

CONDITIONED IMMUNE RESPONSES TO TASTE AND
ENVIRONMENTAL CUES SIGNALLING CYCLOPHOSPHAMIDE

CENTRE FOR NEWFOUNDLAND STUDIES

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CONDITIONED IMMUNE RESPONSES TO TASTE AND ENVIRONMENTAL
CUES SIGNALLING CYCLOPHOSPHAMIDE

BY

• STEVAN JOEL LEWIS, BA

A thesis submitted to the School of Graduate
Studies in partial fulfillment of the
requirements for the degree of
Master of Science

Department of Psychology
Memorial University of Newfoundland

1995

St. John's

Newfoundland



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Abstract

Injection of cyclophosphamide (Cy), an immunosuppressive drug, in conjunction with a conditional stimulus (CS) has repeatedly been shown to produce Pavlovian conditioning. However, reports vary on the critical issue of whether the conditioned response augments the immunosuppressive effect of Cy or counteracts it. In this study, the effects of CS type and post CS reexposure saline injections on the direction of conditioned immune responses were measured using a passive hemagglutination reaction. One hundred and twenty Sprague-Dawley rats were randomly assigned to a conditioning protocol using either a taste CS (saccharin, SAC) or a distinctive environmental CS (Plexiglas tubs). For some animals, CS exposure coincided with intraperitoneal (i.p.) injections of Cy (paired groups) or saline (saline groups). For others, Cy injections occurred 24 h after exposure to the CS (unpaired groups). Groups were further divided following CS reexposure such that half of the animals in each group received an injection of saline. Contrary to some previous reports, both taste and environmental CSs that were paired with injections of Cy support a conditioned immunosuppression of Ab production rather than conditioned immunoenhancement. This conditioned immunosuppression occurred without a measurable conditioned taste aversion, and did not affect Cy-induced reductions in weight gain. Also, injections of Cy reduced fluid consumption 24 h, but not 48 h, later. These findings are inconsistent with some current interpretations of conditioned immune responses and are examined in terms of simultaneous and sequential conditioning procedures.

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I started this degree in September of 1989 and finished it, approximately July 1991, with the exception of about 4 weeks work remaining on my thesis write up. Now, nearly 4 years later, and on its second submission, I hope to rid myself of it once and for all. Obviously, some thank you's for the extended patience, commitment, and support are long over-due.

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(1.0) Introduction.

Evidence for bi-directional communication between the central nervous system (CNS) and the immune system is provided by (a) physiological studies, (b) behavioral influences on immune function, and (c) conditioning paradigms and their effects on immunological reactions. Comprehensive reviews of (a) and (b) are beyond the scope of this paper and can be found in Ader and Cohen (1991), Blalock (1989, 1994), Brown (1991), and Dunn (1989).

(1.1) Conditioning immune responses.

The first attempts to condition an immunobiological response were by two Russian investigators, Metal'nikov and Chorin (1926, cited in Ader, 1981) using Pavlovian conditioning procedures. Pavlovian or classical conditioning involves the association of two stimuli by sequential presentations. The first of these two stimuli is called the conditional stimulus (CS). The CS signals (i.e., precedes) the second stimulus, the unconditional stimulus (UCS). The UCS, as its name implies, elicits relevant activity from the outset. The CS, however, is "neutral" (i.e., it elicits little relevant activity prior to its pairing with the UCS). Pavlov (1927) showed that repeated pairings of a CS (e.g., bell) with a UCS (e.g., food) produced a new response to the CS, a conditioned response (CR, in this case salivation), which occurred in anticipation of the UCS.

Metal'nikov and Chorin paired the scratching or heating of a single area of skin (CS) with the injection of a foreign material into the peritoneum of guinea pigs (US). The injection of this material

unconditionally gives rise to an increase in polymorphonuclear (PMN) leukocytes. After a thirteen day rest period, the animals were then exposed to the CS alone. PMN's increased from a resting level of 0.63 to 62% five hours after reexposure to the CS. Clearly a CR developed to the CS as a result of the pairings.

(1.2) Conditioned immunosuppression.

Ader and Cohen (1975, 1985) rekindled the interest in conditioned immunology with taste aversion studies in which a uniquely flavored drinking solution (CS) was paired with the injection of some toxic drug. The agent is typically, but not always, one that produces nausea. As the animals form an association between the flavor and the toxin, they consume less of that flavored solution. Ader and Cohen paired a strong immunosuppressive drug, Cy (see Note 1), with consumption of different volumes of a SAC solution. This was followed by an extinction trial every third day; that is, the SAC solution was presented but no injection was given. As expected, the magnitude of the initial aversion to SAC and resistance to extinction were correlated with the volume of SAC consumed during conditioning. During the course of the extinction trials, some of the animals died, and mortality rates also tended to vary directly with the volume of SAC solution consumed during conditioning. Ader (1974) hypothesized that these deaths occurred because of a conditioned immunosuppressive response to SAC. Mortality then, was attributed to a compromised immune system that resulted from repeated exposure to the SAC CS. With the integrity of the immune system weakened, these animals were

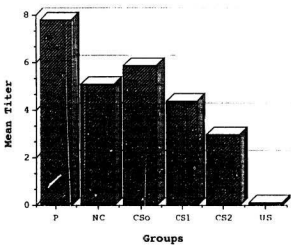
left susceptible to any latent laboratory pathogens.

These speculations led to a controlled experiment to directly assess the conditioned immune response to a taste CS paired with Cy (Ader & Cohen, 1975). Individually caged rats were gradually adapted to drink their daily amount of water during a 15 min period at the same time each day. There were 3 training conditions which differed in their treatment on one conditioning day (Day 0): (1) Conditioned animals (subgroups CS0, CS1, CS2, and US) received a .1% SAC solution for 15 min, paired with a 50 mg/kg i.p. injection of Cy 30 min later; (2) Nonconditioned animals (Group NC) received plain water which was now paired with Cy; and (3) Placebo animals (Group P) received an i.p. injection of an equal volume of vehicle 30 min after drinking SAC solution. On the following two days all animals were provided with plain water during their 15 min drinking period. Three days after the last training trial (Day 3) all animals received an i.p. injection of sheep red blood cells (SRBCs). SRBCs are a benign antigen used to stimulate cell-mediated antibody (Ab) production. Thirty min after immunization, each animal in two subgroups of Conditioned animals (Groups CS1 and CS2) were provided with a SAC solution for 15 min and then injected with saline. A second subgroup (CS0), controlling for the effects of training without presenting the CS, received plain water and a saline injection. A final subgroup (US) was provided with water and Cy to assess the UR produced by Cy (Ader & Cohen, 1975). After immunization, Nonconditioned rats were given SAC-flavored water and injected with saline, whereas Placebo animals received water and no injection. Thus, both Conditioned and Nonconditioned animals

(Groups CS1, CS2, and NC) were exposed to SAC after immunization. Group CS2 was treated like Group CS1, and then given a second SAC-saline pairing 3 days later (Day 6). On the sixth consecutive day after SRBC inoculation (Day 9), blood samples were obtained and titrated by passive hemagglutination (described in methods section).

Groups CS1 and CS2 represent CS reexposure on one or two occasions after antigen inoculation, showing attenuated Ab responses. The Ab response is a measurable part of the immune reaction to previous antigenic stimulation. The critical difference in these results is observed between Group CS0 and Groups CS1 and CS2, illustrating the conditioned effects of a Cy injection (see Figure 1). Conditioned animals that experience a single exposure to SAC following antigen treatment (Group CS1) showed an Ab response that was significantly lower than that of placebo, as well as Nonconditioned animals, and conditioned animals that were not exposed to SAC (Group CS0) (Ader & Cohen, 1975). Placebo treated animals, having experienced no immunosuppressive treatment, had the highest Ab titers and reflect the normal reaction to SRBCs in the absence of any prior exposure to Cy. Animals injected with Cy at the time of immunization (Group US) had the lowest titers, illustrating the suppressive effects of Cy on the reaction to SRBCs. Group NC's Ab titers did not differ from conditioned animals (CS0) who were not reexposed to SAC. That both groups have titers lower than Placebo animals reflects the unconditioned residual immunosuppressive effects of Cy injected 6 days earlier. Groups NC and CS0 are the appropriate control groups against which to evaluate the effects of conditioning and CS reexposure.

Figure 1: Hemagglutinating antibody titers measured 6 days after the injection of SRBC. NC (n=10), nonconditioned rats; CS0 (n=10), conditioned animals that were not reexposed to the CS after immunization; CS1 (n=11) and CS2 (n=9), conditioned animals reexposed to the CS on one or two occasions, respectively; US (n=10), conditioned animals injected with Cy at the time of immunization with SRBC; P (n=10), placebo-treated animals (adapted from Ader & Cohen, 1975).



"These initial results, then, supported the hypothesis that pairing SAC consumption with the injection of an immunosuppressive drug would enable SAC to elicit an immunosuppressive response" (Ader & Cohen, 1985, p. 383).

These same findings have been replicated by Rogers, Reich, Strom, and Carpenter (1976) and Wayner, Flanery, and Singer (1978) with various doses and at different times after inoculation. Other attempts to confirm Ader and Cohen's (1975) results with variants of their basic procedure (e.g., Gorczyński, MacRae, & Kennedy, 1984; Frank & MacQueen, 1988; MacQueen & Siegel, 1989) have not been successful. These investigators obtained instead conditioned immunoenhancement to environmental signals for Cy (see Tables 6 and 7 for a review of studies that have revealed CRs that are not in the same direction as the URs). The inability to produce consistent CRs, despite using the same dose and time-of-day parameters, is a problem.

One factor which may be contributing to these discrepant reports arises from differences in baseline results. After reviewing several studies it has become evident that not all experimental protocols yield equivalent baseline effects. Consequently, concluding either conditioned immunosuppression or immunoenhancement may only be a function of the Ab titers obtained from control and unpaired groups. For example, in Ader and Cohen's (1975) study, Placebo group animals received water paired with an i.p. injection of saline during training and subsequently produce a mean Ab titer value of 7.7. However, when MacQueen and Siegel (1989) conditioned Saline group animals to SAC-Saline during training, they obtained a mean Ab titer value of 4.7. A

difference of 3.0 when using the same assay technique cannot reasonably be attributed to SAC alone. This difference becomes even more significant when Cy trained animals are considered. In MacQueen and Siegel's experiment, group Unpaired animals undergo the sequence SAC -> 24 h delay -> water -> Cy and produce a mean Ab titer of 3.0. Hence, within the same study, animals exposed to Cy produce Ab titers 1.7 units below Saline control animals who never experience Cy. How, then, is a difference of 3.0 possible between the control groups of Ader and Cohen (1975) and those of MacQueen and Siegel (1989) when neither group is exposed to Cy?

Ader and Cohen's (1975) Paired animals receive one SAC-Cy pairing as do MacQueen and Siegel's (1989) Paired animals: their Ab titer values, following one reexposure to the SAC taste cue, are 4.5 and 5.1, respectively. This difference may be attributed to minor methodological variations across laboratories. However, when these Paired group animals are compared to their respective control groups, two opposing CRs are supported. With Ader and Cohen's high baselines (Placebo = 7.7, Non-conditioned = 5.6), they conclude a conditioned immunosuppressive response to the SAC taste CS. Since MacQueen and Siegel's baselines are low (Saline control = 4.7, Unpaired = 3.0), they suggest that SAC controls a conditioned immunoenhancement of Ab production.

Forming accurate predictions about the direction of the CR firstly requires reliable and replicable baseline data. Only then will it be possible to clarify the contribution of taste cues to the regulation of immune function.

(1.3) Conditioned immunoenhancement.

Some studies have demonstrated conditioned compensatory immunoenhancement to signals for Cy-induced antigenic reactivity. For example, Gorczynski et al., (1984) found that taste cues paired with Cy during the light portion of the diurnal (daytime) cycle resulted in conditioned immunosuppression, yet pairings of the same stimuli during the dark portion of the cycle yielded either no CR or a conditioned immunoenhancement. Krank and MacQueen (1988) investigated the CR to environmental or drug state cues that signal Cy and found a compensatory CR. They also tested the effects of a taste cue presented in conjunction with an environmental cue. Despite demonstrating a significant taste aversion, as Ader and Cohen (1975) did, in animals who received both CSs, they found that the combination of the two cues still resulted in conditioned immunoenhancement. In addition, Dyck, Greenberg, and Osachuk (1986) have reported compensatory conditioning to polyinosinic-polycytidylic acid (poly I:C) antigen when measuring natural killer (NK) cell activity of the immune response. In sum, these investigations reveal a discrepancy in CRs when Cy serves as the US. The focus of the experiment proposed here is on the conditions which cause and/or produce the two opposing responses to signals for Cy-induced immunosuppression.

(1.4) Possible sources of opposing responses.

(1.4.1) Injection regime.

In this study, two explanations are advanced that may account

for the divergent results. The first is founded on a review of the literature that indicates one significant procedural inconsistency. It has become apparent that animals who undergo the sequence SRBC -> SAC during the test phase of the experimental design show evidence for a conditioned immunoenhancement of Ab production (e.g., Krank & MacQueen, 1988; MacQueen & Siegel, 1989), whereas animals who experience the sequence SRBC -> SAC -> saline display a conditioned immunosuppressive response (e.g., Ader & Cohen, 1975-1985). The effects of injection procedures alone have also been shown to affect outcomes in a variety of conditioning and non-conditioning studies. We do not know why this post reexposure injection (saline) is critical in determining the direction of the CR.

Experimental paradigms similar to that used by Ader and Cohen (1975) frequently produce a conditioned suppression of immune reactivity (see Table 6). For example, Rogers et al. (1976) have confirmed the results of Ader and Cohen (1975) using an identical protocol, and O'Reilly and Exon (1986) have extended these findings by showing a Cy-conditioned suppression of NK cell activity in rats. McCoy, Rozzman, Miller, Kelly, and Titus (1986) conducted 3 experiments that provide evidence for conditioned immunosuppression in Fisher rats, Balb/c mice, and with delays as long as 6 h between the CS and the US. In all of these experiments, a saline injection followed SAC CS reexposure. The evidence supporting this after-reexposure saline injection (SRBC -> SAC -> saline) is substantial. Data also exist for the converse; that is, the appearance of a compensatory CR when the sequence of events is limited to SRBC -> SAC.

The appearance of a conditioned immunoenhancement to antigenic challenge is observed when investigators have not injected animals following the SAC CS reexposure phase of the experiment (e.g., Krank & MacQueen, 1988; MacQueen & Siegel, 1989). This effect has also been reported with NK cell activity using poly I:C antigen (Dyck et al., 1986; Solvason, Ghanta, & Hiramato, 1988).

The importance of injection procedures (handling) per se is supported by the results of several experiments. For example, Martin (1982, experiment 2) found that 8 saline injections between a pentobarbital -> LiCl phase and a flavor -> pentobarbital conditioning phase did not extinguish an association between handling cues and the injection of pentobarbital. However, when the number of saline injections between the two phases was increased to 22, Cunningham and Linakis (1980) observed a reduced association between handling cues and the injection of pentobarbital. Non-conditioning experiments have also shown that injections of saline alone can alter tryptophan levels in rats (Holder & Huether, 1990), and induce a suppression of NK cell activity (Fride, Skolnick, & Arora, 1990).

(1.4.2) Associative bias.

The second possible source of discrepancy is associative bias in immune conditioning. Krank and MacQueen (1986) and Siegel, Krank, and Hinson (1987) suggest that external CSs (such as environmental cues) produce drug-opposite CRs while taste CSs result in agonist CRs when cy is the US.

Much evidence suggests that Pavlovian conditioning principles

modulate the effects of drugs (for a review, see Siegel, 1983; Siegel et al., 1987); that is, environmental or contextual signals for drug administration acquire the ability to elicit physiological CRs that interact with drug effects. For many effects of drugs, the CR is an anticipatory compensation; for example, the subject with a history of morphine administration (and its analgesic and behavioral sedative effects) displays hyperalgesia (Krank, Binson & Siegel, 1981) and behavioral activation (Binson & Siegel, 1983) CRs opposite to the direct effects of the drug. The role of environmental signals for drug administration has not been considered by Ader and Cohen. They have failed to account for the inability to present the taste CS (SAC) without simultaneously giving exposure to environmental or contextual CSs. For Ader and Cohen, however, the SAC CS proved to be more salient, perhaps due to preexposure to the drinking context, and overshadowed any environmental cues that may have been present. Other investigators (e.g., Krank & MacQueen, 1988) had less habituation to contextual cues, and as a result, context may have overshadowed the taste CS and yielded an immunoenhancement of Ab production.

An explanation for the two opposing CRs is the amount of habituation to the drinking schedule. Ader and Cohen (1975, 1981) typically gave one week of habituation to the drinking schedule used. They repeatedly exposed the animal to the drinking context prior to conditioning. Unintentionally, the CS preexposure effect may have affected conditioning to the drinking context (Lubow & Moore, 1959). CS preexposure reduces the salience of a cue and its ability to enter into an association. Relative to a novel taste cue, if context is

preexposed it would be overshadowed by a taste CS. This latent inhibition explanation would account for the conditioned suppression obtained by Ader and Cohen (1975, 1981).

To account for conflicting findings, Krank and MacQueen (1988) have proposed the following model (see Figure 2). The columns (labelled UCS types) represent the dual unconditional effects of Cy, both nausea and a suppression of Ab production. The rows (labelled CS types) represent the two possible CSs, taste and environmental cues. The type of cue determines the form of the CR, either immunosuppression and immunoenhancement, when it is paired with Cy. When a taste cue serves as the CS it is associated with nausea which produces a taste aversion leading to conditioned immunosuppression of Ab production. Only two exceptions to this pattern exist. In 3 of 5 experiments, MacQueen and Siegel (1989) reported a SAC flavor CS supporting a conditioned immunoenhancement. Gorczyński et al. (1984) also reported conditioned immunoenhancement, but it was observed in only 2 of 8 experiments. These studies are described further in the discussion section. According to Krank and MacQueen's model (1988), immunosuppression is stress-induced where the stress is based on the taste aversion. When an environmental or contextual cue serves as the CS, it is paired with a suppression of Ab production leading to a compensatory CR.

The relative contribution of a particular CS and its corresponding CR should change when one cue is more salient than the other. One method of altering CS salience is habituation. If the environmental CS is preexposed and made less salient than the taste

Figure 2: Associative bias model for determining the direction of the conditioned response (adapted from Krank and MacQueen, 1988).

UCS type

Nausea

**Suppression of
Ab production**

Taste

Conditioned stress-
induced suppression
of Ab production

No conditioning

CS type

Context

No conditioning

Conditioned
compensation
for drug-induced
suppression

CS, then the taste will overshadow the context CS. According to the model, this leads to an immunosuppressive CR. If, however, the taste CS is preexposed, thereby reducing its distinctiveness, the context CS will become more salient and will result in a compensatory CR. The fundamental question arising from these divergent results is what determines the relative contribution of each of the two CSs. Because Cy conditioning can result in either an enhancement or a suppression of immune function, the possibility exists that both CSs may compete. The experiment conducted here assesses immune reactivity to both types of CSs.

(1.5) Summary and hypotheses.

Although there have been many reports of conditioned immunological responses (for a review see Ader & Cohen, 1985; Dunn, 1989), the evidence remains inconclusive with regard to the direction of the CR. Several investigators have obtained results supporting a conditioned immunosuppression of Ab production (e.g., Ader & Cohen, 1975; Gorczynski et al., 1984; Rogers et al., 1976; Wayner et al., 1978); yet the evidence for a compensatory immunoenhancement to antigenic challenge also exists (e.g., Dyck et al., 1986; Gorczynski et al., 1984; Krank & MacQueen, 1988; MacQueen & Siegel, 1989). That two opposing responses occur is both interesting and important; we suggest two reasons that may be responsible for the observed results.

The experiment conducted here examines two issues: (1) does the type of cue (taste or environment) have an effect on the direction of the CR, and (2) have saline injections following CS reexposure

affected subsequent immune reactions? In all of the experiments, the groups and conditions used a composite of those of Ader and Cohen (1975, 1985), Krank and MacQueen (1988), and MacQueen and Siegel (1989); the primary investigators responsible for the opposing views. Other than the use of a limited number of treatment groups, the procedures used in these studies, for the most part, replicate those used by the above authors.

Method

(2.1) Subjects

One hundred and twenty-five experimentally naive male albino rats (Sprague-Dawley) from Charles River breeding farms (St. Constance, Quebec) were randomly assigned to individual suspended stainless steel wire cages (25 cm x 20 cm x 18 cm). These cages were situated in an isolated room to minimize extraneous disruptions. The colony room was maintained on a 12 h light-dark cycle (0700-1900 h) under standard fluorescent illumination at a temperature of 22 ± 2 °C. The animals, weighing 216-264 g upon arrival in the laboratory, were given 14 days of habituation to the colony room prior to the start of the experiment. Continuous access to food (Purina Rat Chow) and tap water was available during habituation. Subjects were weighed every third day between 1400-1600 h, the same time during which animal care activities were conducted.

Of the original 125 subjects, 120 were included in the final analysis. The data from five animals were omitted because none of them were inoculated with SRBCs. These five rats served as a control to assess whether any measure of immune activity was possible without prior antigen exposure.

(2.2) Apparatus and Materials

Cyclophosphamide was purchased from Sigma Laboratories (St. Louis, MO) and reconstituted with injectable 0.9% isotonic saline (9 g

of NaCl per 1000 ml of sterile water) to a concentration of 2% (1 g of Cy per 50 ml of saline). Cyclophosphamide is used medically to prevent cellular proliferation necessary to mount immunological defense (Black & Livingston, 1990; Wilman & Farmer, 1989). Previous studies using this dose (50 mg/kg) have shown it to be below the lethal level.

A 0.1% SAC (Sigma) solution (1 g of SAC per 1000 ml of tap water) served as the distinctive flavor cue and was presented in glass water bottles. Plexiglas tubs (45 cm x 25 cm x 20 cm) with metal wire lids and heat treated Beta-chip bedding (Hardwood Labs, Warrensburg, NY), provided the distinctive environmental cue. These tubs were located on one side of the colony room.

Sheep red blood cells (SRBCs) from Woodlyn Labs (Guelph, Ontario), were used to stimulate the animals immune system and were also a necessary component in the passive hemagglutination reaction. They were prepared in the following manner: defibrinated blood, hematocrit value of 70-80, was centrifuged for 10 min and the supernatant was removed. Approximately 2 ml of sterile physiological saline was added to the blood, and the tube was shaken and centrifuged for another 10 min, at which time the saline/serum layer was removed. This process was repeated three times in order to insure a pure, densely packed, erythrocyte sample. For inoculations, washed cells were suspended in saline to a 1% concentration (1 ml of packed cells per 100 ml of saline). The concentration used in the immune assay was

10% (10 ml of packed cells per 100 ml of saline). Both concentrations were checked for consistent cell dispersion using standard hemacytometer procedures. Suspensions were used within 24 h of preparation and kept refrigerated when not in use.

(2.3) Procedure

Following 14 days of habituation to the laboratory a water deprivation schedule was instituted and maintained until 24 h prior to the collection of blood. The restricted water schedule provided animals with access to tap water in their suspended cages for 60 min during the morning session (0930-1130 h). An additional 30 min access to water was provided between 1600-1800 h each day. Following six days of habituation to the water deprivation regime, the conditioning phase of the experiment began.

The proceeding experimental details describe each of the experimental conditions as though all animals within each group had been trained simultaneously. To ensure randomization the experimental groups were in fact distributed over several days such that 24 animals (four from each of the six groups) were trained on any given day (see Appendix A for a detailed review of the training schedule).

(2.3.1) Phase 1: Conditioning

Each conditioning trial involved four days: Baseline, Conditioning, and two Postconditioning days. During these four days

each animal was injected on two occasions. Baseline days occurred 24 h prior to cue presentation and provided a measure of preconditioning tap water consumption. The next day was a Conditioning day. For the Taste-cue animals (N=60) a distinctive SAC solution was substituted for tap water during the morning drinking session. Env-cue animals (N=60) were removed from their suspended cage and placed in the novel Plexiglas tubs where they received tap water for 60 min. Rats were placed in the same Plexiglas tub on each trial throughout all phases of the experiment. Immediately following cue exposure all animals were injected i.p. with either Cy (50 mg/kg) or an equivalent volume of physiological saline (see Table 1). Of the Taste-cue animals, 20 received SAC paired with Cy (SAC-Paired group), 20 received SAC paired with saline (SAC-Saline group), and 20 more received SAC paired with saline (SAC-Unpaired group). Env-cue animals were similarly divided and injected. Twenty animals were exposed to the environmental cue and received an injection of Cy prior to being returned to their suspended cage (Env-Paired group), 20 received the environmental cue paired with an injection of saline (Env-Saline group), and 20 others received the environmental cue paired with an injection of saline (Env-Unpaired group).

The next day was the first Postconditioning day (PC-1) and all animals were given access to tap water for 60 min. Following tap water removal, rats in the SAC-Paired, SAC-Saline, Env-Paired, and the Env-Saline groups received an i.p. saline injection. Rats from the

Table 1
Experimental Design Showing the Training and Testing
Conditions for each of the Six Groups and their Subgroups

Group	Cond x 1	Pc-1 x 1	Reexposure x 2
Sac-Paired	Sac-Cy	TW-saline	SRBCs -- Sac w/inj
	Sac-Cy	TW-saline	SRBCs -- Sac w/o
Sac-Unpaired	Sac-saline	TW-Cy	SRBCs -- Sac w/inj
	Sac-saline	TW-Cy	SRBCs -- Sac w/o
Sac-Saline	Sac-saline	TW-saline	SRBCs -- Sac w/inj
	Sac-saline	TW-saline	SRBCs -- Sac w/o
Env-Paired	Env-Cy	TW-saline	SRBCs -- Env w/inj
	Env-Cy	TW-saline	SRBCs -- Env w/o
Env-Unpaired	Env-saline	TW-Cy	SRBCs -- Env w/inj
	Env-saline	TW-Cy	SRBCs -- Env w/o
Env-Saline	Env-saline	TW-saline	SRBCs -- Env w/inj
	Env-saline	TW-saline	SRBCs -- Env w/o

Note: Cy = cyclophosphamide. SRBCs = sheep red blood cells. Sac = saccharin. Env = environment. TW = tap water. Pc-1 = postconditioning day one. Cond = conditioning day. Three conditioning trials were each separated by six days, and two reexposure trials were separated by 72 h. SRBCs were injected into rats prior to the first, but not the second, Reexposure day. Under the Reexposure heading, the first subgroup (w/inj) indicates those animals that receive a saline injection after SRBC inoculation and CS Reexposure. Conversely, animals who are not given an injection after CS Reexposure are indicated by (w/o) subgroups. In all groups n = 20. Subgroups always consist of one half of the main groups population (e.g., Sac-Cy w/inj, n = 10).

SAC-Unpaired and the Env-Unpaired groups received an injection of Cy (50 mg/kg) following tap water removal. Groups SAC-Unpaired and Env-Unpaired are 24 h controls to assess the unconditioned effects of Cy injections. On day Pc-1, rats in the Paired and Saline groups were injected with saline (see Table 1). A second Postconditioning day (Pc-2) occurred 48 h after the conditioning trial and provided a measure of tap water consumption by the Unpaired group on the day after their Cy injections. This set of four days (Baseline, Conditioning, Pc-1, and Pc-2) constitutes one complete conditioning trial. Each animal was subject to three conditioning trials, separated by six day intervals to minimize the possibility of cumulative drug effects (Calabresi & Parks, 1985; Shand & Liew, 1980). Days 1-6, 7-12, and 13-18 were conditioning periods 1, 2, and 3, respectively (see Table A-1 for a detailed outline of each subject's treatment schedule).

Day 19 marked the end of three conditioning trials for all animals and the beginning of a 14 day recovery period. During these days, animals received their water bottle for 60 min in their home cage at the same time as on the cue exposure days (0930-1130 h). Water was again presented for 30 min between 1600-1800 h.

(2.3.2) Phase 2: CS Reexposure

Each CS Reexposure trial consisted of three days: Baseline, cue Reexposure, and a Postexposure day. The Postexposure day provided a

measure of tap water consumption following cue Reexposure. On Day 28 rats were inoculated with an i.p. injection of a 1% (v/v) thrice washed SRBC solution (2 ml/kg; approximately 3×10^8 cells/ml) between 0830-0900 h. Animals injected with SRBCs were returned to their suspended cage. At 0930 h, animals were reexposed to the same cue used during conditioning. Taste-cue animals received SAC for 60 min while Env-cue animals were transferred to the Plexiglas tubs and received tap water for 60 min. Thus, in accordance with the procedure of Ader and Cohen (1975), Krank and MacQueen (1988), and MacQueen and Siegel (1989), animals were presented with their respective cue on the day of antigenic challenge; this constitutes the first Reexposure day. For half of the animals in all groups, an i.p. saline injection (2 ml/kg) followed cue Reexposure; the others did not receive a saline injection following Reexposure (refer to Table 1). Cue Reexposure was repeated 72 h later (Day 31), as were the saline injections, but the SRBC inoculations were not repeated. Free access to water was reinstated 24 h prior to the blood assay to ensure adequate blood volume for the immune assay.

(2.3.3) Phase 3: Euthanasia and Blood Collection

Blood samples (approximately 4 cc) were obtained three days following the second Reexposure day. Beginning at 1130-1230 h, animals were removed from their home cage and anesthetized with pentobarbital (65 mg/kg). Rats were then transported in suspended

wire cages to another section of the facility. Blood was collected with a cardiac puncture under semi-aseptic conditions using a 5 cc syringe and 18 G needle when subjects no longer responded to a moderate tail pinch (about 12 min after injection of pentobarbital). Animals were then immediately sacrificed via an i.p. injection of an additional 65 mg/kg of pentobarbital.

(2.4) Serum Preparation and Passive Hemagglutination

Whole blood obtained from anesthetized rats was placed into sterile heparin-rinsed test tubes and centrifuged at 600 x g for 20 min. The top serum layer was then drawn off with pasteur pipettes and placed in another test tube to be centrifuged again, thereby removing as many cells from the serum as possible. When the serum was clear, after having been centrifuged twice, it was removed and heated in a water bath for 30 min at 56 °C. One hundred microlitres of denatured rat sera was then placed in a well which contained an equal amount of saline. The solution was stirred and 100 microlitres was removed and deposited in another well which also contained 100 microlitres of saline. Again, this mixture was stirred and 100 microlitres drawn off; this process was repeated until 12 such serial dilutions had been prepared. Fifty microlitres of a 10% SRBC solution was then added to each of the twelve wells. The wells were then covered with parafilm and remained stationary for four hours while the hemagglutination reaction occurred.

Each rat's sera was prepared in duplicate and scored by two observers who were unaware of the rats group assignment. The intersample correlation coefficients (Pearson product moment correlation) for observers A and B were $[r(119) = 0.924, p < .001]$ and $[r(119) = 0.930, p < .001]$, respectively on two ratings each of 120 reactions. Additionally, the interrater correlation coefficient was $[r(119) = 0.916, p < .001]$.

(2.5) Statistical Treatment of Data

Three types of data are reported: Ab titers, fluid consumption, and body weights. Analysis of variance (ANOVA) procedures were used to access differences among experimental and control groups. When a significant difference was obtained Newman-Keuls method of multiple comparisons was used (Ferguson, 1976). Significant three-way interactions were analyzed in accordance with the procedures described by Keppel and Zedeck (1989). The acceptable significance level for all analyses was set at .05.

Results

(3.1) Immune Response

Contrary to preconceptions underlying this experiment, the only type of conditioning observed was a suppression of immune activity and this suppression was equally strong to either CS type. The use of injections during reexposure also had no effect.

Table 2 shows the average passive hemagglutination titer for each of the three groups of Taste-cue and Env-cue animals. Rats experiencing the sequence SAC → Cy or Env → Cy on three conditioning trials displayed the lowest Ab titers when tested with subsequent cue reexposure. A 2 x 3 x 2 (Cue [SAC or Env] x Group [Paired, Unpaired, or Saline] x Injection [Saline or No Saline]) ANOVA revealed a significant main effect of Group [$F(2, 108) = 20.12, p < .001$]. All other comparisons were not significant ($ps > .41$) (see Table 3). Newman-Keuls pairwise tests (Ferguson, 1976) on the means of the significant main effect revealed that the Paired group had reliably lower Ab titers than both the Saline ($q = 8.89, F_{.99}(3, 117) = 4.20$) and the Unpaired ($q = 3.41, F_{.95}(2, 117) = 2.80$) groups. The Unpaired group also had reliably lower Ab titers than the Saline group ($q = 5.48, F_{.99}(2, 117) = 3.70$). The difference between the Ab titers of the Paired group and the Unpaired group is clear evidence of conditioned immunosuppression, and the difference between the Unpaired group and the Saline group reflects the residual unconditional effect of three Cy injections. These results are not consistent with the

Table 2
 Mean (\pm SEM) Antibody Titer (\log_2) as a Function of
 Training Condition and Conditional Stimulus (CS) Type

CS type	Group		
	Paired	Unpaired	Saline
Taste (Sac)	2.487 \pm .186	3.069 \pm .230	3.963 \pm .344
Environment	2.125 \pm .299	2.975 \pm .123	4.383 \pm .409

Note: N=120. Sixty animals were assigned to each CS type. Paired, Unpaired, and Saline groups consisted of 20 rats per cue. The Injection factor was omitted in this table because its inclusion offered no additional information (see Table 3 ANOVA).

Table 3
Analysis of Variance (ANOVA) on the Passive
Hemagglutination Antibody Titers

Source of Variation	df	Sum of Squares	Mean Square	F	p
Cue	1	0.00	0.00	0.00	.961
Group	2	70.91	35.45	20.12	<.001
Cue x Group	2	3.16	1.58	0.89	.411
Injection	1	0.24	0.24	0.14	.707
Cue x Inj	1	0.06	0.06	0.03	.849
Group x Inj	2	0.61	0.30	0.17	.841
Cue x Gr x Inj	2	0.07	0.03	0.02	.979
Error	108	190.25	1.76		

Note: Factors are Cue = 2, Group (Gr) = 3, and Injection (Inj) = 2.

reports of Krank and MacQueen (1988) and MacQueen and Siegel (1989) insofar as none of these investigators obtained immunosuppression to an environmental CS.

The five animals that were not inoculated with SRBCs but were otherwise treated as Saline group rats, produced Ab titers of zero. This demonstrates that with the technique employed here, previous antigen experience is necessary if the immune system is to be activated.

(3.2) Fluid Consumption

SAC and tap water consumption were recorded to the nearest tenth of a ml for each day of the experiment. Mean SAC consumption for the Taste-cue rats and mean tap water consumption for the Env-cue rats is presented for the 4 days of each Conditioning trial in Table B-1. Table B-2 summarizes the fluid consumption data from the CS Reexposure phase of the experiment. The Baseline, Conditioning, Reexposure, and Postconditioning data are analyzed separately since each of these days provide a measure of fluid consumption that describes a particular feature of an animal's training schedule. Each type of day is discussed under individual sub-headings that correspond chronologically to the order of the experiment.

In summary, fluid consumption for all animals did not differ on Baseline days but a pronounced SAC aversion was evident in SAC-Paired animals following a single SAC -> Cy pairing. This aversion persisted

over both CS Reexposure days. No aversion was observed in Env-Paired animals to tap water; rather, consumption remained consistent throughout all phases of the experiment. Postconditioning data suggested that injections of Cy reduced fluid consumption 24 h, but not 48 h, later. During the Reexposure phase the SAC-Paired group showed an aversion to the flavor cue, but the Env-cue rats did not show any reduction in tap water consumption while in the novel Plexiglas tubs. The manipulation of following CS Reexposure with an injection of saline did not affect fluid consumption.

(3.2.1) Baseline

A $2 \times 3 \times 2 \times 6$ (Cue x Group x Injection x Day (within factor)) ANOVA of Baseline fluid consumption revealed a significant effect over Days [$F(5, 540) = 10.14, p < .001$], and a Group x Injection interaction [$F(2, 108) = 3.18, p < .045$] (see Table B-3). The Day effect indicates that some fluctuation in tap water consumption occurred throughout the experiment. The sixth Baseline day used in the analysis is a measure of fluid consumption 48 h after the second Reexposure day, and is a Baseline measure to the extent that it precedes the next experimental manipulation (immune assay) by 24 h. Analysis of the Group x Injection interaction did not reveal any systematic effects of the injection regime on fluid consumption 24 h prior to each of the three Conditioning days and the two Reexposure days. The simple main effects in the Group x Injection

interaction are presented in Table B-4 and the subsequent Newman-Keuls comparisons are presented in Table B-5. That all groups were comparable in terms of fluid consumption prior to fluid re-presentation suggests that all animals had recovered from the unconditional effects of the drug.

(3.2.2) Conditioning

As summarized in Table B-1, SAC consumption by the three groups of SAC-cue animals was comparable on the first Conditioning day. Over the course of conditioning, consumption of SAC by SAC-Saline and SAC-Unpaired animals increased and stabilized ($M = 22.2$ ml), but consumption decreased for SAC-Paired animals following a single injection of Cy ($M = 7.9$ ml). The three groups of Env-cue rats did not show any significant changes in tap water consumption ($M = 12.8$ ml) over the three conditioning trials. A $2 \times 3 \times 2 \times 3$ (Cue x Group x Injection x Day [within factor]) ANOVA of Conditioning day fluid consumption revealed a significant Cue x Group x Day interaction [$F(4, 216) = 30.46, p < .001$] (see Table B-6). Subsequent analyses were based on $2 \times 3 \times 2$ (Cue x Group x Injection) ANOVAs of each of the three Conditioning days (Keppel, 1982).

(3.2.2.1) Conditioning Day-1

A significant effect of Cue [$F(1, 108) = 47.22, p < .001$] was obtained on Conditioning Day-1 (see Table B-7). No other differences

were significant ($ps > .50$). This cue effect reflects greater consumption of a novel SAC flavored solution ($M = 16.8$ ml) relative to plain tap water ($M = 11.1$ ml) in a distinct environment.

(3.2.2.2) Conditioning Day-2

A significant Cue x Group interaction [$F(2, 108) = 31.67, p < .001$] was obtained on Conditioning Day-2 (see Table B-8). Subsequent analysis of the simple main effects revealed a significant difference between the three groups (Paired, Unpaired, and Saline) when SAC was the cue [$F(2, 216) = 101.45, p < .001$] (see Table B-9). Newman-Keuls comparisons of the means (refer to row Cond-2 of Table B-1) revealed that the SAC-Paired group consumed reliably less SAC than either the SAC-Saline ($q = 19.06, F_{.99}(3, 57) = 4.28$) or the SAC-Unpaired group ($q = 15.16, F_{.99}(2, 57) = 3.76$). SAC-Unpaired animals also consumed reliably less SAC than SAC-Saline animals ($q = 3.89, F_{.99}(2, 57) = 3.76$). These comparisons indicate that the SAC-Paired animals had an aversion to SAC based on a single injection of Cy on Conditioning Day-1, and that the SAC-Unpaired rats had a weak SAC aversion despite the 24 h interval separating their exposure to SAC and the injection of Cy. This effect was not observed in the SAC-Unpaired animals during subsequent exposure to SAC on Conditioning Day-3 and Reexposure Day-2, but was evident on Reexposure Day-1. The instability and magnitude of the aversion may reflect some sensitization by the Unpaired animals to the taste Cue. No significant differences existed in the tap water

consumption of Env-cue animals on Conditioning Day-2 (see Table B-9; refer to row Cond-2 of Table B-1 for the means).

(3.2.2.3) Conditioning Day-3

A significant Cue x Group interaction [$F(2, 108) = 116.36, p < .001$] was obtained on Conditioning Day-3 fluid consumption (see Table B-10). Analysis of the simple main effects revealed a significant difference between the Paired, Unpaired, and Saline groups when SAC was the cue [$F(2, 216) = 247.43, p < .001$] but not when the environment was the cue (see Table B-11). Newman-Keuls comparisons of the means (refer to row Cond-3 of Table B-1) revealed that the SAC-Paired group consumed reliably less SAC than either the SAC-Unpaired ($q = 27.49, F_{.99}(3, 57) = 4.28$) or the SAC-Saline group ($q = 26.99, F_{.99}(2, 57) = 3.76$). The SAC-Unpaired and SAC-Saline groups did not differ ($q = .499, F_{.95}(2, 57) = 2.83$). These data are additional evidence for a conditioned taste aversion in the SAC-Paired animals. The three groups of Env-Cue animals drank comparable amounts of tap water whether or not it was followed by an injection of Cy (refer to row Cond-3 of Table B-1). That fluid consumption does not differ between Env-Paired, Env-Unpaired, and Env-Saline rats suggests that fluid consumption does not reveal any conditioning effects to environmental cues. Conditioning is revealed by the conditioned Ab titer data for Env-cue animals that was described earlier.

(3.2.3) Postconditioning

(3.2.3.1) Postconditioning Day-1

Postconditioning Day-1 (Pc-1) refers to the day immediately following a conditioning day; thus, there are three Pc-1 days. A $2 \times 3 \times 2 \times 3$ (Cue x Group x Injection x Day [within factor]) ANOVA of fluid consumption 24 h after conditioning revealed a significant effect of Group [$F(2, 108) = 34.52, p < .001$]. No other differences were obtained ($ps > .09$) (see Table B-12). Subsequent Newman-Keuls analyses of the combined Pc-1 days demonstrated that the Paired group ($M = 15.1$ ml) drank reliably less tap water than either the Unpaired ($M = 18.8$ ml) ($q = 8.78, F_{.99}(3, 117) = 4.20$) or the Saline group ($M = 18.6$ ml) ($q = 8.40, F_{.99}(2, 117) = 3.70$). The fluid consumption of the Saline and the Unpaired group did not differ ($q = .377, F_{.95}(2, 117) = 2.80$). These comparisons indicate that injections of Cy reduce fluid consumption 24 h later.

(3.2.3.2) Postconditioning Day-2

The second Postconditioning day (Pc-2) occurred 48 h after the Conditioning day. A $2 \times 3 \times 2 \times 3$ (Cue x Group x Injection x Day [within factor]) ANOVA of Pc-2 fluid consumption revealed a significant Injection x Day interaction [$F(2, 216) = 3.73, p < .025$] and Group [$F(2, 108) = 24.31, p < .001$] effect (see Table B-13). Newman-Keuls analyses on the Group effect showed that the Unpaired group ($M = 14.5$ ml) drank reliably less fluid than either the Paired

group ($M = 18.3$ ml) ($q = 8.48$, $F_{.99}(3, 117) = 4.20$) or the Saline group ($M = 18.0$ ml) ($q = 7.89$, $F_{.99}(2, 117) = 3.70$). Consumption did not differ between the Paired and the Saline group ($q = .587$, $F_{.95}(2, 117) = 2.80$). These findings replicate the findings observed on Pc-1 days in that injections of Cy reduce fluid consumption 24 h, but not 48 h, later.

Analysis of the Injection x Day interaction revealed a significant difference between Pc-2 days only when the Injection factor was Saline injection [$F(2, 216) = 5.78$, $p < .01$] (see Table B-14). Newman-Keuls comparisons of the means revealed that animals drank significantly more tap water on the second Pc-2 day ($M = 17.7$ ml) than on the third Pc-2 day ($M = 15.9$ ml) ($q = 4.79$, $F_{.99}(3, 177) = 4.20$). No other comparisons were significant. The presence of an Injection x Day interaction cannot be interpreted because the manipulation, the Injection factor, has yet to be introduced. It is only during the Reexposure phase of the experiment that the presence or absence of a saline injection becomes important. This significant difference must therefore reflect a sampling error.

(3.2.4) CS Reexposure

Each rat experienced two Reexposure days in which the previously conditioned CS was presented alone. For half of the animals in each group, reexposure was followed by an injection of saline (refer to Table 1). A $2 \times 3 \times 2 \times 2$ (Cue x Group x Injection x Day [within

factor]) repeated measures ANOVA of fluid consumption on the two Reexposure days revealed a significant Cue x Group X Day interaction [$F(2, 108) = 9.34, p < .001$] (see Table B-15). Subsequent analyses were based on $2 \times 3 \times 2$ (Cue x Group x Injection) ANOVAs of each of the two Reexposure days.

(3.2.4.1) Reexposure Day-1

A significant Cue x Group interaction [$F(2, 108) = 131.49, p < .001$] was obtained on Reexposure Day-1 (see Table B-16). Subsequent analysis of the simple main effects revealed a significant difference between the Paired, Unpaired, and Saline groups when SAC was the cue [$F(2, 108) = 413.91, p < .001$] but not when environment was the cue (see Table B-17). Newman-Keuls comparisons of the means (refer to row Rx-1 of Table B-2) revealed that the SAC-Paired group drank reliably less SAC than either the SAC-Saline ($q = 37.67, F_{.99}(3, 57) = 4.28$) or the SAC-Unpaired group ($q = 32.14, F_{.99}(2, 57) = 3.76$). SAC-Unpaired animals also consumed reliably less SAC than SAC-Saline animals ($q = 5.53, F_{.99}(2, 57) = 3.76$). This later finding indicates that the Unpaired group may have had a slight aversion to the SAC relative to the Saline control group.

(3.2.4.2) Reexposure Day-2

Analysis of the second Reexposure day revealed a significant Cue x Group interaction [$F(2, 108) = 115.63, p < .001$] (see Table B-18).

Subsequent analysis of the simple main effects revealed a significant difference between the three groups (Paired, Unpaired, and Saline) when SAC was the cue [$F(2, 108) = 553.81, p < .001$] but not when environment was the cue (see Table B-19). Newman-Keuls comparisons of the means (refer to row Rx-2 of Table B-2) revealed that the SAC-Paired group consumed reliably less SAC than either the SAC-Unpaired ($q = 41.66, F_{.99}(3, 57) = 4.28$) or the SAC-Saline group ($q = 39.79, F_{.99}(2, 57) = 3.76$). SAC-Unpaired animals did not differ reliably from the SAC-Saline animals ($q = 1.87, F_{.95}(2, 57) = 2.83$). These comparisons, like those obtained on Reexposure Day-1, illustrate the persistence of a conditioned flavor aversion in the SAC-Paired group.

Further analysis of the simple main effects in the 3-way interaction (see Table B-15) were performed to assess the changes in SAC consumption across Reexposure days. The Unpaired group showed a significant increase in SAC consumption from the first to the second Reexposure day [$F(2, 108) = 50.46, p < .001$], whereas subjects in the Paired and the Saline group did not change (see Table B-20; refer to rows Rx-1 and Rx-2 of Table B-1 for means).

(3.2.5) Postexposure - 24 h after CS Reexposure

For each CS Reexposure day there was Postexposure day 24 h later. A $2 \times 3 \times 2 \times 2$ (Cue x Group x Injection x Day [within factor]) repeated measures ANOVA of tap water consumption on the two Pe-1 days revealed a significant Cue x Group interaction [$F(2, 108) =$

3.38, $p < .03$] (see Table B-21). No other differences were revealed ($ps > .08$). Subsequent analysis of the simple main effects revealed a significant difference between the Paired, Unpaired, and Saline groups when SAC was the cue [$F(2, 108) = 3.23, p < .05$] but not when the environment was the cue (see Table B-22). Newman-Keuls comparisons of the combined means (refer to rows Pe-1.1 and Pe-1.2 of Table B-2) revealed that the SAC-Paired group ($M = 19.2$ ml) consumed reliably more tap water than the SAC-Saline ($M = 16.8$ ml) ($q = 3.59, F_{.95}(3, 57) = 3.40$) group. SAC-Paired animals did not differ significantly from the SAC-Unpaired group ($M = 18.1$ ml) ($q = 1.63, F_{.95}(2, 57) = 2.83$) nor did the SAC-Unpaired group differ reliably from the SAC-Saline ($q = 1.95, F_{.95}(2, 57) = 2.83$) group. These comparisons illustrate that the SAC-Paired animals consumed slightly higher amount of tap water 24 h after cue reexposure when the cue was not followed by an injection of Cy.

(3.3) Weight Changes

Table 4 summarizes the mean body weights (g) of the 6 groups on three days. The three selected days correspond to those analyzed by Frank and MacQueen (1988). A $2 \times 3 \times 2 \times 3$ (Cue \times Group \times Injection \times Day [within factor]) repeated measures ANOVA of the body weights revealed a significant Cue \times Day interaction [$F(2, 216) = 6.24, p < .002$] but not a Group effect [$F(1, 216) = 0.44, ns$] (see Table 5). Analysis of the simple main effects revealed that body weights did not

Table 4
 Mean Body Weight by Group (g)

	DAY		
	<u>Arrival</u>	<u>72h After Conditioning</u>	<u>After Reexposure</u>
Sac-Paired	238.95	380.65	414.45
Sac-Unpaired	243.37	390.95	429.05
Sac-Saline	240.98	389.05	425.10
Env-Paired	242.43	401.30	444.45
Env-Unpaired	238.44	390.20	440.99
Env-Saline	237.76	391.10	425.55

Note: Arrival = weight 24 h after placement in the colony room.
 After Conditioning = weight 72 h after Conditioning Day-3. After
 Reexposure = weight taken 24 h after Rx-2 and prior to reinitiation
 of ad lib tap water.

Table 5
 Analysis of Variance (ANOVA) on
 Weight Data

Source of Variation	df	Sum of Squares	Mean Square	F	p
Cue	1	3944.85	3944.85	3.82	.052
Group	2	912.08	456.04	.44	.643
Cue x Group	2	5950.82	2975.41	2.88	.061
Injection	1	140.25	140.25	.13	.712
Cue x Inj	1	468.54	468.54	.45	.501
Group x Inj	2	2093.45	1046.72	1.01	.365
Cue x Gr x Inj	2	2210.46	1105.23	1.07	.345
Day	2	240244.16	1201220.08	4059.34	<.001
Cue x Day	2	3697.99	1848.99	6.24	<.002
Group x Day	4	1034.70	258.67	.87	.480
Cue x Gr x Day	4	1591.79	397.94	1.34	.254
Inj x Day	2	62.89	31.48	.10	.899
C x Inj x Day	2	362.96	181.48	.61	.542
Gr x Inj x Day	4	714.57	178.64	.60	.660
C x Gr x Inj x D	4	955.09	238.77	.80	.521
Error (b)	108	111256.29	1030.15		
Error (w)	216	63917.55	295.91		

Note: Factors are Cue (C) = 2, Group (Gr) = 3, Injection (Inj) = 2, and Day (D) = 3.

differ between the two cue groups upon arrival in the laboratory [$F(1, 216) = 0.24$, ns], but that Taste-cue animals weighed significantly less than the Env-cue animals 72 h after Conditioning Day-3 [$F(1, 216) = 5.43$, $p < .025$] and 24 h after Reexposure Day-2 [$F(1, 216) = 20.15$, $p < .001$] (see Table B-23). This finding is not surprising since the observed SAC aversion would result in reduced fluid intake and weight gain. These results provide no evidence to support Krank and MacQueen's (1988) claim that animals receiving paired injections of Cy gain more weight than those receiving the drug in an unpaired manner.

Discussion

In the present results, (1) both taste and environmental CSs paired with injections of Cy produced conditioned suppression of Ab production, (2) unreinforced saline injections did not affect the magnitude of conditioning, and (3) conditioning does not aid in the resistance to Cy-induced reductions in weight gain. The conditioned suppression produced by environmental cues confirmed that conditioned changes in Ab production could be obtained without a measurable conditioned taste aversion.

Two explanations of why conditioning sometimes enhanced the immune response and sometimes had the opposite effect of suppressing it were advanced in the Introduction. One was that saline injections resulted in immunoenhancement. Animals undergoing the sequence SRBC -> SAC during the test phase of the experimental design show evidence for a conditioned immunoenhancement of Ab production (e.g., Krank & MacQueen, 1988; MacQueen & Siegel, 1989), whereas animals experiencing the sequence SRBC -> SAC -> saline display a conditioned immunosuppressive response (e.g., Ader & Cohen, 1975-1985). However, the present results show no effect of Postexposure saline injections on immune reactivity.

The second explanation for the opposing CRs is the notion of associative bias in immune conditioning. It was suggested by Krank and MacQueen (1986, 1988) that CS modality contributes to the expression of the various immunological CRs. This hypothesis is consistent with the work of Siegel and colleagues who often report that environmental CSs produce opposing, and usually compensatory, CRs to counteract the unconditional effects of pharmacological agents (see

Siegel et al., 1987). The associative bias model (Krank & MacQueen, 1988) was not supported by our data since conditioning to both environmental and taste cues produced similar agonistic conditioning.

(4.1) Environmental Data.

(4.1.1) Immune Response.

Evidence for a conditioned suppression of immune function is revealed by the lower Ab titers of the Env-Paired animals that were reexposed to the environmental cue for Cy administration. The Env-Paired group had lower levels of antibodies than animals that were either injected with saline following cue exposure (Env-Saline group) or had explicitly unpaired training with the environmental CS and Cy (Env-Unpaired group). Animals in the Env-Saline group, having experienced no immunosuppressive treatment, had Ab titers greater than the Env-cue and the Env-Unpaired group (refer to Table 2). That Env-Unpaired animals have Ab titers lower than Env-Saline animals reflects the residual effects of three Cy injections given during the conditioning phase. The unconditional suppression observed in the Unpaired animals has been reported by others (cf. Ader, 1981; Ader & Cohen, 1975, 1982; Górczynski, MacRae, & Kennedy, 1982; Krank & MacQueen, 1988; MacQueen & Siegel, 1989; O'Reilly & Exon, 1986).

The finding that animals receiving Cy injections paired with an environmental CS show immunosuppression upon subsequent CS reexposure is uncommon (see Tables 6 and 7). Krank and MacQueen (1988) and MacQueen and Siegel (1989) have instead reported that environmental cues signalling Cy administration evoke compensatory immune responses to counteract the disruptive effects of the immunosuppressive drug.

Table 6

Summary of Research Articles where the CS and the US
have been Presented Simultaneously.

	SIMULTANEOUS	
	CR = UR	CR ≠ UR COMPENSATORY
TASTE or TASTE + LiCl		
ODOR or ODOR + LIGHT		
ENV or ENV + SAC	Gorczyński (1992, exp. 1) Gorczyński et al. (1982, exp. 1) MacQueen et al. (1989, exp. 1)	Krank & MacQueen (1988, exp. 1) Krank & MacQueen (1988, exp. 2) Krank et al. (1992, exp. 1) [†] Krank et al. (1992, exp. 2) [†] MacQueen & Siegel (1989, exp. 4)
PENT		

Note: CR = conditioned response, UR = unconditioned response, LiCl = lithium chloride, ENV = environment cue, SAC = saccharin, PENT = pentobarbital. [†] = No real measure of conditioning.

Table 7

Summary of Research Articles where the CS and the US
have been Presented Sequentially.

SEQUENTIAL		
	CR = UR	CR ≠ UR COMPENSATORY
TASTE or TASTE + LiCl	<p>Ader & Cohen (1975, exp. 1, 2) Ader & Cohen (1982, exp. 1) Ader, Cohen, & Bovbjerg (1982, exp. 1) Ader, Cohen, & Grota (1979) Bovbjerg et al. (1984, exp. 1) Bovbjerg et al. (1987, exp. 1) Bovbjerg, Ader, & Cohen (1982, exp. 1) Buske-Kirschbaum, Kirschbaum, Stierle, Lehnert, & Hellhammer (1992, exp. 1) Dyck et al. (1990, exp. 1, 2) Gorczyński & Kennedy (1985, exp. 1) Gorczyński et al. (1984, exp. 1) Gorczyński et al. (1984, exp. 1, 2, 5, 6, 8) Grochowicz et al. (1991, exp. 1) Hiramoto et al. (1987, exp. 1, 2) Jenkins, Chadwick, & Nevin (1983, exp. 1, 2) Klosterhalfen & Klosterhalfen (1985, exp. 1) Klosterhalfen & Klosterhalfen (1987, exp. 1-4) Klosterhalfen & Klosterhalfen (1990, exp. 1, 2) Kusnecov et al. (1983, exp. 1) Lewis (1989, exp. 1) Lewis (1991, pilot study) McCoy et al. (1986, exp's 1-3) Neveu et al. (1987, exp's 1-3) O'Reilly & Exon (1986, exp. 1) Rogers et al. (1976, exp. 1) Roudebush & Bryant (1991, exp. 1) Solvason et al. (1988, exp. 1) Solvason et al. (1991, exp. 2) Wayner et al. (1978, exp. 1)</p>	<p>Gorczyński et al. (1984, exp. 4, 7) MacQueen & Siegel (1989, exp. 1-3)</p>

Table 7 continued

Summary of Research Articles where the CS and the US
have been Presented Sequentially.

ODOR or ODOR + LIGHT	Dark et al. (1987, exp. 1) Dyck et al. (1990, exp. 3) Ghanta, Hiramoto, Solvason, Soong, & Hiramoto (1990, exp. 1) Hiramoto et al. (1993, exp. 1) Russell et al. (1984, exp. 1) Solvason et al. (1988, exp. 2) Solvason et al. (1991, exp. 1) Spector et al. (1987, exp. 1)	Dyck et al. (1986, exp. 1)
ENV or ENV + SAC	Gorczynski & Kennedy (1984, exp. 2) Krank (1991, personal communication) Lewis (1989, exp. 2) Lysle et al. (1988, exp. 1) Sato, Flood, & Makinodan (1984, exp. 1)	
PENT		MacQueen & Siegel (1989, exp. 5) MacQueen et al. (1990, exp. 1,2)

Note: CR = conditioned response, UR = unconditioned response, LiCl =
lithium chloride, ENV = environment cue, SAC = saccharin, PENT =
pentobarbital.

Considering the variable nature of immune responses, it is likely that both immunoenhancement and immunosuppression exist and that their relative strengths depend on procedural details.

The environmental conditioning procedure used in this experiment differs from that used by other investigators studying conditioned immune responses. In this experiment, rats were removed from their suspended home cage and placed in the novel Plexiglas tube where they received tap water for 60 min. Immediately following cue exposure animals were injected i.p. with either Cy or saline and returned to their suspended home cage. Hence, the pairing order of these events was sequential. In contrast, Krank and MacQueen (1988) and MacQueen and Siegel (1989) exposed their animals to the environmental cue, removed them after 15 min exposure, injected them, and returned them to the environmental CS for an additional 45 min and 15 min, respectively. Hence, the pairing order of their conditioning regime was simultaneous. It is possible that simultaneous pairings of an environmental cue with an injection of CY favor compensatory conditioning while sequential pairings of the environmental cue with Cy favor conditioned suppression.

(4.1.2) Sequential Conditioning with Environmental or Odor CSs.

As shown in Table 7, when environmental or compound environmental + SAC CSs are paired sequentially with immunomodulatory USs, the conditioned response is overwhelmingly in the same direction as the unconditioned response. If, on the basis of considerations of the biological roles of odor and compound odor + light cues (Garcia, Hankins, & Rusiniak), one expects them to have a role similar to that

of environmental cues, such CRs are in the same direction as the UR with one exception. Dyck et al. (1986) presented mice with a compound odor + light CS paired with an injection of the immunostimulatory agent poly I:C on 4 occasions. Upon cue reexposure they observed decreased NK cell activity, a result opposite to that produced by injections of poly I:C alone. Interestingly, Dyck et al. (1990) in more recent studies using taste and odor cues paired with recombinant interleukin-1B (stimulates serum corticosterone production) have not found evidence for compensatory conditioning. Instead, they found that sequential pairings with taste aversion and odor conditioning paradigms produced CRs that mimicked the URs unconditional effects. Moreover, many groups of investigators using odor CSs with various immunomodulatory USs including poly I:C (Rogers et al., 1976; Solvason et al., 1988; Solvason et al., 1991; Spector, 1987), BSA antigen (Dark, Peeke, Ellman, Salfi, 1987; Russell et al., 1984), and spleen cells (Ghanta et al., 1987; Hiramoto, Hiramoto, Solvason, & Ghanta, 1987) have observed CRs that are in the same direction as the URs.

(4.1.3) Simultaneous Conditioning with Environmental or Odor CSs.

The data for simultaneous presentations using environmental CSs is not as clear as the data for sequential presentations using environmental or odor CSs (refer to Table 6). Krank and MacQueen (1988) and MacQueen and Siegel (1989) are the only investigators to obtain conditioned immunoenhancement to environmental CSs. Though Krank, Jacob, O'Neill, and Finley (1992) are included in Table 6, it is difficult to compare their results as their study was terminated prematurely because of unexplained deaths in 17% of their animals.

Postmortem examinations of their animals revealed that deaths may have been due to pathogen (s) introduced from a contaminated cyclosporine (Csp) injection. Their evidence for claiming a compensatory response was based not on the usual immune reactivity measure to CS reexposure, but on the finding that animals in the paired group showed little evidence of peritonitis and an enhanced ability to survive Csp injections relative to animals in the unpaired group. Because this study lacks the usual conditioned immune measures taken after CS reexposure, it is difficult to interpret and compare the results to other studies of conditioned immune responses.

Krank and MacQueen (1988) examined conditioned changes in the Ab response to SRBC in mice given Cy paired with environmental cues or with environmental cues plus a taste CS (SAC). A difference between the CS groups was not obtained, nor was a difference between groups reexposed to the CSs and saline treated mice. The Ab response of paired mice reexposed to the CS was greater than that of paired mice which were not reexposed to the CS and unpaired mice. According to Ader & Cohen (1991), these data do not actually provide evidence for a conditioned enhancement of Ab production, but allow only the inference of a compensatory mechanism. The authors also acknowledge that no direct measures of compensatory conditioning were obtained.

MacQueen and Siegel (1989, experiment 4) have claimed evidence for conditioned enhancement of the Ab response to SRBCs when conditioned rats were reexposed to an environmental CS previously paired with Cy. The methodology and results of this experiment are consistent with those of Krank and MacQueen (1988) and provide only inferential evidence for a compensatory CR (Ader & Cohen, 1991).

Interestingly, in a different study published the same year by MacQueen et al. (1989) using an environmental audio-visual CS paired with an injection of antigen (egg-albumin), the CR to CS reexposure was not compensatory. In that study, the CR was equivalent to the UR. According to a footnote in the article, the environmental audio-visual CS was based on that used in MacQueen & Siegel (1989, experiment 4). Thus, the critical difference between these two investigations by the same author is the US; in one it was Cy and in the other egg-albumin. It is not clear how in one study the CR is compensatory yet in the other the CR mimics the UR? We suggest that if compensatory CRs are valid responses in studies of immune conditioning then they should at least be consistent and relative to the unconditional effects of the US.

Two studies utilizing environmental or environmental plus SAC CSs with simultaneous presentations are provided by Gorczyński et al. (1982) and Gorczyński (1992) (see Table 6). Gorczyński et al. (1982) skin grafted mice at 40-day intervals (CS + US) or were sham-grafted (CS only). The CS in this case was the environmental cues associated with the grafting technique (handling, shaving, pentobarbital anesthetic, etc.) and the US was alloantigen only. After 3 conditioning trials all mice were sham-grafted. Animals that experienced paired CS plus US showed a conditioned increase in the cytotoxic T lymphocyte precursors (CTLp) for alloantigens of the grafted tissue. Though this CR was only observed in 50-60% of the paired group, it is evidence of the CR being in the same direction as the UR.

Only one study has reported compensatory conditioning when the

CS was an environmental or odor cue paired sequentially with an immunomodulating agent. The data for environmental or odor cues paired simultaneous with immunomodulating USs are not so consistent. Of those that have reported compensatory CRs when the CS and US are presented simultaneously it is (a) difficult to interpret and compare their results as no direct measures of immune conditioning were obtained, or (b) not entirely clear that the observed results are indeed compensatory. These problems are further confounded by the observation that the same investigator has reported evidence for both compensatory and non-compensatory conditioning when the experimental protocol is essentially the same with the exception of a different US. Finally, still other studies have reported that environmental CSs presented simultaneously with either alloantigen or physical rotation stress USs, produce CRs in the same direction as the UR (Gorczyński et al., 1984). Clearly, further research is required to assess the importance of sequential and simultaneous pairings on the direction of the CR to environmental cues.

(4.1.4) Fluid Consumption.

Additional evidence of conditioning was not revealed in the tap water consumption of the Env-cue rats. On each Baseline day, rats drank comparable amounts of tap water (refer to Tables B-1 and B-2). The first Conditioning day revealed a slight reduction in tap water consumption for all Env-cue rats, and tap water consumption was reduced in the Env-paired animals throughout all exposures to tap water in the novel Plexiglas tubs. Over the course of conditioning, the three groups of Env-cue rats did not show any significant changes

in tap water consumption. Hence, no measurable conditioning effects were revealed through tap water consumption in the Env-Paired animals. These results are consistent with the suggestion that conditioned taste aversions are not necessary for the production of conditioned immunosuppressive responses.

In all groups, injections of Cy reduced fluid consumption 24 h, but not 48 h, later. This robust effect is attributed to the gastrointestinal toxicity induced by Cy and has not been reported previously in studies with rats. This behavioral measure parallels a toxicological finding that showed complete elimination of Cy in the urine samples of rats 24 h after intravenous treatment at a dose of 1 mg/kg⁻¹ (Sessink, van den Broek, & Bos, 1991). A similar rate of elimination is observed in humans; nausea and vomiting occur about 2-6 h after drug administration and lasts less than 24 h (Chabner, Myers, & Oliverio, 1977).

The absence of a tap water aversion was expected since conditioned aversions to tap water are obtained only after unusual training procedures are employed (cf. Elkins, 1974). In addition, given the animals previous experience with tap water during the habituation phase, it is unlikely that tap water would possess sufficient saliency to form an association with Cy. It is not possible to compare these findings with those of other investigators that have assessed environmental conditioning. In fact, no degree of toxicity can be ascertained in MacQueen and Siegel's (1989, experiment 4) study because water was freely available throughout the experiment. Frank and MacQueen (1988) do not report tap water consumption.

(4.2) Taste Aversion Dissociation Literature.

That Env-cue animals show no evidence for a conditioned aversion to tap water is consistent with the suggestion that the conditioning of immunopharmacological effects does not depend on taste aversion learning (Ader & Cohen, 1981; Bovbjerg, Ader, & Cohen, 1984; Klosterhalfen & Klosterhalfen, 1987, 1990). Still, some investigators (Cunningham, 1985; Kelley, Dantzer, Mormede, Salomon, & Aynaud, 1985) have argued that conditioned immunosuppressive responses are the result of stresses arising from a taste aversion procedure. Our data (1) supplement the dissociation literature by showing that a conditioned immune response is possible without observing a conditioned taste aversion, and (2) question the associative bias model (Krank & MacQueen, 1988) insofar as the model relies on taste aversions to explain immunosuppressive CRs.

Though the effects of conditioning are revealed in the Ab titers of the Env-cue animals, a second measure of conditioning would be useful. Since conditioned aversions to tap water are (a) difficult to obtain, (b) independent of conditioned immune changes, and (c) probably unrelated to environmental CSs, we recommend that future studies include a measure of place preference/avoidance to the environmental cue.

(4.4) Flavor Data:

(4.4.1) Immune Response.

Evidence for a conditioned suppression of immune function is revealed by the lower Ab titers of the SAC-Paired animals that had SAC consumption paired with an injection of Cy. SAC-Paired

animals had lower Ab titers than animals that were either injected with saline following cue exposure (SAC-Saline group) or had explicitly unpaired training with SAC and Cy (SAC-Unpaired group). The SAC-Saline animals, having experienced no immunosuppressive treatment, had higher Ab titers than the SAC-Unpaired group, which received Cy 24 h after cue presentation, and the SAC-Paired group (refer to Table 2). The unconditioned suppression observed in the SAC-Unpaired group is similar to that observed in the Env-Unpaired group.

Establishing reliable baseline titers in animals not exposed to Cy injections is critical to the interpretation of the CR. Some investigators have reported compensatory CRs as a function of low baseline effects. In this study, both SAC-saline and Env-Saline groups showed similar mean titers (3.9 and 4.3, respectively) that do not differ significantly from those of Krank and MacQueen (1988) and MacQueen and Siegel (1989). In fact, our baselines are more conservative than those previously reported yet we are still able to observe conditioned suppression in paired group animals.

(4.4.2) Sequential Conditioning with Taste or Odor CSs.

Conditioned suppression of Ab production is consistent with most investigations that have paired a taste CS with an injection of Cy (see Table 7). Two exceptions to this highly reproducible effect (cf. Gorczyński et al., 1984; experiments 4 and 7; MacQueen and Siegel, 1989; experiments 1-3) have reported that the CR to a SAC taste cue is a compensatory enhancement of immune activity. It is difficult to accept these reports given the wealth of consistent data to the

contrary. It is noteworthy, however, that the conditioned compensatory effect observed by Gorczynski et al. (1984) was not reliable, and was attributed to the level of exogenous stress imposed during conditioning. It is possible that increased stress could have initiated the release of corticosterone (cf. Flores et al., 1990; Lysle et al., 1990) and in turn an increase in natural killer cell production, but it was not evident from the reported methodology that experiments 4 and 7 (Gorczynski et al., 1984) were more stressful than the other experiments.

MacQueen and Siegel (1989; experiments 1-3) also obtained a compensatory CR to a SAC taste CS previously paired with Cy. Ader and Cohen (1991) have stated that these studies, like Krank and MacQueen's (1988), permit only the inference of an enhanced response on the basis of the failure to observe immunosuppression. Ader and Cohen suggest that these studies are questionable because of (a) the absence of a group to define unconditional Cy effects in some experiments, (b) the observation of a compensatory response following a single CS-US pairing, and (c) the compensatory effect observed after a single pairing was not modified by multiple conditioning trials.

(4.4.3) Simultaneous Conditioning with Taste or Odor CSs.

As shown in Table 6, our review of the literature did not reveal any studies that presented taste cues and immunomodulating USs simultaneously.

(4.4.4) Fluid Consumption.

Additional evidence of conditioning to SAC was found in the

fluid consumption data of the Taste-cue rats. On each Baseline day, animals drank comparable amounts of tap water (refer to Tables B-1 and B-2). The first Conditioning day revealed a reduction of fluid consumption when SAC replaced the usual tap water. Over the course of conditioning, consumption of SAC by the SAC-Saline and the SAC-Unpaired animals increased and stabilized, but consumption of SAC decreased in the SAC-Paired group following a single injection of Cy. On both CS Reexposure days the SAC-Paired group drank less SAC than either the SAC-Unpaired or the SAC-Saline animals. This observation confirms that Cy administration supports a conditioned taste aversion. Consistent with the Env-cue animals, injections of Cy reduced fluid consumption 24 h, but not 48 h, later.

The acquisition of a conditioned SAC aversion is consistent with the findings of other investigators (cf. Adar & Cohen, 1975, 1965; Bobbjerg, Kim, Siskind, & Weksler, 1987; Krank & MacQueen, 1988; MacQueen & Siegel, 1989). The absence of extinction of the aversion contrasts with the results obtained by MacQueen and Siegel (1989; experiments 1 and 2). They reported that rats in the paired groups displayed extinction of their SAC aversion on the second reexposure day. This difference may have occurred because, in their experiment, rats that acquired a SAC aversion were not given additional access to drinking water. Given the rats dehydrated state, consumption of SAC may have been inevitable.

(4.5) Weight Changes.

Analysis of the weight data did not reveal any effect of conditioning with Cy. This result is consistent with that reported by

MacQueen and Siegel (1989; experiments 4 and 5) who provided their animals with free access to food and tap water throughout the experiment.

In contrast, Krank and MacQueen (1988) found that an additional compensatory effect of the environmental signal for Cy was present in the weight changes among groups. Specifically, they observed that mice receiving Cy gained less weight than saline control animals unless the Cy was administered in a paired manner. They suggested that weight gain is less affected by signalled injections of Cy, than by unsignalled injections, because the cumulative effect of compensatory responses allows the animal to better cope with the drug's adverse impact. Moreover, Krank, Hinson, and Siegel (1984) reported a similar conditioned weight gain effect when morphine was the US. In both of the above investigations, tap water and food availability were limited to 1 h access 48 h prior to each conditioning trial. Ad lib access was reinstated following the completion of each conditioning trial.

One procedural difference that may account for the weight change observed by Krank and MacQueen (1988) is that the mice in their experiment were never adapted to a fluid deprivation schedule. Rather, food and water were available ad lib until 2 days prior to the start of a conditioning trial. On these occasions, water was available for only 1 h per day. At the completion of each conditioning trial all animals were returned to the home cages and given water. Our animals received predictable access to water each day: at the time of cue exposure and between 2-4 h later that day. Similarly, water access was predictable in MacQueen and Siegel's

(1989) experiments (4, 5): it was continuous. The relationship between the water schedule and weight data is unclear, though our failure to find a compensatory weight gain may be masked by all of the additional water that our animals and the animals of MacQueen and Siegel (1989) received each day. It is also possible that the slightly harsher deprivation regime used by Krank & MacQueen (1988) may have allowed a demonstration of an effect of conditioning on weight gain.

(4.6) General Summary

Two explanations were advanced in the introduction to account for the discrepant results found in studies of conditioned immune response. In the results presented here, the role of Postexposure saline injections and CS modality were not significant factors affecting the direction of the CR. It was then suggested, based of the different conditioning regimes used with environmental cues, that simultaneous pairings of an environmental cue with an injection of Cy favor compensatory conditioning while sequential pairing of an environmental with Cy favor conditioned suppression. Following a review of the available literature (refer to Tables 6 and 7), it was determined that both taste and environmental CSs paired sequentially with immunomodulating agents produce CRs that are almost exclusively in the same direction as the unconditioned response. The data for simultaneous presentations are not so clear, and explanations as to why compensatory conditioning was observed are discussed.

Future studies of conditioned immune response need to employ strict conditioning regimes that allow fair comparisons across CS

types and methods of presentation. In addition, we suggest measuring a behavioral factor such as conditioned place aversion to further confirm the effects of conditioning with environmental CSs. If these experiments were conducted again we would include in addition to the groups used here (1) a taste + Cy group paired simultaneously, (2) an environmental + Cy group paired simultaneously, and (3) an environmental + Cy group paired simultaneously and tested for a conditioned place preference or aversion.

Note 1:

Various drugs suppress T-cell mediated immunity; alkylating drugs, such as cyclophosphamide, disrupt DNA synthesis and prevent cellular proliferation necessary to mount immunological defense. This effect is quite general extending to virtually all forms of immunological reactions and, indeed to the replication of cells outside the immune system. Moreover, alkylating agents induce nausea, vomiting and dizziness.

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APPENDIX A

Sequence and Distribution of Experimental Events

Since it is not possible to train or test 120 animals on any given day, the following is provided as an addendum to the collapsed description given in the Methods section. Prior to Day 1, there was a 14 day adjustment period to the colony room followed by six days of habituation to the restricted water schedule. Under Event heading the numbers in brackets correspond to the animals trained on that day. A table outlining the randomization of group assignment by number follows the sequence of events. Under the heading Start Time, the time at which 24 animals were to begin allocation for cue exposure is indicated. Available time from 0930 h until the designated Start Time was used to place water on the cages of animals trained 24 h earlier. Similarly, time following Start Time + 12 min was used to place tap water on the cages of the remaining rats. Thus, each of five sets of 24 rats received the experimenter's attention for 12 min or 30 sec/rat. This schedule was strictly adhered to ensure that each animal received only 60 min of cue exposure and/or water each day. Following the recovery period, SRBC inoculations were given beginning at 0830 h. Times after the slash signify the onset of the usual training time. Rx1 = first reexposure to cue and Rx2 = second reexposure to cue. Reinstatement of ad lib water always occurred 24 h prior to euthanasia.

<u>Day</u>	<u>Event</u>		<u>Start Time</u>
Day 1	Train	(1-24)	0930 h
Day 2	Train	(25-48)	0942 h
Day 3	Train	(49-72)	0954 h
Day 4	Train	(73-96)	1006 h
Day 5	Train	(97-120)	1018 h
Day 6	water only		0930 h
END OF CONDITIONING TRIAL 1			
Day 7	Train	(1-24)	0930 h

Day 8	Train	(25-48)	0942 h
Day 9	Train	(49-72)	0954 h
Day 10	Train	(73-96)	1006 h
Day 11	Train	(97-120)	1018 h
Day 12	water only		0930 h

END OF CONDITIONING TRIAL 2

Day 13	Train	(1-24)	0930 h
Day 14	Train	(25-48)	0942 h
Day 15	Train	(49-72)	0954 h
Day 16	Train	(73-96)	1006 h
Day 17	Train	(97-120)	1018 h
Day 18	water only		0930 h

END OF CONDITIONING TRIAL 3

14 DAY RECOVERY PERIOD

Day 28	SRBC + Rx1	(1-24)	0830 h/ 0930 h
Day 29	SRBC + Rx1	(25-48)	0830 h/ 0942 h
Day 30	SRBC + Rx1	(49-72)	0830 h/ 0954 h
Day 31	SRBC + Rx1	(73-96)	0830 h/ 1006 h
	Rx2	(1-24)	0930 h
Day 32	SRBC + Rx1	(97-120)	0830 h/ 1018 h
	Rx2	(25-48)	0942 h
Day 33	Rx2	(49-72)	0954 h
	ad lib water	(1-24)	1130 h
Day 34	Rx2	(73-96)	1006 h
	Euthanasia	(1-24)	1130 h
	ad lib water	(25-48)	1130 h
Day 35	Rx2	(97-120)	1018 h
	Euthanasia	(25-48)	1130 h
	ad lib water	(49-72)	1130 h
Day 36	Euthanasia	(49-72)	1130 h
	ad lib water	(73-96)	1130 h
Day 37	Euthanasia	(73-96)	1130 h
	ad lib water	(97-120)	1130 h
Day 38	Euthanasia	(97-120)	1130 h

Table A-1
Randomization of Groups with Animal Number by Conditioning Day

<u>Day 1</u>	<u>Animal #</u>	<u>Day 4</u>	<u>Animal #</u>
Sac-Paired	1,2,3,4	Env-Paired	73,74,75,76
Sac-Unpaired	5,6,7,8	Env-Unpaired	77,78,79,80
Sac-Saline	9,10,11,12	Env-Saline	81,82,83,84
Env-Paired	13,14,15,16	Sac-Paired	85,86,87,88
Env-Unpaired	17,18,19,20	Sac-Unpaired	89,90,91,92
Env-Saline	21,22,23,24	Sac-Saline	93,94,95,96
<u>Day 2</u>		<u>Day 5</u>	
Sac-Unpaired	25,26,27,28	Env-Unpaired	97,98,99,100
Sac-Saline	29,30,31,32	Env-Saline	101,102,103,104
Env-Paired	33,34,35,36	Sac-Paired	105,106,107,108
Env-Unpaired	37,38,39,40	Sac-Unpaired	109,110,111,112
Env-Saline	41,42,43,44	Sac-Saline	113,114,115,116
Sac-Paired	45,46,47,48	Env-Paired	117,118,119,120
<u>Day 3</u>			
Sac-Saline	49,50,51,52		
Env-Paired	53,54,55,56		
Env-Unpaired	57,58,59,60		
Env-Saline	61,62,63,64		
Sac-Paired	65,66,67,68		
Sac-Unpaired	69,70,71,72		

Note: Paired = Cyclophosphamide Paired. Env = Environment. Sac = Saccharin. With the above randomization, time-of-day variations were strictly counterbalanced over experimental days.

Table B-1
 Mean Fluid Consumption (ml) of Groups over Days
 during the Conditioning Phase

DAY	Sac-Paired	Sac-Unpair	Sac-Saline	Env-Paired	Env-Unpair	Env-Saline
Baseline-1	19.17	19.56	18.74	19.85	20.22	19.18
Cond-1	17.20	16.59	16.89	11.82	10.42	11.35
PC-1.1	15.93	19.05	17.83	15.88	18.65	19.55
PC-2.1	17.88	14.11	17.74	19.21	15.75	17.89
Baseline-2	18.67	19.59	18.33	19.88	19.42	19.97
Cond-2	7.91	19.61	22.62	13.20	12.81	13.57
PC-1.2	13.31	19.12	17.89	15.88	19.47	19.05
PC-2.2	19.17	14.23	17.59	19.20	14.03	19.30
Baseline-3	19.65	20.36	19.32	19.95	20.71	20.59
Cond-3	2.42	23.63	23.24	14.10	13.95	14.47
PC-1.3	14.77	18.59	18.24	14.64	18.26	19.60
PC-2.3	17.71	14.68	17.15	16.64	14.21	18.57

Note: Baseline - (1, 2, 3) = Fluid consumption 24 h prior to conditioning days 1, 2, and 3, respectively.
 Cond = Conditioning day. PC-1 = postconditioning day one. PC-2 = postconditioning day two. The number
 preceding the decimal on PC days indicates the conditioning trial to which the data belong (e.g., PC-1.1 is
 the data for day PC-1 of the first conditioning trial).

Table B-2
Mean Fluid Consumption (ml) of Groups over Days
during the CS Reexposure Phase

<u>DAY</u>	<u>SAC-PAIRED</u>	<u>SAC-UNPAIR</u>	<u>SAC-SALINE</u>	<u>ENV-PAIRED</u>	<u>ENV-UNPAIR</u>	<u>ENV-SALINE</u>
Baseline-4	17.92	18.14	18.07	17.60	19.98	18.08
RX-1	1.35	19.72	22.88	13.39	14.29	14.31
Pe-1.1	19.77	18.36	17.22	17.44	17.43	17.71
Baseline-5	18.63	17.45	17.98	17.25	18.55	19.10
RX-2	1.63	25.46	24.39	12.83	12.91	14.51
Pe-1.2	18.77	17.98	16.50	17.07	17.26	17.95
Baseline-6	17.68	19.21	17.28	17.19	17.94	18.88

Notes: Baseline - (4, 5, 6) = fluid consumption 24 h prior to Rx-1, Rx-2, and the day of the immune assay, respectively. Pe-1 = postexposure day one. The number preceding the decimal on Pe days indicates the reexposure day to which the data belong (e.g., Pe-1.1 is the data for day Pe-1 of the first reexposure trial).

Table B-3
 Analysis of Variance (ANOVA) on Tap Water Consumption
 on the Six Baseline Days

Source of Variation	df	Sum of Squares	Mean Square	F	P
Cue	1	51.16	51.16	1.64	.202
Group	2	49.96	24.98	0.80	.451
Cue x Group	2	42.97	21.48	0.68	.503
Injection	1	1.79	1.79	0.05	.810
Cue x Inj	1	63.88	63.88	2.05	.155
Group x Inj	2	198.59	99.29	3.18	<.045
Cue x Gr x Inj	2	143.55	71.77	2.30	.104
Day	5	407.09	81.41	10.14	<.001
Cue x Day	5	19.38	3.87	0.48	.789
Group x Day	10	51.37	5.13	0.64	.779
Cue x Gr x Day	10	90.20	9.02	1.12	.341
Inj x Day	5	85.61	17.12	2.13	.060
C x Inj x Day	5	18.25	3.65	0.45	.809
Gr x Inj x Day	10	105.60	10.56	1.31	.218
C x Gr x Inj x D	10	118.59	11.85	1.47	.144
Error (b)	108	3363.72	31.14		
Error (w)	540	4334.44	8.02		

Note: Factors are Cue (C) = 2, Group (Gr) = 3, Injection (Inj) = 2, and Day (D) = 6.

Table B-4
 Partitioning of the Group x Injection interaction
 obtained on the Six Baseline Days

Source of Variation	df	Sum of Squares	Mean Square	F	p
Group at No Saline Injection	2	170.71	85.35	10.63	<.001
Group at Saline Injection	2	77.81	38.90	4.84	<.01
Error (w)	540	4334.44	8.02		

Fc (2, 540) = 6.91, p <.001

Fc (2, 540) = 4.66, p <.01

Table B-5
 Newman-Keuls Pairwise Tests of the Group x Injection
 interaction obtained on the Six Baseline Days

	Paired-N	Saline-Y	Unpaired-N	Unpaired-Y	Paired-Y	Saline-N
Paired-N	_____	1.59 (2, 114)	5.24 ** (3, 114)	5.29 ** (4, 114)	5.57 ** (5, 114)	5.96 ** (6, 114)
Saline-Y		_____	3.64 (2, 114)	3.69 * (3, 114)	3.97 * (4, 114)	4.37 * (5, 114)
Unpaired-N			_____	.050 (2, 114)	.320 (3, 114)	.724 (4, 114)
Unpaired-Y				_____	.279 (2, 114)	.670 (3, 114)
Paired-Y					_____	.395 (2, 114)
Saline-N						_____

Note: Group-N = denotes subgroups that do not receive a saline injection following CS reexposure. Group-Y = denotes subgroups that receive a saline injection following CS reexposure. Numbers in brackets below obtained Studentized range Q are df. The first number, r, refers to the number of steps between given means while the second value is based on $p(n-1) = 6(20-1)$.

* $p < .05$

** $p < .01$

Table B-6
 Analysis of Variance (ANOVA) on Fluid Consumption
 of the Three Conditioning Days

Source of Variation	df	Sum of Squares	Mean Square	F	p
Cue	1	1316.37	1316.37	46.82	<.001
Group	2	2450.79	1225.39	43.59	<.001
Cue x Group	2	2659.31	1329.65	47.30	<.001
Injection	1	14.48	14.48	0.51	.474
Cue x Inj	1	38.14	38.14	1.35	.246
Group x Inj	2	29.51	14.75	0.52	.593
Cue x Gr x Inj	2	22.34	11.17	0.39	.673
Day	2	101.06	50.53	4.24	.015
Cue x Day	2	181.32	90.66	7.61	<.001
Group x Day	4	1775.53	443.88	37.50	<.001
Cue x Gr x Day	4	1449.80	362.45	30.46	<.001
Inj x Day	2	11.31	5.65	0.47	.622
C x Inj x Day	2	29.88	14.94	1.25	.286
Gr x Inj x Day	4	21.35	5.33	0.44	.773
C x Gr x Inj x D	4	10.77	2.69	0.22	.923
Error (b)	108	3035.99	28.11		
Error (w)	216	2570.20	11.89		

Note: Factors are Cue (C) = 2, Group (Gr) = 3, Injection (Inj) = 2, and Day (D) = 3.

Table 10
 Analysis of Variance (ANOVA) on Fluid Consumption
 on Conditioning Day One

Source of Variation	df	Sum of Squares	Mean Square	F	p
Cue	1	972.99	972.99	47.22	<.001
Group	2	20.61	10.30	0.50	.607
Cue x Group	2	3.44	1.72	0.08	.920
Injection	1	9.35	9.35	0.45	.501
Cue x Inj	1	2.72	2.72	0.13	.716
Group x Inj	2	13.72	6.86	0.33	.717
Cue x Gr x Inj	2	4.58	2.29	0.11	.894
Error	108	2225.26	20.60		

Note: Factors are Cue = 2, Group (Gr) = 3, and Injection (Inj) = 2.

Table 11
 Analysis of Variance (ANOVA) on Fluid Consumption
 on Conditioning Day Two

Source of Variation	df	Sum of Squares	Mean Square	F	p
Cue	1	371.71	371.71	19.79	<.001
Group	2	1250.52	615.26	32.77	<.001
Cue x Group	2	1189.55	594.77	31.67	<.001
Injection	1	0.24	0.24	0.01	.909
Cue x Inj	1	64.23	64.23	3.42	.067
Group x Inj	2	36.33	18.16	0.96	.383
Cue x Gr x Inj	2	5.13	2.56	0.13	.872
Error	108	2027.66	18.77		

Note: Factors are Cue = 2, Group (Gr) = 3, and Injection (Inj) = 2.

