotine and Disease

The effects of smoking, as related to the development of a myriad of health conditions, is widely documented. The nicotine in tobacco smoke is a major risk factor in coronary and peripheral vascular disease, cancer, chronic obstructive pulmonary disease (COPD) and peptic ulcer disease. Although these conditions have been associated with the additive and synergistic effects of the large number of chemical constituents in tobacco, nicotine alone has also been implicated in the development of many physical diseases (Benowitz, 1986). Although nicotine alone is not a known carcinogen, it is known to interact with other chemicals, thus rendering it a co-carcinogen. Nicotine when associated with benzo(a)pyrene, another tobacco constituent, has been associated with the development of skin cancer in mice (Bock, 1980).

The potential mediating factor for the increased susceptibility to certain disease states associated with smoking may be a compromising of immune system functioning resulting from exposure to tobacco smoke, and perhaps specifically nicotine. Ferson, Edwards,

Lind, Milton and Hersey (1979) report decreased IgG, IgA and NK activity in smokers, as compared to nonsmokers. Furthermore, similar inhibition of immune system function (IgG, IgA and NK activity) was evident in melanoma patients, who were smokers that recently had the tumour removed, and had not yet commenced chemotherapy (Ferson, et al., 1979). The authors speculate that the high incidence of malignancy in smokers, may be linked to compromises of certain aspects of immune system function important in restricting tumour growth (Ferson, et al., 1979).

Herscowitz and Cooper (1979) demonstrated that postnatal exposure to tobacco smoke in mice produced a progressive decline, with age, of splenic plaque forming cells (PFC) to SRBC, as compared to nonexposed mice. Specifically, when animals were exposed to tobacco smoke for various periods of time, beginning 24 hours postnatally, no differences in their ability to produce adequate splenic PFC responses were evident (Herscowitz & Cooper, 1979). However, beginning at day 10, this immunological proficiency began to decline. Specifically, smoke exposed mice showed a 30 percent reduction at postnatal day 10, 60 percent reduction at postnatal day 14, and 90 percent reduction at 10 weeks of age as compared to nonexposed mice (Herscowitz & Cooper, 1979). As such, this study further corroborates

the immunosuppressive nature of tobacco smoke.

As previously discussed, Nair et al. (1990) demonstrated the ability of nicotine alone to exert immunosuppressive properties *in vitro*. This primarily included a reduction in NK activity (Nair et al., 1990). As such, while many studies indicate that tobacco smoke, with its vast quantity of chemical constituents, produces immunosuppression, Nair et al. (1990) have supported that nicotine alone possesses potent immunosuppressive properties.

Nicotine Teratogenesis

Nicotine has been reported to increase complications associated with pregnancy, as well as exert harmful effects on the developing fetus. Maternal modulation of nicotine and the levels of nicotine found in fetal plasma vary across different species. Abel (1984) indicates that nicotine levels in mouse fetal tissue tend to be lower than maternal tissue levels. However, in the rat, higher plasma levels of nicotine are reached in fetal tissue than in maternal plasma. Also, both nicotine and its major metabolites have been detected in the amniotic fluid of human smokers (Abel, 1984). Furthermore, Abel (1984) points out that the presence of nicotine has been detected in the breast milk of women smokers. Thus, the effects of nicotine may not only reach the infant in the prenatal

environment, but in the early perinatal environment as well.

Smoking during pregnancy has long been associated with an increased likelihood of spontaneous abortion, lower fetal birth weight, increased perinatal mortality such as sudden infant death syndrome (SIDS), some physical malformations, and behavioral deficits (Abel, 1984).

The effects of smoking during pregnancy and prenatal nicotine exposure on subsequent behavioral factors in the exposed offspring have been examined. Specifically, infants born to mothers who smoked performed more poorly on tasks of simple learning such as, head turning and sucking (Abel, 1984). Follow-up studies of such children indicate impairments in reading and mathematics at 11 years of age (Butler & Goldstein, 1973). Abel (1984) also reviews that infants born to smokers are more likely to display behavioral hyperactivity. This finding has subsequently been documented in rats prenatally exposed to low doses of nicotine (0.3 mg/kg/day), under more controlled empirical conditions (De Lellis, 1987). Furthermore, De Lellis (1987) reports that rats prenatally exposed to nicotine may be differentially affected on classically conditioned two-way avoidance learning.

In light of research which has implicated

cigarette smoking with a wide array of diseases in the actual smoker, children prenatally exposed to tobacco smoke, have also been studied for increased susceptibilities to a host of medical conditions. Abel (1984) reports that smoking during pregnancy may be a mediating factor in SIDS; specifically in women who smoked six or more cigarettes per day. It is suggested that these infants may exhibit brain stem anomalies leading to respiratory difficulties (Abel, 1984), as well as impaired fetal lung development (Moessinger, 1989), resulting from nicotine exposure in utero.

Children born to smokers have also been found to be admitted to hospital more often than children of non-smokers. Incidence of admissions due to pneumonia and bronchitis is especially elevated in these children (Abel, 1984). In one study of 12,734 children, it was found that maternal, and not paternal smoking, was significantly implicated in hospital admissions due to bronchitis and other respiratory tract illnesses, in the first five years of life. Furthermore, rates of admissions for lower respiratory tract illness were found to be as high in children born to mothers who stopped smoking immediately after delivery, as in those mothers who smoked continuously both during and after pregnancy (Taylor & Wadsworth, 1987). This suggests that maternal smoking influences the incidence of

respiratory illness in children, primarily through a teratogenic effect, as opposed to through exposure of passive smoke perinatally. Similar findings have been reported more recently by Moessinger (1989). While Moessinger (1989) indicates that it is possible that early postnatal susceptibility to respiratory tract infection may be due to impaired fetal lung development, Taylor & Wadsworth (1987) indicate that this effect may also be mediated by impaired immune system development, resultant from exposure to cigarette smoke in utero.

Empirical evidence also exists, linking prenatal exposure to smoking with an increased susceptibility to cancer. Results from animal studies indicate that in utero exposure to tobacco smoke is linked to the development of cancer, even though tumours were not observed until the animals had reached half of their expected lifetime (Nicolov & Chernozemski, 1979). As such, it is possible that the teratogenic effects of nicotine, in contributing to disease states, may lay dormant and not become evident until well into the organism's postnatal development. Abel (1984) also postulates that the increased susceptibility to cancers and other illnesses, in offspring of mothers who smoked during pregnancy, may be related to an impairment of immunological functioning resulting from in utero

exposure to cigarette smoke.

While many studies have examined the nature of smoking during pregnancy, and prenatal nicotine exposure on the development of many different disease states, no research to date has examined the long term effects of this exposure on the adult's subsequent immune system functioning. As such, the present study will examine the effects of prenatal nicotine exposure on immune system functioning in the adult rat. Furthermore, given the findings of De Lellis (1987) implicating prenatal nicotine exposure in influencing classically conditioned two-way avoidance learning, possible CNS factors and associated learning mechanisms will also be addressed through the use of the conditioned immunosuppression paradigm. Finally, by examining the effects of nicotine exposure at different trimesters of pregnancy, a possible critical period effect for immune deficiency may be discerned.

Method

Subjects

Twenty-six experimentally naive, female Satinder's Heterogeneous Stock (SHS) rats, weighing approximately 180-250 grams, were used to breed approximately 150 experimental animals. This genetic line is a four-way cross among other genetically distinct rat lines. These

are: Roman high avoidance (RHA), Roman low avoidance (RLA), Maudsley reactive (MR), and Maudsley non-reactive (MNR) rat lines (Satinder, 1980).

All females were housed individually, in standard laboratory cages with food and water available ad libitum. Room temperature was kept at 22°C +/- 1°C, and relative humidity was maintained at approximately 50 percent. All animals were maintained on a 12 hour light/dark cycle (lights on at 8:00 a.m.).

Breeding

All females were paired with a SHS male rat, for the purposes of breeding. Genetic backgrounds of each dam/sire pairing were controlled for. That is, care was taken to insure that each dam/sire pairing originated from unrelated litters, and thus shared minimal genetic similarity. As such, this pairing procedure avoided the potential confounds of genetic inbreeding. All pairs were allowed to mate overnight. Each morning after pairing, cages were checked for the presence of a vaginal sperm plug. When a plug was detected, this was designated as day 0 of pregnancy. On day 0, the male was removed and the female was assigned to one of five nicotine exposure conditions, matching for body weight.

Prenatal Nicotine Exposure

Nicotine (0.6 mg/kg/day) was administered to pregnant rats by subcutaneous (s.c.) injection.

One-third of the dose (0.2 mg/kg) was given at 9:00 a.m., and the remaining two-thirds (0.4 mg/kg) was administered at 5:00 p.m. Females were exposed to the nicotine in one of five possible experimental conditions: the first trimester (days 1-7), second trimester (days 8-14), third trimester (days 15-21), all trimesters (days 1-21), or during none of the trimesters (non-injected control group).

In order to control the potential effects of drug exposure on maternal food and fluid consumption, and the possible confound of differential nourishment on the developing offspring, several possible prospective control procedures can be employed. One such procedural control is pair-feeding, in which food and water consumption of control animals is matched with those of experimental animals (Abel, 1981). However, this technique may, in itself, introduce new and different confounds. For example, pair-fed animals always lag one day behind experimental animals on the controlled levels of food and fluid intake. This arises because the amount of food and water given to control animals is directly dependent on the amounts consumed on the previous day, by experimental animals. This may have serious implications, should drug exposure or the necessary lags in paired-feeding, coincide with, or fail to coincide with certain critical periods of

development (Satinder, 1985). This is of specific importance when examining teratogenic effects using a rat model, given the brevity of the total length of gestation in the rat (i.e. 21 days).

Satinder (1985) points out that an effective means of establishing controls for food and fluid intake, is to monitor daily food and fluid consumption of females during gestation, as well as body weights, which can later be employed as statistical covariates. As such, daily body weights, food consumption, and fluid consumption were recorded for use as statistical controls, by analysis of covariance.

Postnatal Treatment of Offspring

On day 20 of pregnancy, pregnant females were transferred to stainless steel birthing cages, with high grade spruce wood shavings as bedding. The day of birth was designated as postnatal day 0.

In order to isolate prenatal effects from effects which may result from postnatal changes in maternal care of offspring, due to maternal drug exposure, the need to employ cross fostering practices has been proposed. However, Joffe (1969) proposes that cross fostering may, in itself, introduce new confounds and therefore influence behavior in the offspring. Kodama (1982) has reported that cross fostering itself, altered behavioral development by influencing the

appearance certain reflexes, freezing and hyperactivity. Also, Ackerman, Hofer and Weiner (1977) demonstrated that by 30 days of age, fostered pups weighed less, and did not survive food deprivation as readily as pups raised by their biological mothers. Furthermore, cross fostering implicitly incurs an added expense of twice the number of dams, as well as the sensitive issue of destruction of the pups born to surrogate mothers (Satinder, 1985).

As such, Satinder (1985) proposes several methodological alternatives, which may theoretically eliminate the need for cross fostering practises. Specifically, possible changes in milk production and feeding, and its potential effects on postnatal development, can be evaluated by recording body weight at birth, at regular intervals after birth, and at weaning. Also, differences in the survival rates of offspring can also be assessed. Should direct observation of maternal behavior provide evidence of differential maternal care of offspring, then cross fostering practices may be warranted (Satinder, 1985). However, previous evidence from this laboratory has revealed that prenatal exposure to nicotine does not differently influence maternal care of offspring (De Lellis, 1987). Hence, in light of these theoretical concerns, cross fostering of pups was not used here.

Therefore, on day 1 after birth, all dams and pups were weighed, litter sizes and total litter weights were recorded. All litters were coded such that the experimenter was unaware of the prenatal treatment of each specific litter. All dams and litters were checked daily, but otherwise remained undisturbed, except for the purposes of cage cleaning, and testing of developmental signs and reflex in the pups. Pups were weighed every seventh day until the time of weaning (postpartum day 28). From birth until weaning, no differences in maternal care of the offspring were observed.

Upon weaning, all experimental animals were housed individually, in standard laboratory cages with food and water available ad libitum. Room temperature was maintained at 22°C +/- 1°C, and relative humidity was kept at approximately 50 percent. All animals were maintained on a 12 hour light/dark cycle (lights on at 8:00 a.m.).

Procedure and Design

This portion of the study (developmental and reflex testing) employed a 2 (sex) X 5 (gestational period of nicotine exposure) factorial design. Approximately 15 male and 15 female offspring from each nicotine exposure condition completed each cell of the design. In order to control for litter effects (i.e.

genetic background), each cell contained animals from a minimum of three different litters. That is, each experimental cell contained male and/or female animals from no less than three different litters, for that specific nicotine exposure condition.

Developmental Signs and Reflexes

All offspring were tested on several developmental signs from birth until approximately day 35 (adapted from Jensh, 1981). All litters were coded so that the experimenter was unaware of the prenatal treatment of each litter. The age of each animal at the time of manifestation of each developmental sign and reflex, was recorded as per the following criteria (**numbers in parentheses indicate the day at which assessment of the characteristic began**):

Developmental Measures:

(a) Pinna Detachment: Unfolding of the pinnae of both ears into the fully erect position. (**day 2**)

(b) Primary Coat of Downy Hair: Initial presence of downy hair. (**day 8**)

(c) Incisor Eruption: Eruption of upper two incisors. (**day 6**)

(d) Development of Fur: Initial presence of true fur. (**day 8**)

(e) Eye Opening: Complete uncovering of the membranes over both eyes. (**day 10**)

(f) Sexual Development in Males: Beginning at day 28, males were examined for overt descent of the testes.

(g) Sexual Development in Females: Each female's vaginal orifice was examined for rupture of the vaginal membrane. Slight pressure applied to under the base of the tail, revealed an open vagina. (day 28)

Developmental Reflexes

(a) Righting Reflex: Each pup was placed on its dorsal side, on a flat, level surface. The righting reflex was considered present if the animal could orient itself to the upright position within three seconds, on three consecutive trials. The day on which the reflex was determined to be present was recorded. (day 2)

(b) Negative Geotaxis: Each animal was required to complete a 180° turn, within 30 seconds, after being placed on an incline plane of 30°, facing downwards. Once again, three consecutive successful trials indicated the presence of the reflex. The day on which the reflex was determined to be present was recorded. (day 10)

Conditioned Immunosuppression

At 90 days of age, animals were tested for the acquisition of conditioned taste aversion accompanied with conditioned immunosuppression. This age was

necessary to insure that any maternally (i.e. passively) acquired immunoglobulins were no longer present in the offspring, and that any antibody detected through serological testing, was that produced exclusively by the experimental animal (Brambell, 1958).

One-third (n=5) of the animals in each prenatal nicotine group, were randomly assigned to the conditioned group, one-third to a placebo control group, and one-third to a non-conditioned control group. Therefore, a 2 (male vs. female) X 5 (trimester of nicotine exposure) X 3 (behavioral conditioning group) design was employed for the conditioned immunosuppression phase of the experiment.

Materials

In diverging from the traditional design of Ader and Cohen (1975) this study used a 5% sucrose solution (w/v) for taste aversion conditioning, instead of saccharin. Ader and Cohen (1981) have reported successful taste aversion conditioning and conditioned immunosuppression using sucrose. Furthermore, successful conditioning has been reported from this laboratory using a 5% sucrose solution as the CS (Porter, 1987). The 5% sucrose solution (w/v) served as the conditional stimulus (CS) for taste aversion and conditioned immunosuppression.

Cyclophosphamide monohydrate (CY; Sigma Chemicals, St. Louis, MO), in 0.9% saline, injected intraperitoneally (75 mg/kg, i.p.), served as the unconditional stimulus (UCS) for nonconditioned immunosuppression. Seventy-five mg/kg of CY has been reported to be enough drug to produce both taste aversion and immunosuppression in all (i.e. male and female) experimentally tested rats (Ader & Cohen, 1981). A solution of 1% sheep red blood cells (SRBC; Woodlyn Labs, Guelph, Ontario), was washed three times with 0.9% phosphate buffered saline, and then suspended in 0.9% phosphate buffered saline. This cell suspension served as an immunological antigen. Sheep blood cell suspensions were prepared regularly, as per specified expiry dates, and were stored at 4°C, until needed for experimental purposes.

Baseline

An initial baseline period lasted 3 days. On day 0 rats were supplied with food ad libitum, with water supplied in two calibrated drinking tubes. All data measures were recorded in the morning, at 9:00 a.m. On day 1, food and water intake and body weights were recorded. On day 2, food and water intake and body weights were again recorded. On day 3, all water bottles were removed after data were recorded, marking the end of the baseline period, and the start of the

preconditioning phase.

Preconditioning Phase

In order to establish a fluid intake regimen, animals were restricted to two daily periods of fluid intake lasting one hour each; one hour in the morning (9:00 a.m. - 10:00 a.m.), and one hour in the afternoon (4:00 p.m. - 5:00 p.m.). These two drinking periods were maintained for the remainder of the study. The preconditioning phase lasted seven days. Body weights, food consumption and fluid intake were recorded on each day of the preconditioning phase.

Conditioning Phase

Conditioned Group. On the first day of conditioning (day 0) the two calibrated drinking tubes were attached to each cage; both containing a 5% (w/v) sucrose solution. These remained in place for the one hour morning drinking period. Fluid consumption, food intake and body weights were recorded on each day of conditioning. Thirty minutes following removal of the bottles in the morning drinking period, animals in this group were given an injection of 75 mg/kg (i.p.) of CY.

Ader and Cohen (1975) and subsequent studies have employed several subgroups within the conditioned group. All conditioned groups received an initial saccharin and cyclophosphamide pairing, but varied in the subsequent reexposure to the CS. For example, some

animals were never reexposed to the CS (CS_o), while others were reexposed on one or more occasions (Ader & Cohen, 1975). This methodology has been employed in a plethora of research examining conditioned immunosuppression, which have well documented and established the presence of a conditioning effect when saccharin or sucrose is paired with CY. Furthermore, previous research from this laboratory has successfully produced conditioned immunosuppression and taste aversion without inclusion of the CS_o subgroup (Porter, 1987). Hence, the present study used a single conditioning group, which was initially conditioned and reexposed to the CS on only one occasion.

On days 1 and 2 of the conditioning phase, animals were given a free choice of water and 5% sucrose solutions, in order to assess overall fluid preference. On these days, however, no CY injection was given. On day 3 of conditioning, the same protocol as for day 0 of conditioning was followed, except that on day 3, animals were injected i.p. with 2 ml/kg of the 1% SRBC suspension, instead of CY. This was done to initiate a primary humoral immune response. On days 4 and 5, animals were again exposed to a free choice of water and 5% sucrose (w/v), as per days 1 and 2, in order to further assess fluid preference. No injections were given on these days. Therefore, the present study

included two fluid preference exposure days following each injection day (i.e. CY and SRBC). That is, on the two days following initial conditioning and the two days following SRBC injection, a free choice fluid exposure was used in order to assess overall fluid preference.

Bovbjerg, Ader and Cohen (1984) have demonstrated that exposure to 4 extinction trials was insufficient to produce extinction of either conditioned immunosuppression or conditioned taste aversion. Animals in that study which received 9 extinction trials showed extinction of immunosuppression but not of taste aversion (Bovbjerg et al., 1984). As such, the presentation, in the present study, of the CS on the four free choice fluid preference trials and one day 3 nonpaired exposure, would likely not produce extinction of taste aversion or immunosuppression. The digressions from the Ader and Cohen (1975) paradigm have been used previously in this laboratory to successfully condition the suppression of a primary humoral response (Porter, 1987).

Finally, on days 6-8 (postconditioning phase), both drinking tubes were filled with water only. This was done in order to minimize the number of extinction trials for conditioned immunosuppression and taste aversion through further exposure to the sucrose

solution.

In addition to the morning drinking period, all animals were also provided with drinking tubes for the one hour, afternoon drinking period. However, during this period, both tubes contained only water. This drinking period was not used for experimental purposes, but rather served to minimize relative fluid deprivation in the experimental animals.

On day 9, six days following antigen exposure, animals were anesthetized using 0.5 ml/kg of 0.25 mg/ml euthanyl (i.p.). Approximately 4-5 mls of blood were drawn, via cardiac puncture, using a 20 gauge needle attached to a 10 ml tuberculin syringe. All animals were then sacrificed by CO_2 asphyxiation.

Nonconditioned Group. All procedures for the nonconditioned group were the same as those for the conditioned group, except that nonconditioned animals received only water in their drinking tubes on conditioning (day 0) and antigen exposure days (day 3). This group served to isolate the effects of the CY on immune responsiveness.

Some data suggests that primary stimulus generalization, in the form of olfactory cues, may influence the manifestation of taste aversion by decreasing the magnitude of the conditioned response, or by decreasing overall fluid intake in control

animals (Ader, 1977). As such, nonconditioned animals were housed in a separate room from that of both the conditioned group and the placebo group, in order to eliminate the possibility of a conditioned taste aversion to the sucrose solution, through olfactory cues.

Placebo Group. The placebo group was treated with the same protocol employed with the conditioned group, except that these animals received an i.p. injection of 0.9% physiological saline solution in place of CY, on day 0. The use of this group assisted in isolating the effects of the 5% sucrose solution in the acquisition of conditioned taste aversion.

Hematological Analysis

Blood was allowed to clot overnight at 4°C, and was then centrifuged at 4°C and 3000 RPM for 15 minutes. Serum was removed and stored at -40°C, for later testing of antibody concentration, by hemagglutinating antibody titer.

Hemagglutinating Antibody Titer

On the day of serological testing, 150μl of each rat's serum was transferred to a 12 x 75 glass culture tube. In order to inactivate complement, these samples were placed in a water bath at 57°C for 30 minutes (Ader & Cohen, 1975).

All antibody titers were done in disposable

polystyrene, 96-well, U-shaped, microtitration plates. Plates were prepared for an 11 serial, twofold dilution of each serum sample, in ratios of: 1:1, 1:2, 1:4, through 1:1024. The final well of each sample contained only phosphate buffered saline, and served as a negative control. Beginning with the second well, 50μl of phosphate buffered physiological saline was added to each well, using a repeater pipette.

Final plate preparations were done by adding 100μl of the complement inactivated serum, to the first well of each row on the microtiter plate. Serial dilutions were prepared by taking 50μl of undiluted serum (1:1) from the first well, and mixing it with the 50μl of buffered saline in the second well (1:2). Once mixed, 50μl of the solution in the second well was then removed, and mixed with the buffered saline in the third well (1:4). This same process was repeated for each of the next wells, until 11 serial dilutions (1:1024) were completed. Upon mixing the serum/saline mixture in the final well (1:1024), 50μl of solution from the final well was removed and discarded leaving 50μl of diluted serum. Therefore, each of the first 11 wells contained 50μl of the serial twofold serum/saline dilution, with the first well containing pure serum, and the twelfth well containing only saline.

Once all the appropriate dilutions were prepared,

50μl of 1% SRBC was added to each well of the plate, including the final well of saline only. All plates were then covered and placed at 21°C, for one hour. At that time, each well was visually examined for the presence of agglutination.

Titers for determination of antibody concentration were performed in duplicate for each sample ($r = +.79$). The mean of each pair of titer results was recorded for each rat. Antibody concentration was recorded as the reciprocal of the highest dilution (endpoint of titer) producing definite hemagglutination, and expressed as a power of the base two (Kwapinski, 1965). The criteria for the determination of the endpoint of each hemagglutinating antibody titer were adapted from Moore, Humphreys and Levett-Moseley (1972).

Results

Statistical Analysis

Antibody concentration was calculated and recorded as the reciprocal of the highest dilution producing definite hemagglutination and expressed as a power of the base two (Kwapinski, 1965). Sucrose solution preference was measured as the percentage of total morning fluid intake. Developmental signs and reflexes were measured in terms of average age of acquisition per group.

Data obtained from measurements of developmental signs and reflexes were statistically analyzed using a two-way analysis of variance and for **Sex X Prenatal Drug Exposure Group** (1st, 2nd, 3rd, all trimesters, control). Taste aversion data were analyzed using a **2 X 5 X 3 X (9)** repeated measures multivariate analysis of variance for **Sex X Prenatal Drug Exposure Group X Conditioning Group** (conditioned, nonconditioned, placebo) **X (Conditioning Phase)** (baseline, preconditioning, conditioning day 0, day 1, etc;). Immunological data was analyzed by a 2 X 5 X 3 factorial analysis of variance for **Sex X Prenatal Drug Exposure Group X Conditioning Group** (conditioned, nonconditioned, placebo). When required analysis of simple effects, covariance and post hoc analyses were also performed.

Part I: Immunological Measures

Immunological functioning was measured by hemagglutinating antibody titer (Ader & Cohen, 1975; Kwapinski, 1965).

Mean antibody titers for males and females are depicted in Figure 1. Results indicate that females produced significantly higher titers than did males [$F(1,156)= 7.594$; $p < .007$]. When body weight for the postconditioning period was used for as a covariate, sex differences for antibody titers were no longer

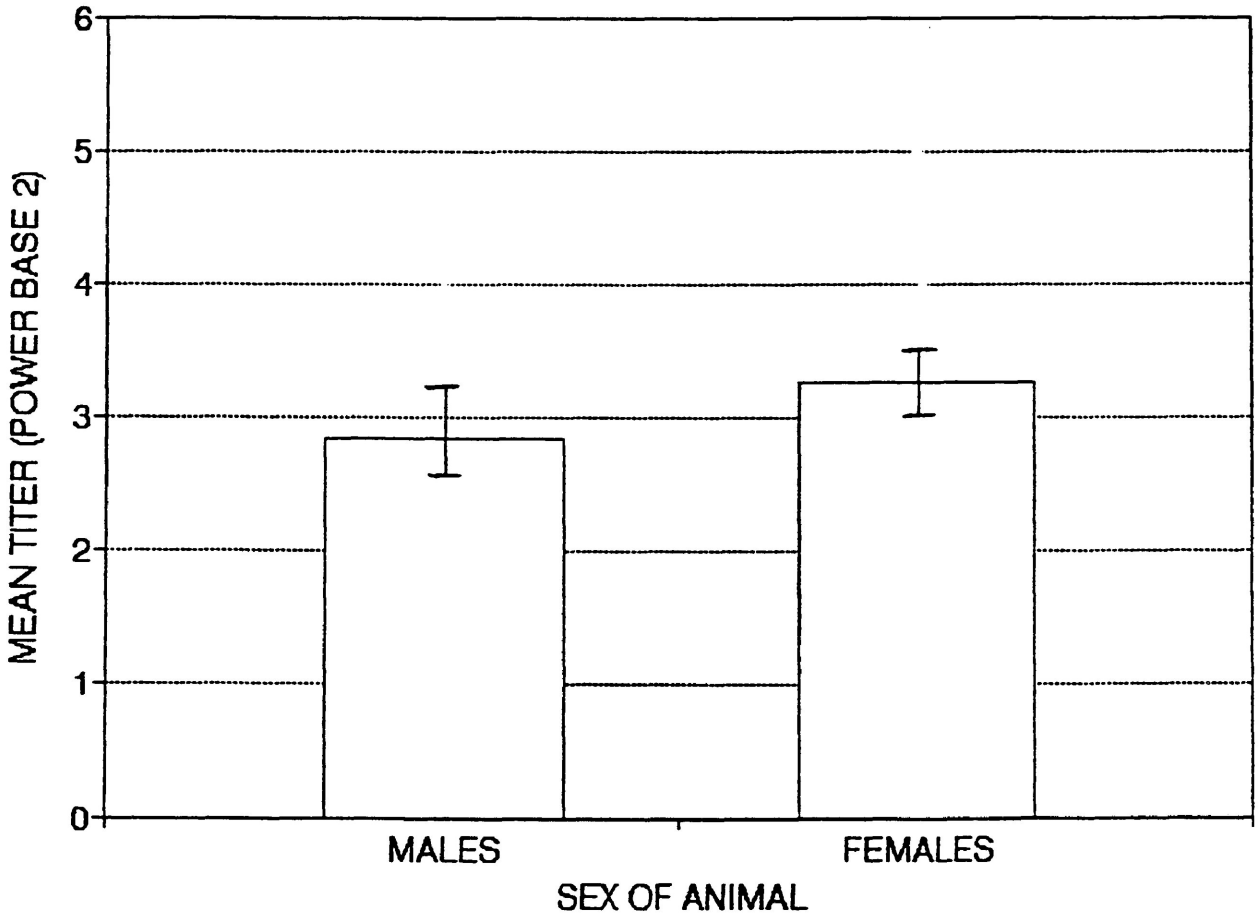

Figure 1. Mean Antibody Titers and Standard Errors for Male and Female Rats During Immunosuppression Conditioning.

significant [F (1,156)= 3.692; p < .057].

Mean antibody titers for trimester group are presented in Figure 2. There were no significant antibody titer differences found for nicotine trimester group. Furthermore, inclusion of relevant covariates such as: body weight, sex, food consumption, and fluid intake, failed to yield significant trimester group differences.

Mean antibody titers for conditioning groups are shown in Figure 3. There were significant differences in antibody

titers for conditioning groups [F (2,156)= 168.376; p < .0001]. Furthermore, inclusion of body weight as a covariate failed to dramatically influence this effect [F (2,156)= 156.138; p <.0001]. The use of sex of animal, as a covariate, also failed to account for this effect [F (2,156)= 168.528; p < .0001].

Scheffe comparisons of cell means revealed that placebo animals produced significantly elevated antibody titers (i.e. 5.55) compared to those of both conditioned and nonconditioned animals. Contrary to expectation, antibody titers of nonconditioned animals were relatively low (i.e. 1.85), and did not differ significantly from those of conditioned animals (i.e. 1.72; see Figure 3). This is contrary to much of the literature on behavioral conditioning of a primary

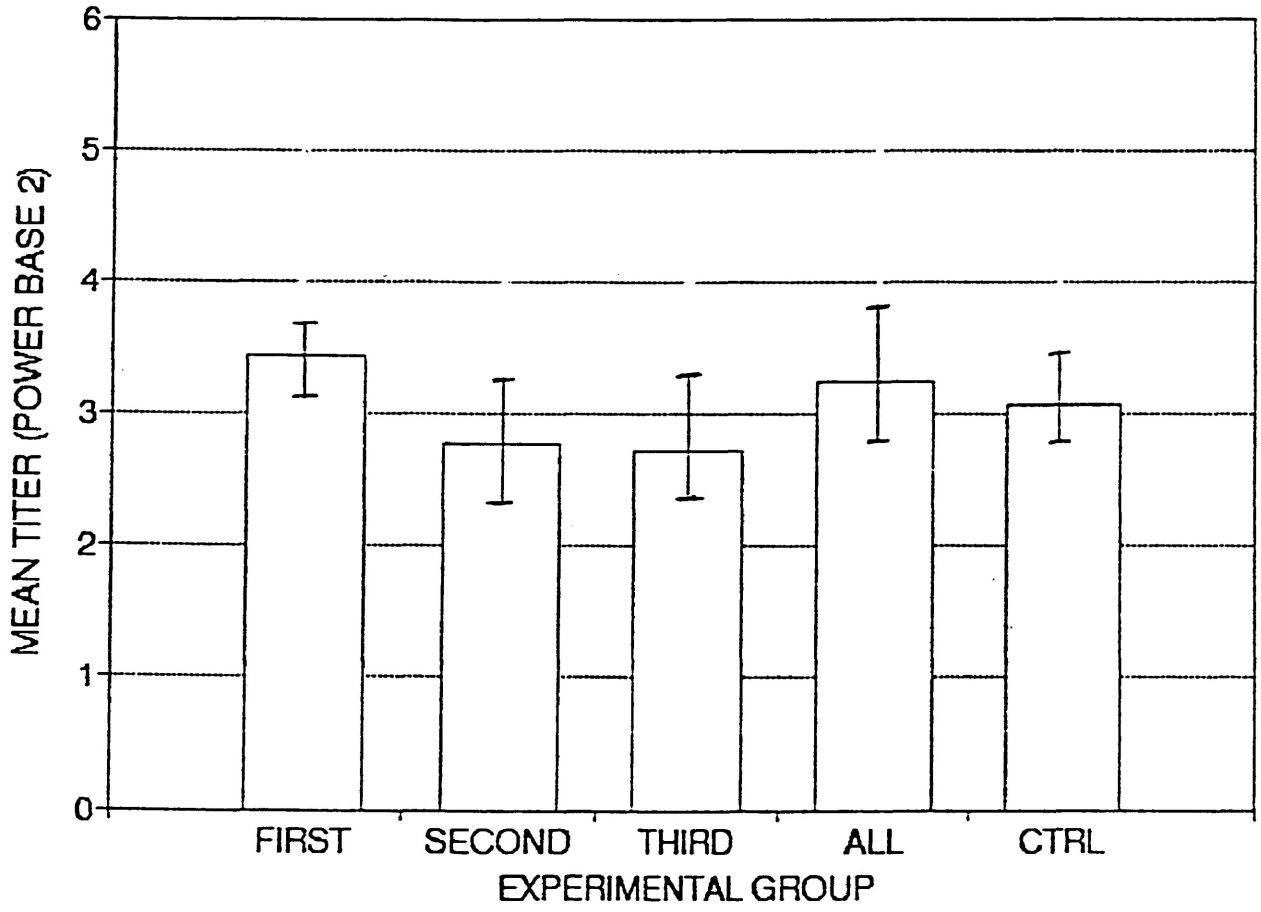

Figure 2. Mean Antibody Titers and Standard Errors for Rats Prenatally Exposed to Nicotine (0.6 mg/kg/day) During Different Periods of Gestation.

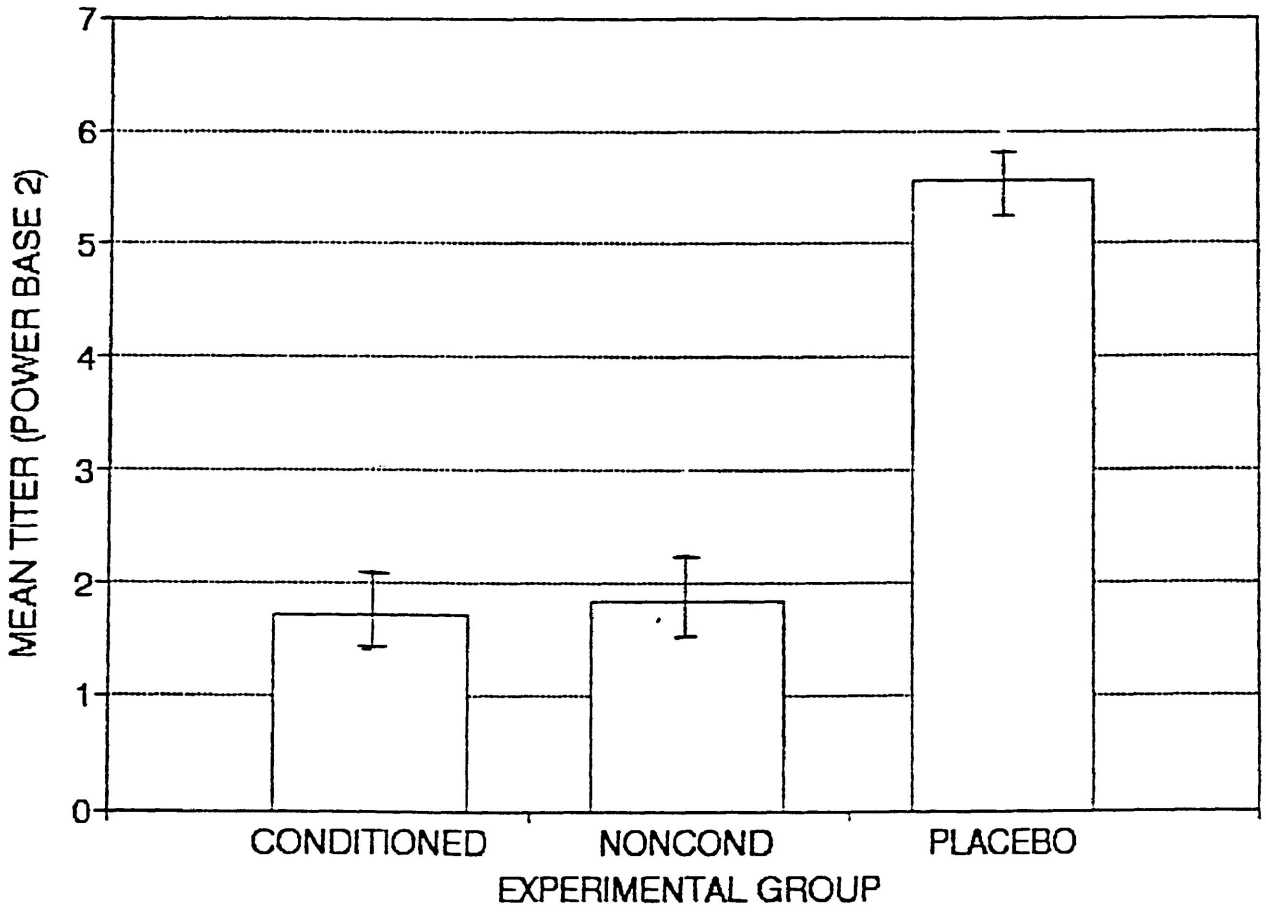

Figure 3. Mean Hemagglutinating Antibody Titers and Standard Errors, 6 Days after SRBC Inoculation for Conditioned, Nonconditioned and Placebo Control Animals.

humoral immune response, which indicates that nonconditioned animals typically produce titers significantly higher than those of conditioned animals, but still significantly lower than those of placebo controls (Ader, 1980; 1981; Ader & Cohen, 1975; Cohen, Ader, Green & Bovbjerg, 1979; Porter, 1987; Rogers, Reich, Storm & Carpenter, 1976).

Finally, there were no significant interaction effects for sex, trimester of exposure or conditioning group on the production of antibody titers.

Part II: Conditioned Taste Aversion

In order to assess the extent of conditioned taste aversion, animals were given a free choice between plain water and a sucrose solution (5% w/v), during the one-hour morning drinking period. Sucrose preference was determined by calculating percentage of sucrose solution consumed relative to the total fluid consumed (sucrose + water), for the morning drinking period. Since animals were exposed to the free choice paradigm on only days 1, 2, 4 and 5 of conditioning, only data from these days were used in overall analyses for determination of acquisition and maintenance of taste aversion. Data from the baseline, preconditioning and post conditioning phases were calculated as the mean total fluid consumed during those periods. Data from conditioning day (Day 0) and antigen day (Day 3) fluid

consumption were recorded as total fluid intake for those days; since both bottles on each cage contained the same drinking solution. In order to assess the roles of sex, trimester of nicotine exposure, and conditioning on fluid intake and acquisition of taste aversion, all data were analyzed using a sex (2) X conditioning (3) X trimester group (5) repeated measures analysis of variance, for the nine phases of taste aversion conditioning (i.e. baseline, preconditioning, conditioning day 0, day 1, etc;). When appropriate, simple effect, covariance and post hoc analyses were performed.

Means for sucrose preference (i.e. free choice on conditioning days 1, 2, 4 and 5) for prenatal nicotine exposure group are depicted in Figure 4. Means for total fluid intake on days when both bottles contained the same solution (i.e. baseline, preconditioning, day 0, day 3 and post conditioning) are found in Table 1.

F-ratios for repeated measures analyses are found in Table 2. There were no trimester group differences found for any of the nine conditioning phases of the study (see Figure 4 and Tables 1 and 2). Furthermore, no significant interaction effects were revealed (see Table 2).

Analysis by repeated measures MANOVA revealed significant sex differences across the 9 conditioning

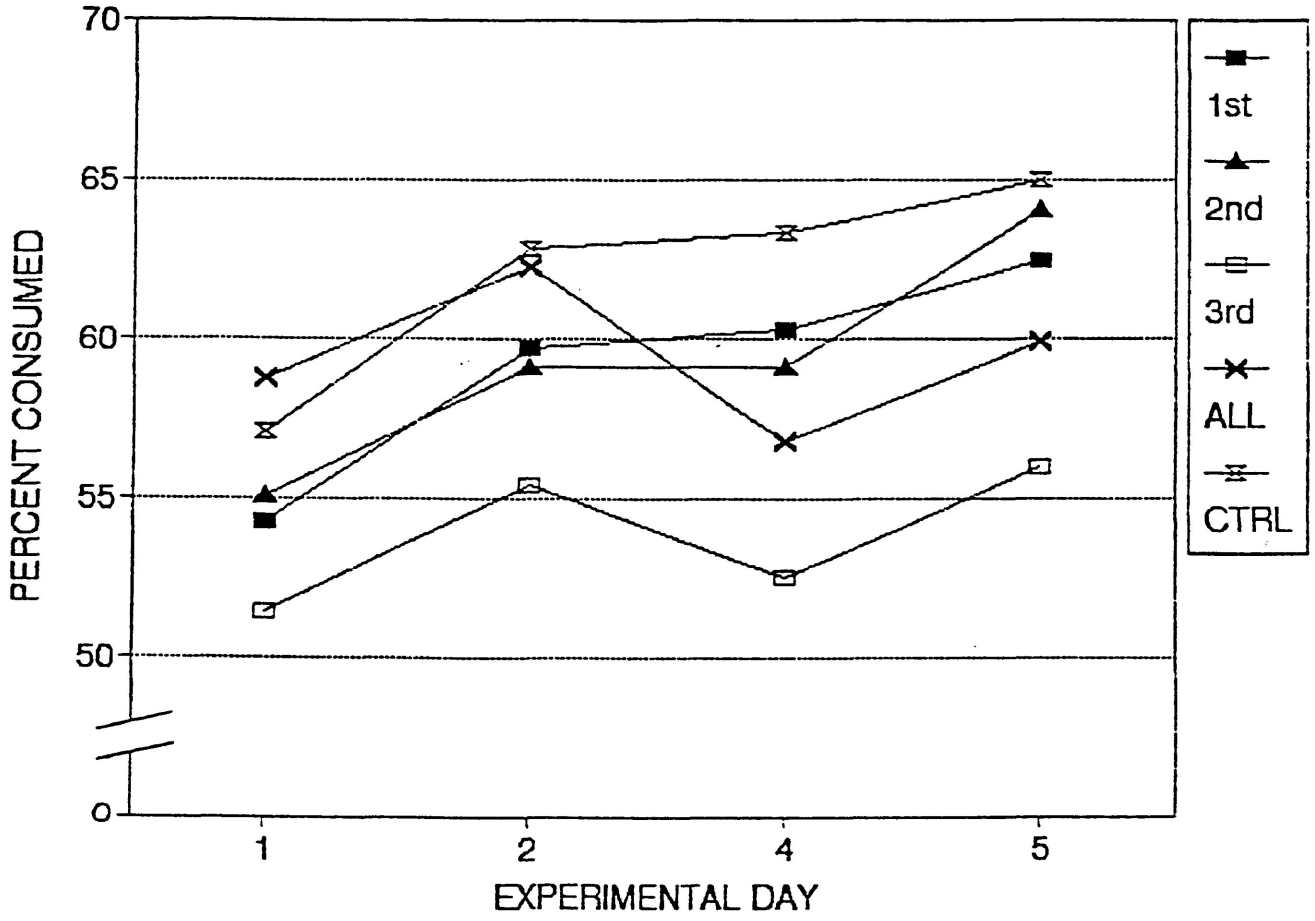

Figure 4. Percentage of Sucrose Solution Consumed on Conditioning Days 1, 2, 4 and 5 for Animals Prenatally Exposed to Nicotine (0.6 mg/kg/day) During Different Periods of Gestation.

Table 1

Mean Total Fluid Consumption (mls) for Baseline, Preconditioning, Day 0, Day 3, and Post-Conditioning Phases for Animals Prenatally Exposed to Nicotine (0.6 mg/kg/day) During Different Periods of Gestation

	Baseline (Water 24hr)	Precond (Water 1hr)	Day 0 (Sucr.)	Day 3 (Sucr.)	Postcond (Water 24hr)
Trimester					
First					
mean	32.8	11.7	15.0	11.8	11.9
(S.E.)	(1.4)	(0.5)	(0.7)	(1.5)	(0.6)
Second					
mean	34.9	13.4	16.5	13.1	14.2
(S.E.)	(2.3)	(0.5)	(0.7)	(1.6)	(0.7)
Third					
mean	34.0	13.6	16.8	11.6	12.8
(S.E.)	(1.4)	(0.5)	(0.6)	(1.5)	(0.5)
All					
mean	37.4	12.5	15.6	11.5	14.6
(S.E.)	(1.4)	(0.5)	(0.8)	(1.4)	(0.6)
Control					
mean	34.6	12.2	15.8	12.0	12.6
(S.E.)	(1.1)	(0.5)	(0.8)	(1.3)	(0.6)

NB: First=Days 1-7; Second=Days 8-14; Third=Days 15-21

Table 2

F-Ratios for Between and Within Subject Effects for Repeated Measures Analyses of Taste Aversion Conditioning

Variation Source	df	F	Significance of F
Between Subjects			
Sex	1,156	.00	.966
Trimester	4,156	1.69	.157
Conditioning	2,156	594.01	.0001
Sex X Tri	4,156	1.02	.398
Sex X Con	2,156	2.88	.060
Tri X Con	8,156	1.75	.093
Sex X Tri X Con	8,156	1.09	.375
Within Subjects			
Phase*	8,156	575.32	.0001
Sex X Phase	8,156	7.65	.0001
Tri X Phase	32,156	.68	.911
Con X Phase	16,132	169.81	.0001
Sex X Tri X Phase	32,156	1.04	.408
Sex X Con X Phase	16,156	1.39	.168
Tri X Con X Phase	64,156	1.09	.289
Sex X Con X Tri X Phase	64,156	.88	.727

Note: * Phase refers to conditioning phase of study (i.e. baseline, preconditioning, day 0, day 1, day 2, day 3, day 4, day 5, postconditioning).

Table 3

Group Means, F-Ratios and Covariates for Sex Differences in Fluid Intake During Taste Aversion Conditioning (days with both bottles same solution)

	Baseline (mls)	Precond (mls)	Day 0 (mls)	Day 3 (mls)	Postcond (mls)
Males					
Mean	37.90	14.34	17.74	13.61	15.18
(S.E.)	(1.0)	(0.3)	(0.4)	(1.1)	(0.4)
Females					
Mean	31.63	10.97	14.03	10.31	11.16
(S.E.)	(0.8)	(0.3)	(0.5)	(0.7)	(0.3)
Main Effect (Males vs Females)					
Sex F=	20.98*	77.68*	44.56*	27.40*	82.49*
with BW F=	0.90	1.97	1.21	1.95	0.31

Notes: Significance of F * p < .01.

Since no significant differences were evident for conditioning days 1, 2, 4 and 5 F-Ratio's for these days are not included in this table.

d.f. sex (1,156)

BW = Body weight for each respective phase of conditioning.

phases (see Table 2). Simple effects tests indicated significant sex differences for total fluid consumed during the baseline phase, preconditioning phase, conditioning day 0, conditioning day 3, and post-conditioning phase of the study, with males consuming greater amounts than females. As evident in Table 3, all of these significant differences disappeared when body weight for each specific experimental phase was used as a covariate (see Table 3).

Analysis with repeated measures MANOVA revealed significant conditioning group differences for fluid intake for the 9 conditioning phases (see Table 2). Analyses for each the conditioning phase are found in Table 4. There were no significant conditioning group differences found during baseline, preconditioning or postconditioning. Significant differences for conditioning group were evident on days 0, 1, 2, 3, 4 and 5 (see Table 4).

Scheffe comparison of means, for day 0 fluid intake, revealed that nonconditioned animals consumed significantly less fluid (14.48 mls), than both conditioned (15.98 mls) and placebo animals (16.98 mls). This was expected, since nonconditioned animals received water only on day 0, while conditioned and placebo animals received the novel and sweet 5% sucrose solution.

Table 4

F-Ratios for Conditioning Groups (Conditioned vs Nonconditioned vs Placebo) for Each Conditioning Phase of Taste Aversion Conditioning

Conditioning Phase	df	F	Significance of F
Baseline	2,156	.880	.417
Preconditioning	2,156	3.277	.041
Day 0 (CY)	2,156	6.981	.001
Day 1	2,156	213.459	.0001
Day 2	2,156	259.950	.0001
Day 3 (SRBC)	2,156	284.874	.0001
Day 4	2,156	309.516	.0001
Day 5	2,156	359.939	.0001
Postconditioning	2,156	.283	.754

Group means for sucrose preference days (days 1, 2, 4 and 5) for each conditioning group are depicted in Figure 5. Scheffe test for comparisons of means for day 1 indicated that all groups differed significantly from each other in sucrose preference, with placebo animals consuming 89.9% sucrose solution and nonconditioned and conditioned animals consuming 63.2% and 12.4% respectively (see Figure 5). Day 2 Scheffe mean comparisons revealed that the differences between nonconditioned and placebo groups, seen on day 1, were no longer present (see Figure 5).

Conditioning group differences were still evident on day 3 with similar Scheffe comparisons to those of conditioning day 0. All groups differed significantly from each other in total fluid intake. However, unlike the data on day 0, conditioned animals consumed little of the sucrose solution (2.73 mls), while placebo animals consumed 19.7 mls of sucrose solution. As with day 1, nonconditioned animals consumed significantly less fluid (13.37 mls) than placebo animals, once again attributable to the sweetness of the placebo group's sucrose solution in contrast to the nonconditioned group receiving water only on this day.

Day 4 mean comparisons revealed a similar pattern to that of day 1. As seen in Figure 5, all groups differed significantly from each other, with placebo

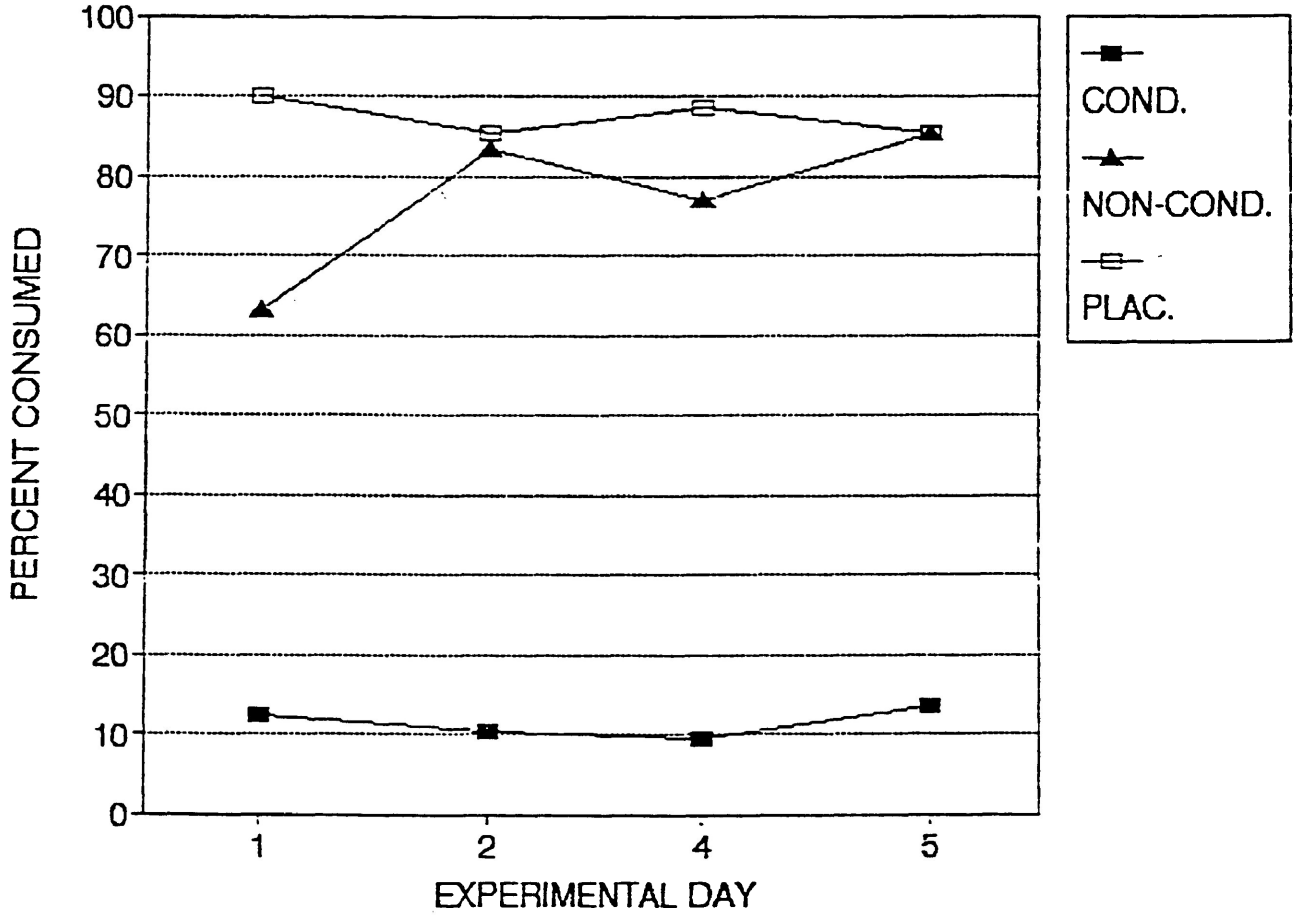

Figure 5. Percent Sucrose Solution Consumption on Conditioning Days 1, 2, 4, and 5 for Conditioned, Nonconditioned and Placebo Control Animals.

animals demonstrating the greatest preference for sucrose (88.43%), followed by nonconditioned (76.99%), and conditioned animals (9.45%). Scheffe comparisons for day 5 indicated that placebo and nonconditioned animals consumed a significantly greater percentage of the sucrose solution (see Figure 5).

Conditioned Immune Response

In order to assess the relationship between sucrose preference (CS) and the degree of immunosuppression (CR), Pearson product moment correlations were performed for conditioned animals (i.e. sucrose/CY exposed). Positive correlations would indicate that animals showing higher preferences for sucrose would also have produced the highest antibody titers. Conversely, animals demonstrating a low preference for the sucrose solution would have produced the lowest antibody titers. Therefore, conditioned taste aversion would be associated with conditioned immunosuppression. Correlation coefficients are presented in Table 5.

Contrary to expectation, results from Table 5 indicate no significant positive correlations between fluid preference and antibody titers for conditioned animals. This is suggestive of little relationship between taste aversion and immune reactivity, and as such conditioned immunosuppression.

Table 5

Pearson Correlation Coefficients for Relationships Between Antibody Titer and Experimental Sucrose Preference Days, for Conditioned Animals (Sucrose/CY Exposed)

	Day 1	Day 2	Day 4	Day 5
Titer	.25	.08	.11	.05
Percent sucrose (S.E.)	12.38 (1.6)	10.48 (2.7)	9.45 (1.9)	13.49 (2.4)

Part III: Reproductive Measures

Reproductive measures for dams in each prenatal drug exposure group are shown in Table 6. These data include: breeding success (i.e. number paired vs number of litters born), litter size at birth and weaning, total litter weights at birth and weaning, number of still births per group, and ratio of male to female pups at birth and weaning.

These data revealed that average litter size differed as a function of nicotine exposure during different drug exposure periods [F (4,199)=10.061; p < .001]. Post-hoc Scheffe comparisons of means indicated that females exposed in the first trimester had significantly smaller litters than females exposed in either the second trimester, all trimesters, or nonexposed controls. Furthermore, Scheffe comparisons indicated that third trimester exposed dams gave birth to significantly smaller litters than second trimester exposed dams or controls (see Table 6).

Group data for total litter weights are also found in Table 6. Overall litter weights were also found to differ as a function of timing of nicotine exposure [F (4,199)=5.57; p < .001]. This level was significance

Table 6

Mean Reproductive Measures at Birth and Weaning of Pregnant Rats Exposed to Nicotine (0.6 mg/kg/day) During Different Periods of Gestation

	Pairs	Litters	Still births	Males	Females	LS	LW
Trimester							
First							
birth	7	5	1	5.6	3.6	9.2	58.0
weaning				5.6	3.4	9.0	584.9
Second							
birth	4	3	0	5.0	5.7	10.7	63.9
weaning				5.0	5.7	10.7	643.1
Third							
birth	4	4	0	5.8	4.0	9.5	56.9
weaning				5.8	4.0	9.5	623.3
All							
birth	6	5	0	3.8	5.3	10.4	62.4
weaning				3.8	5.3	10.4	663.6
Control							
birth	5	4	0	5.8	5.0	10.5	63.8
weaning				5.8	5.0	10.5	644.3

Note: Tri 1=Days 1-7 Tri 2=Days 8-14 Tri 3=Days 15-21

BW = body weight

LS = litter size

LW = litter weight

was still present when degrees of freedom for total number of litters born (n=21) was used, as opposed to total number experimental animals [F (4,21)=5.57 p < .001]. When litter size was used as a covariate, the significance of group differences among average litter weights increased [F (4,199)= 10.50; p < .0001].

Post-hoc Scheffe analyses revealed that total litter weights (at birth), born to females exposed during the third trimester, were significantly lower than those exposed in the second trimester, and controls. These effects were no longer significant with total litter weights at weaning [F (4,20)= .230; p < .91].

There were no significant group differences for female body weights on days 0 or 21 of pregnancy, or on the first day after birth. Furthermore, no group differences were found for percent maternal body weight increase, from day of sperm plug detection (pregnancy day 0) to postpartum day 1 (see Table 7).

Part IV: Developmental Signs and Reflexes

Given the potential effect of prenatal nicotine on the growth and development of offspring, and the role of body weight in contributing to the manifestation of

Table 7

Body Weights of Pregnant Females Delivering Litters After Exposure to Nicotine (0.6 mg/kg/day) During Different Periods of Gestation

		BW Day 0+	BW Day 21	BW post birth*	Percent increase++
Trimester					
	Female				
	1	216	300	254	17.6
	2	220	330	258	17.3
1st	3	183	279	225	22.9
	4	189	289	222	17.5
	5	219	307	248	13.2
Means		205.4	301.0	241.4	17.7
(S.E.)		(8.0)	(8.7)	(7.5)	(1.5)
	1	211	302	235	11.4
2nd	2	212	314	230	8.4
	3	223	312	246	10.3
Means		215.3	309.3	237.0	10.0
(S.E.)		(3.9)	(3.7)	(4.7)	(0.9)
	1	201	289	223	10.9
3rd	2	189	265	224	18.5
	3	221	306	240	8.6
	4	215	297	240	11.6
Means		206.5	289.3	231.8	12.4
(S.E.)		(7.2)	(8.8)	(4.8)	(2.2)
	1	216	311	235	8.8
	2	197	295	220	11.7
ALL	3	195	265	209	7.2
	4	210	302	240	14.3
	5	183	271	202	10.4
Means		200.2	288.8	221.2	10.5
(S.E.)		(5.8)	(8.9)	(7.3)	(1.2)

Table 7 continues...

Table 7 (cont.)

		BW Day 0+	BW Day 21	BW post birth*	Percent increase++
Trimester					
	Female				
CTRL	1	185	277	207	11.9
	2	211	304	245	16.1
	3	186	298	230	23.7
	4	191	292	218	14.1
Means (S.E.)		193.3 (6.1)	292.8 (5.8)	225.0 (8.2)	16.5 (2.6)

Note: Tri 1=Days 1-7 Tri 2=Days 8-14 Tri 3=Days 15-21

BW = body weight

+ = Day 0 indicates day on which sperm plug detected.

* = post birth indicates 24 hours after pups littered.

++ = percent increase from plug date to BW post birth.

many developmental signs and reflexes individual body weights were recorded at seven day intervals, until weaning at 28 days of age (De Lellis, 1987).

Body Weights

Mean body weight measures by sex and trimester of nicotine exposure are shown in Table 8. Table 9 lists F-ratios for body weight measures at postnatal days 1, 7, 14, 21, 28, and 90. Given the high degree of relationship between litter size and body weight (r=+.90 at birth; r=+.67 at weaning), F-ratios for analysis of covariance, using litter size at birth and total litter weight at birth, as covariates, are also shown in Table 9. No significant interaction effects were found on any of the developmental signs or reflexes, and as such are not reported in these tables.

Males, at postnatal day 1, were significantly heavier than females [F (1,199)=10.09; $p < 002$]. The significant difference between male and female body weights, on day 1, was found to increase, when both litter size and total litter weight were used as covariates [F (1,199)= 14.864; $p < .0001$]. It should be noted however, that the amount of within group variability for male and female body weights, on day 1,

Table 8

Mean Body Weights (gms) by Sex, From Birth Until Weaning and 90 Days of Age, of Rats Prenatally Exposed to Nicotine (0.6 mg/kg/day) During Different Trimesters of Gestation

	Tri 1	Tri 2	Tri 3	All	Ctrl	
Day 1						Means
Males	6.56	6.11	6.11	6.15	6.14	6.24
Females	6.24	5.88	5.89	5.78	6.01	5.93
Mean	6.44	5.99	6.02	5.92	6.08	6.09
Day 7						
Males	13.79	12.83	12.60	12.55	13.14	13.05
Females	12.57	12.35	12.24	12.09	12.44	12.31
Mean	13.33	12.57	12.45	12.27	12.82	12.69
Day 14						
Males	25.40	22.74	24.31	22.97	24.09	24.08
Females	21.60	21.95	23.48	22.59	22.03	22.35
Mean	23.97	22.32	23.96	22.73	23.15	23.24
Day 21						
Males	39.44	35.20	38.27	36.09	37.37	37.55
Females	36.62	34.38	36.44	35.20	35.07	35.47
Mean	38.37	34.76	37.50	35.54	36.33	36.55
Day 28						
Males	67.30	61.06	67.07	65.05	64.13	65.30
Females	61.21	59.59	63.58	63.22	58.05	61.36
Mean	65.00	60.28	65.60	69.93	61.38	63.39
Day 90						
Males	299.9	317.8	309.7	306.5	333.1	313.1
Females	186.3	191.1	194.5	186.9	193.3	190.3
Mean	244.8	250.5	254.1	250.4	265.5	252.9

Note: Tri 1=Days 1-7 Tri 2=Days 8-14 Tri 3=Days 15-21

Table 9

F-Ratios, Covariates and p-Values by Age for Body Weights of Animals Exposed to Nicotine (0.6 mg/kg/day) at different Gestational Trimesters

	Day 1	Day 7	Day 14	Day 21	Day 28	Day 90
Trimester Exposed	4.392*	2.734	1.625	3.663*	2.933	3.300
LS & LW as covariate	0.214	4.288*	2.091	0.431	2.195	3.369*
Sex of Animal	10.087*	9.852*	14.899*	8.399*	11.94*	986.08*
LS & LW as covariate	14.864*	13.567*	19.824*	10.547*	13.549*	N/A
Sex X Tri	0.272	0.570	2.409	0.425	0.809	1.440
sex as covariate	0.493	0.470	2.525	0.125	0.404	N/A

Notes: Significance of F * p < .01.

d.f. days 1-28 for trimester (4,199), sex (1,199) and interaction (4,199).

d.f. day 90 for trimester (4,156), sex (1,156) and interaction (4,156).

LS = Litter Size

LW = Total Litter Weight

N/A = Data not available

was very small (S.E.M. = 0.068 and 0.067 respectively), and that these small differences in variability may account for significant F-ratios (see Tables 8 and 9).

As shown in Table 8, sex differences in body weight were also significant at postnatal day 7, where males began to demonstrate the expected heavier weights as compared to females [F (1,199)= 9.825; p < .002]. Finally, as evident in Table 8, this pattern of males heavier than females was found to continue, as expected, from day 7 through weaning and until postnatal day 90 [F (1,156)=986.07; p < .0001].

Body weights differences among trimester groups yielded several meaningful results. Specifically, significant group differences were noted on postnatal days 1, 7 and 21. Although significant group differences for body weight on day 1 were found for nicotine trimester groups [F (4,199)= 4.392; p < .002], the significance disappeared when litter size and total litter weight were included as covariates [F (4,199)= .214; p < .93].

However, as evident in Table 9, using litter size and total litter weights as covariates, failed to account for trimester differences in body weight on

postnatal day 7 [F (4,199)= 4.288; p < .002]. Scheffe test for comparison of means revealed significantly elevated weights for first trimester exposed animals, as compared to rats exposed in all trimesters.

Significant differences in body weights were also evident on day 21. However, this effect was not significant when litter size and litter weight were used as covariates (see Table 9).

Significant body weight differences for trimester group were also evident at postnatal day 90, only when sex was used as a covariate [F (4,156)= 3.369; p < .01]. Due to the large percentage of overall variance attributable to sex at this age, Scheffe comparisons of means were not possible. As such, cell mean comparisons were conducted using a factorial analysis of covariance with sex as a covariate. Comparisons of means revealed that first trimester exposed animals weighed less than did control rats [F (1,63)= 6.789; p < .012; see Table 8].

Developmental Signs

Group means for developmental signs are shown in Table 10.

Pinna Detachment. There were no significant sex

Table 10

Mean Age in Days for the Appearance of Developmental Signs and Reflexes of Rats Prenatally Exposed to Nicotine (0.6 mg/kg/day) During Different Trimesters of Gestation

	Tri 1	Tri 2	Tri 3	All	Ctrl	
Pinna Detachment						Means
Males	2.43	2.40	2.45	2.35	2.30	2.389
Females	2.41	2.47	2.50	2.28	2.37	2.386
Mean	2.42	2.44	2.47	2.31	2.33	2.388
Incisor Eruption						
Males	11.14	11.13	11.27	11.25	11.65	11.30
Females	10.82	10.71	11.13	11.28	11.21	11.07
Mean	11.02	10.91	11.21	11.27	11.45	11.19
Primary Coat						
Males	8.0	8.0	8.0	8.0	8.0	8.0
Females	8.0	8.0	8.0	8.0	8.0	8.0
Mean	8.0	8.0	8.0	8.0	8.0	8.0
Eye Opening						
Males	14.25	14.47	14.73	14.55	14.87	14.56
Females	14.29	14.47	14.75	14.25	14.84	14.49
Mean	14.27	14.47	14.74	14.37	14.86	14.53
Crawling						
Males	7.29	7.20	7.27	7.35	7.13	7.25
Females	7.41	7.12	7.19	7.38	7.11	7.26
Mean	7.33	7.16	7.24	7.37	7.12	7.25
Walking						
Males	11.04	10.80	11.18	10.45	10.52	10.81
Females	11.06	10.94	11.06	10.44	10.63	10.76
Mean	11.04	10.88	11.13	10.44	10.57	10.79

Table 10 continues....

Table 10 (cont.)

	Tri 1	Tri 2	Tri 3	All	Ctrl	
Sexual Development						Means
Males	25.00	25.27	25.00	25.25	25.00	25.08
Females	34.41	35.06	35.06	35.13	34.42	34.85
Mean	28.55	30.47	29.24	31.33	29.26	29.80
Righting Reflex Totals						
Males	2.96	5.13	4.77	3.45	3.39	3.81
Females	3.24	5.00	5.25	4.81	3.89	4.48
Mean	3.07	5.06	4.97	4.29	3.62	4.13
Negative Geotaxis						
Males	10.11	10.13	10.00	10.05	10.00	10.06
Females	10.18	10.06	10.06	10.09	10.00	10.08
Mean	10.13	10.09	10.03	10.09	10.00	10.07

Note: Tri 1=Days 1-7 Tri 2=Days 8-14 Tri 3=Days 15-21

differences or trimester group differences for pinna detachment.

Incisor Eruption. There were no significant sex differences found for eruption of incisors [F (1,199)= 5.108; $p < .03$]. When body weight at day 7 was used as a covariate, significant sex differences for incisor eruption were found [F (1,199)=10.683; $p < .001$], with the incisors of males developing later than those of females.

Trimester group differences for incisor eruption were evident [F (4,199)=3.315; $p < .02$], with this level of significance increasing when litter size and body weight at day 7 were used as covariates [F (4,199)=3.234; $p < .012$]. Comparisons of means revealed that second trimester animals erupted their incisors earlier than animals exposed in all trimesters, or controls (see Table 10).

Primary Coat. No significant differences for sex or trimester group were evident for the development of a primary coat.

Eye Opening. There were no significant sex differences for eye opening, although significant differences were found for trimester group [F (4,199)=

6.445; $p < .0001$]. The significance of this effect increased when body weight at postnatal day 14 and litter size were used as covariates [F (4,199)=7.398; $p < .00001$].

Post hoc Scheffe comparisons of means revealed that first trimester animals opened their eyes significantly sooner than third trimester and control animals. Also, animals exposed in all trimesters demonstrated eye opening sooner than controls (see Table 10).

Sexual Development. Due to the differential latencies for the manifestation of overt signs of sexual development between males and females, sex differences for this variable were not addressed. There were differences in sexual development for trimester group [F (4,199)=3.003; $p <.02$], with this level of significance increasing when body weight on day 28 was used as a covariate [F (4,199)= 3.301; $p < .01$]. This level of significance was found to further increase when both body weight at postnatal day 28 and litter size were used as covariates [F (4,199)= 3.872; $p < .005$].

Developmental Reflexes

Group means for developmental reflexes of negative geotaxis and righting reflex are found in Table 10.

Negative Geotaxis. There were no significant differences for sex or trimester group on measures of negative geotaxis. Furthermore, the use of body weights at postnatal day 7 and 14, litter size, and/or righting reflex as covariates, all failed to change the level of significance.

Righting Reflex. Significant sex differences were found for righting reflex [F (1,199)=8.538; p < .004], with males demonstrating this reflex sooner than females. However, this effect disappeared when litter size and body weight at day 7 were used as covariates [F (1,199)= 2.678; p < .103].

Significant trimester group differences were revealed for righting reflex [F (4,199)=15.023; p < .0001]. Using litter size and body weight at day 7 as covariates did not alter this level of significance [F (4,199)= 15.507; p < .0001].

Scheffe test for comparisons of means revealed that first trimester animals demonstrated the righting reflex significantly earlier than second and third

trimester rats, as well as animals exposed in all trimesters. Also, control animals demonstrated this reflex sooner than second and third trimester animals (see Table 10).

Part V: Additional Conditioning Variables

Body Weights

Trimester group differences for body weight were present during the baseline phase (age 90 days) of the study [F (4,156)= 3.30; $p < .013$], with first trimester animals having the lowest body weights and control animals having the highest body weights (see Tables 8 and 9). Trimester differences for body weight were not evident on any of the other conditioning phases of the study (see Table 11).

As expected, sex differences in body weight were evident throughout all the conditioning phases of the study (see Table 11).

Mean body weights for conditioning groups during each conditioning phase are depicted in Figure 6. As expected, no two groups differed significantly in body weight during the baseline, preconditioning or conditioning day 0 phases of the study. Body weights differed significantly on days 1, 2, 3 and 4 (see

Table 11

F-Ratios for Sex, Trimester Group and Conditioning Group Differences in Body Weights During Taste Aversion Conditioning

	Base	Pre	Day 0	Day 1	Day 2
Main Effect					
Sex	986.1*	959.5*	1005.4*	976.8*	963.5*
Tri	3.300	1.173	1.058	0.991	0.833
Con	0.261	0.539	1.436	6.510*	7.912*

Notes: Significance of F * $p < .01$.

d.f. for sex (1,156), trimester (4,156) and conditioning (2,156).

no significant interaction effects were found.

Table 11 continues...

Table 11 (cont.)

	Day 3	Day 4	Day 5	Post
Main Effect				
Sex	949.9*	999.9*	923.2*	943.1*
Tri	1.237	1.029	0.988	0.654
Con	14.23*	5.757*	4.208	3.309

Notes: Significance of F * p < .01.

d.f. for sex (1,156), trimester (4,156) and conditioning (2,156).

no significant interaction effects were found.

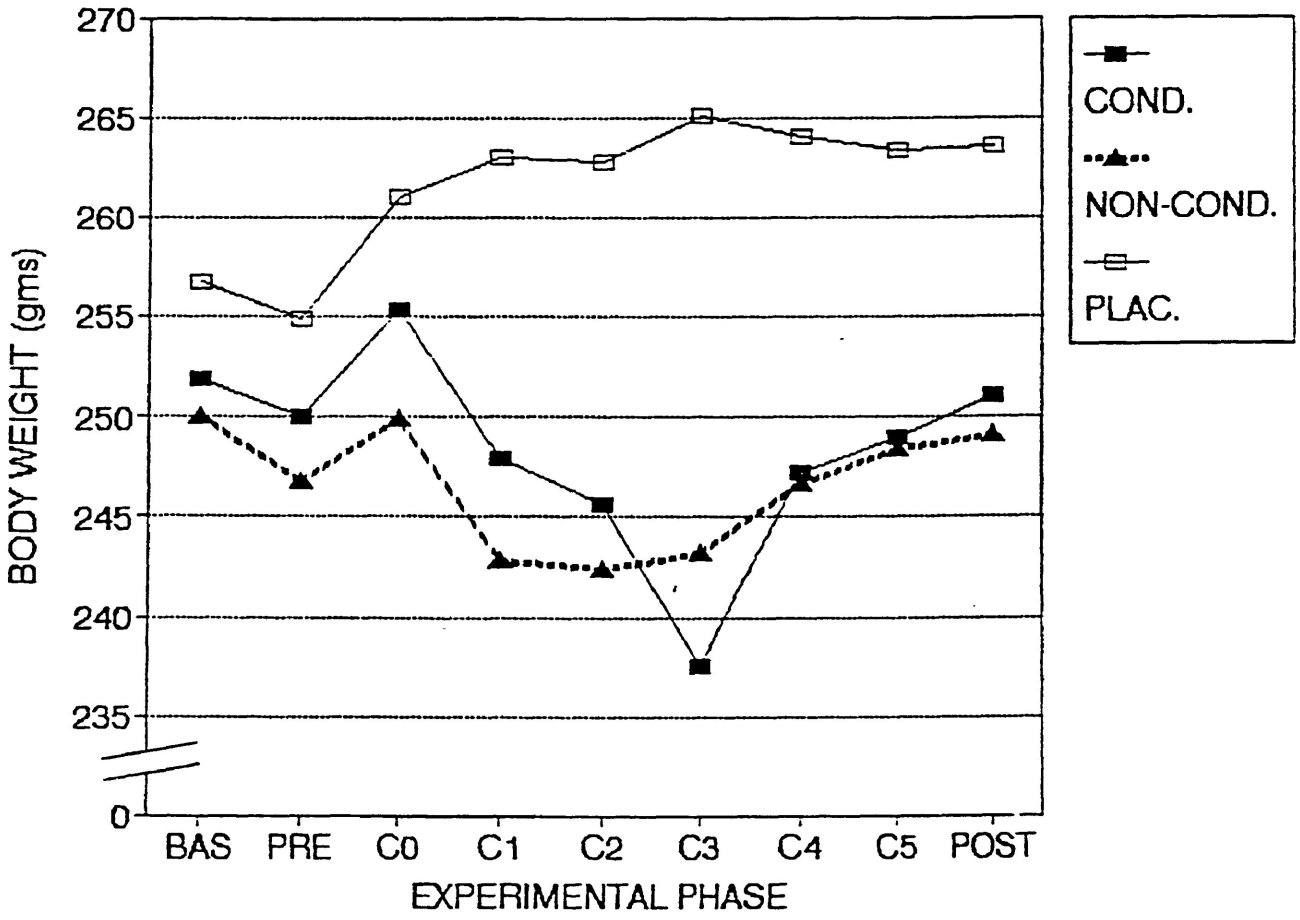

Figure 6. Mean Body Weights for Conditioning Groups During Each Phase of Taste Aversion/Conditioned Immunosuppression Conditioning.

Table 11 and Figure 6). Post hoc analyses revealed that both cyclophosphamide (CY) injected groups (conditioned and nonconditioned) differed significantly from placebo animals, but not from each other. Although group differences on conditioning day 5 and during postconditioning were no longer significant, conditioned and nonconditioned animals still maintained body weights lower than those of placebo controls (see Figure 6). Theoretically, these phases of the study may have been far enough removed temporally from the CY induced nausea on day 0, that these animals established more typical food consumption patterns at that time.

Food Consumption

As expected, sex differences in food consumption were evident throughout all phases of conditioning. However, when body weight for each given phase was used as a covariate for that experimental phase, the significant sex difference disappeared, for all phases of conditioning. Also, there were no trimester group differences found during any of the conditioning phases.

Conditioning group means for food consumption are shown in Figure 7. Conditioning effects on food

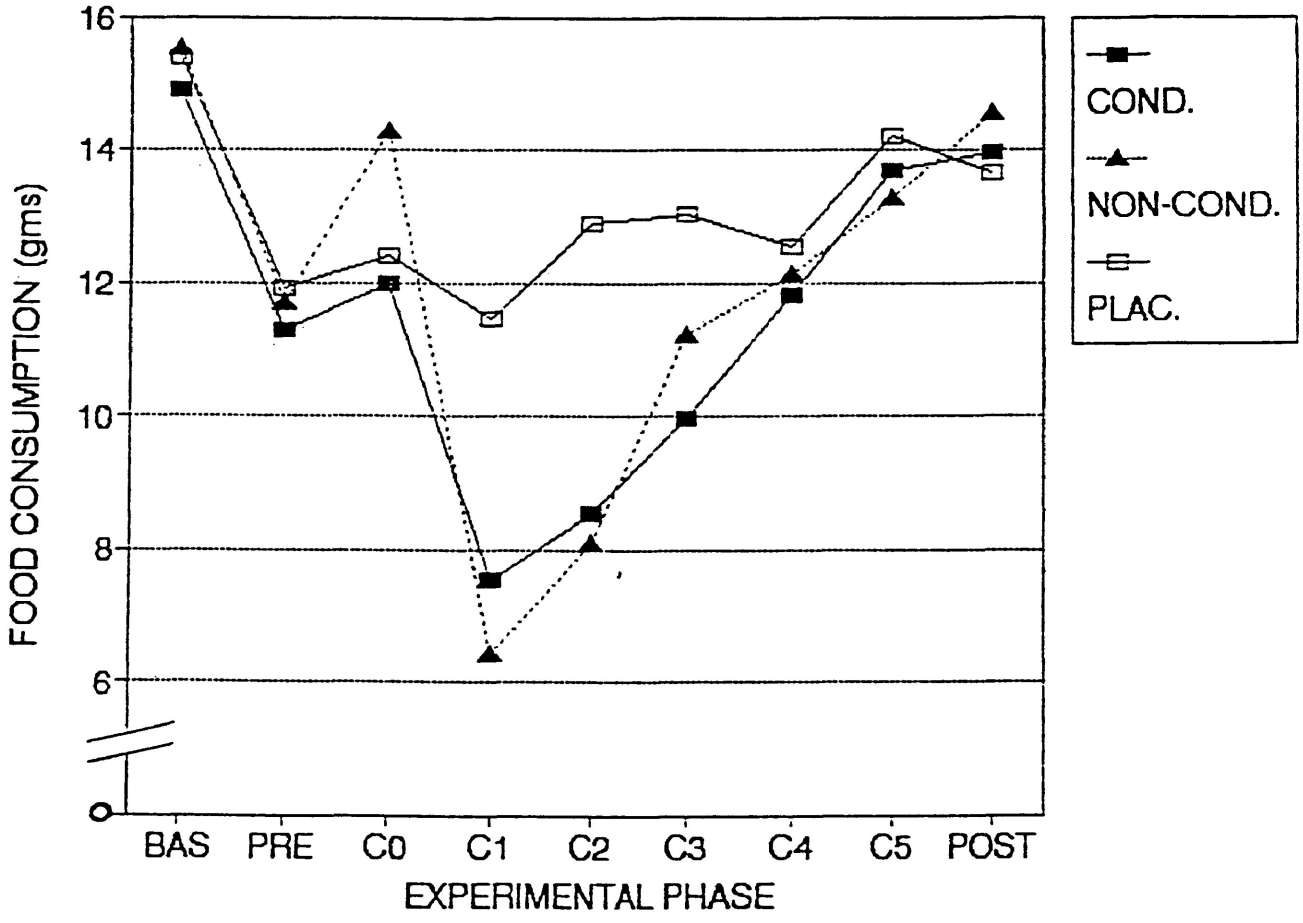

Figure 7. Mean Food Consumption for Conditioning Groups During Each Phase of Taste Aversion/Conditioned Immunosuppression Conditioning.

consumption were evident at various phases, and almost parallelled those of body weights. That is, no group differences were found during baseline, preconditioning or conditioning day 0 (see Figures 6 and 7). On day 1, CY exposed animals showed significantly reduced food intake, and a corresponding decrease in body weight. This decrease in food consumption and body weight slowly increased to nonsignificant levels by day 5 for body weight, and day 4 for food consumption.

Statistically significant conditioning group differences for food consumption were evident on day 1 [F (2,156)= 73.327; p < .0001], day 2 [F (2,156)= 69.194; p < .0001], and day 3 [F (2,156)= 15.613; p < .0001]. In each case the use of body weight as a covariate failed to account for this effect. In each of these cases, post hoc analyses revealed a similar pattern to that of body weights. That is, conditioned and nonconditioned animals differed significantly from placebo controls, but not from each other. Although a significant difference was not evident on day 4, this pattern was still present and corresponded to increases in body weights seen on day 5 (see Figures 6 and 7).

Discussion

Taste Aversion

Animals given the novel sucrose solution followed by the administration of cyclophosphamide (CY) demonstrated a marked aversion to the sucrose solution, when subsequently provided the sucrose solution. As seen in Figure 5, conditioned animals, provided with a free choice between water and sucrose, demonstrated an aversion to the sucrose solution, one, two, four, and five days following initial conditioning. This was evident in that conditioned animals consumed almost equal amounts of sucrose solution as placebo animals (16.27 mls vs 16.98 mls) on the initial day of conditioning (day 0); while on the first day following conditioning, conditioned animals consumed only 12% sucrose relative to water, as compared to 90% in placebo animals. By the fifth day after conditioning, the consumption of sucrose by conditioned animals never exceeded 15%, as compared to 85% for placebo controls (see Figure 5). This aversion to a novel drinking solution is similar to findings reported in other studies of conditioned taste aversion and conditioned immunosuppression (Ader & Cohen, 1975; Ader, et al.,

1982; Cohen, et al., 1979; Rogers et al., 1976). Furthermore, these results are a replication of those previously reported from this laboratory by Porter (1987), using the same conditioning paradigm.

Finally, this study did not demonstrate any marked extinction of taste aversion in conditioned animals, which showed only a 3% increase in sucrose preference, over the four days of free choice fluid exposure (see Figure 5). Extinction of conditioned taste aversion may have been more evident if conditioned animals were provided with more than five extinction trials. However, as reported by Bovbjerg et al. (1984), as many as 18 extinction trials were insufficient to produce any extinction of taste aversion conditioning. Given that six days following antigen inoculation has been reported to be optimal for antibody production (Ader & Cohen, 1981), further extinction trials were not included in this study.

Immunological Responses

Sex differences. The data from this study present several interesting findings concerning the immunological responses. As demonstrated by Porter (1987), the present study revealed a similar sex

difference in antibody titers, in which females consistently produced elevated antibody titers as compared to males (see Figure 1). However, Porter (1987) failed to control for variability resulting from sex differences in body weight. The present study revealed that sex differences in antibody titers covaried with sex differences in body weight. Speculation on the possible underlying mechanisms of elevated titers in female animals is beyond the scope of this study. As such, further research may assist in understanding the mediating factors involved in sex differences in titers in response to CY exposure.

Trimester differences. The present study did not reveal any effects of prenatal nicotine exposure on the production of antibody to sheep red blood cell (anti-SRBC). That is, no impairments of antibody production were evident in animals prenatally exposed to nicotine (see Figure 2). This finding is contrary to several authors that indicate that in utero exposure to tobacco smoke, and more specifically nicotine, may have compromising effects on the immune system (Abel, 1984; Moessinger, 1989; Nair, et al., 1990; Taylor & Wadsworth, 1987).

Several explanations for a lack of teratogenecity of nicotine on immune system functioning, in the present study, are plausible. Firstly, interspecies differences may play a role in the effects of prenatal nicotine on subsequent immune system functioning. That is, the aforementioned studies (Abel, 1984; Moessinger, 1989; Nair, et al., 1990; Taylor & Wadsworth, 1987), which report potential teratogenic effects of tobacco smoke and/or nicotine on the immune system, all employed human subjects or human tissue samples. As yet, no specific studies have been reported on the development of a rat model of nicotine teratogenecity on immune system capabilities. As such, the failure of the present study to demonstrate immunological deficits, as a function of prenatal nicotine exposure, may reflect interspecies differences between humans and rats.

Secondly, previous data from this laboratory has demonstrated that the teratogenic dose of nicotine employed (0.6 mg/kg/day), is high enough to produce functional deficits, but still low enough to spare any physical deformities in Satinder's Heterogeneous Stock (SHS) animals (De Lellis, 1987). Given the role of the

central immunological organs of the immune system (thymus gland and bone marrow), for optimal immunological reactivity, it is possible that the present dose of nicotine was not high enough to affect the functional development of these organs, or other principle immune system factors; and therefore no deleterious effects on immune system capabilities were evident.

Thirdly, the age of the present population of subjects may also play a role in these results. That is, since these animals were inoculated in adulthood (90 days of age), it is possible that the teratogenic effects of nicotine on the immune system were no longer present. Taylor and Wadsworth (1987) collected human data up to five years of age. These authors report possible immunological deficits as demonstrated by increased numbers of admissions to hospital for bronchitis and upper respiratory tract infections, in children of mothers who smoked while pregnant. However, follow-up data on adult immune system status in these individuals is not presented or available. Current studies in this lab are investigating a possible dose and age effect for the possible teratogenic effects of

nicotine on the immune system.

Fourthly, the use of the hemagglutinating antibody titer procedure provides a crude and relative (i.e. nonquantitative) measure of immunological activity. As such, any sensitive immunological deficits which may have been present, in this data, could have been overlooked. Therefore, future research employing more sophisticated, accurate and quantitative techniques such as enzyme linked immunosorbant assay (ELISA) or radioimmunoassay, may be warranted.

Finally, given the vast nature and complexity of the immune system, it is possible that some other area of immunological activity may have been affected by the nicotine dose employed here. However, since the present study only measured one aspect of immunological functioning, it is possible that the teratogenic effects of nicotine on the immune system were overlooked.

Therefore, it is not to say that prenatal nicotine exposure has no influence on the functioning of the immune system, but rather that further investigation is necessary to ascertain if any effects may extend to other areas of the immune system and immunological

activity.

Conditioned immunological effects.

Evidence of conditioned immunological effects were difficult to unequivocally determine in the present study, due to the observed suppression of titers in both conditioned and nonconditioned animals (see Figure 3). Also, as evident in Table 5, the present study did not demonstrate a definitive relationship between taste aversion and immunosuppression. Contrary to expectation, nonconditioned animals did not produce titers significantly greater than those of conditioned animals (see Figure 3). Conditioned and nonconditioned animals both produced suppressed anti-SRBC titers compared to those of placebo controls, and yet not significantly different from each other. A plethora of studies have demonstrated that nonconditioned animals typically produce titers significantly higher than those of conditioned animals, but still significantly lower than placebo controls (Ader & Cohen, 1975; Ader, et al., 1982; Cohen, et al., 1979; Rogers et al., 1976). Previous research from this laboratory has also demonstrated the expected pattern of anti-SRBC antibody titers, using the same SHS genetic line of animals, and

the same conditioning procedure (Porter, 1987).

Direct inferences from the present data regarding, conditioned immunosuppression, could have been made had the inclusion of a comparison group of conditioned animals not reexposed to the CS (CS_o) been included. In retrospect, replication of this study with the inclusion of a group of conditioned animals not reexposed to the CS (CS_o), may be warranted.

However, Wayner et al. (1978) reported that nonconditioned animals did not differ significantly from cyclophosphamide (CY) exposed conditioned animals, in the production of anti-SRBC antibody titers. Furthermore, both conditioned and nonconditioned animals produced significantly lower titers to those of placebo controls (Wayner, et al., 1978). The extensive effects of CY on the functioning of the immune system has been extensively researched. Kerckhaert, Hofhuis and Willers (1977) have reported that a single injection of CY can suppress humoral immune functioning for up to seven days following administration. This may then be followed by a sharp rise in antibody synthesis on the eighth or ninth day following CY administration (Kerckhaert, 1977). Furthermore, Kerckhaert, et al.

(1977) indicate that this effect of CY is dose dependent. As such, the almost equal levels of immunosuppression seen in both conditioned and nonconditioned animals in this study, may reflect the extensive immunosuppressive effect of the specific CY dose employed. As such, the high dose of CY coupled with the short interval between conditioning and immunization, may account for the suppressed antibody titers seen in conditioned and nonconditioned animals.

It has been postulated that acute elevations in adrenocorticotropic hormone (ACTH), resulting from acute and nonspecific stress during taste aversion conditioning, may result in the elevation of corticosteroids to immunosuppressive levels. As a result, subsequent reexposure to the CS may result in a conditioned release of these immunosuppressive hormones. Ader (1976) has found that taste aversion conditioning, using CY, does result in the acute release of immunosuppressive levels of corticosteroids. Furthermore, it has been reported that the successful blockade of CY induced conditioned immunosuppression is possible through adrenalectomy (Gorczynski, MacRae & Kennedy, 1983).

However, elevations in corticosteroid levels with taste aversion inducing, nonimmunosuppressive drugs have been found to not produce conditioned immunosuppression. Ader and Cohen (1975) found that lithium chloride (LiCl) was a successful unconditional stimulus for taste aversion, but failed to produce any conditioned immunosuppressive effects, which were only conditioned using CY. Furthermore, Ader and Cohen (1975) did not find any elevations in steroid levels with LiCl induced taste aversion. Also, later studies which reported elevations in corticosterone levels using LiCl, also failed to report any conditioned immunosuppression (Ader, 1976).

Taken together, it would appear that conditioned immunosuppression may not be mediated by the nonspecific release of immunosuppressive corticosteroid hormones. However, this is not to say that conditioned immunosuppression is not dependent on some other underlying physiological phenomenon. Brittain and Wiener (1985) postulate that future research may examine the role autonomic nerves and other sympathetic nervous system mechanisms, in possibly understanding the mediating factors in conditioned immunosuppression

phenomena.

Conclusions

The complete nature of the conditioned immunosuppression phenomenon is not fully understood. From the present study, the influence of prenatal nicotine exposure on either the acquisition or extinction of conditioned immunosuppression cannot be determined, since unequivocal determination of observable conditioned immunosuppression was not possible. This however is not to say that exposure to nicotine in utero is exempt in exerting potential immunomodulatory effects in adulthood. Rather, more intensive, controlled and specialized analysis is necessary to isolate any specific and subtle immunological changes which may result from in utero exposure to pharmacological agents.

In retrospect, several methodological aspects of the current paradigm could have been conducted differently, to allow more conclusive empirical inferences. Replication of the present study using a slightly higher nicotine dose (eg. 1.0 mg/kg/day), may produce more readily observable immunological effects. Furthermore, comparison of prenatal nicotine effects on

conditioned immunosuppression across two or three separate age groups (eg. 30 days, 60 days, 90 days) may provide insight into possible developmental factors. Also, reexposure to the CS, and subsequent taste preference would be assessed after immunization, therefore minimizing the overall number of extinction trials. Finally, inclusion of a conditioned group not reexposed to the CS (CS_o) would be essential in establishing a more empirically valid frame of reference.

Hence, the potential effects of prenatal nicotine exposure on immunological and conditioned immunological effects may be revealed, through the inclusion of these stricter methodological considerations. Future research may require the testing of individual hypotheses through more long term systematic analysis.

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