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# Behaviorally Conditioned Suppression of Murine T-Cell Dependent but not T-Cell Independent Antibody Responses

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SCHULZE, G. E., R. W. BENSON, M. G. PAULE AND D. W. ROBERTS. *Behaviorally conditioned suppression of murine T-cell dependent but not T-cell independent antibody responses.* PHARMACOL BIOCHEM BEHAV **30**(4) 859–865, 1988.—The aversive and immunosuppressive effects of cyclophosphamide (CY, 250 mg/kg IP), an unconditioned stimulus (UCS), were paired with the presentation of a novel saccharine flavored drinking solution (SAC), a conditioned stimulus (CS), in female Balb/c mice. The objective was to determine the temporal relationship between presentation of the CS (SAC) and immunization with sheep red blood cell (SRBCs), a T-cell dependent antigen, and type III pneumococcal polysaccharide (S3), a T-cell independent antigen, on subsequent antibody responses. Reexposure to the CS or UCS occurred on days −4, −2, 0, +2, or +4 relative to immunization. Primary antibody responses in each group were measured six days following immunization. A strong association between the CS and the UCS developed, producing flavor aversions as evidenced by decreased SAC consumption. CY administration by itself consistently suppressed both types of antibody responses. CS presentation (i.e., SAC) had no significant effect on anti-S3 antibody response. However, the anti-SRBC response was significantly depressed following CS exposure. Exposure to the CS only on days −4 or +2 relative to immunization resulted in statistically significant suppression of antibody response to SRBC's while exposure on days −2, 0, and +4 resulted in anti-SRBC antibody suppression that did not reach significance. These results support the hypothesis that conditioning of antibody responses is relatively specific for T-cell dependent antigens, and that the timing of CS presentation relative to immunization is important in conditioning a suppression of antibody responses.

Behaviorally conditioned immune suppression T-cell antibody responses Cyclophosphamide

CENTRAL nervous system (CNS) modulation of immune function has been consistently demonstrated [1,7]. Immune responses appear to be influenced by CNS processes through both afferent and efferent pathways [13]. During immunization, for example, increased electrical activity in the rat ventromedial hypothalamic nuclei is reported to occur [8]. Similarly, immunosuppression can be induced by anterior hypothalamic lesions [29]. Furthermore, functional sympathetic enervation of the thymus and spleen was suggested when 6-hydroxydopamine and alpha-methyltyrosine administration resulted in enhanced immune response to sheep red blood cells (SRBC) [29]. The existence of an immune-neuroendocrine network has been postulated and would bring the concept of a self-regulated immune system into conformity with other known CNS-influenced bodily functions [8].

Classical conditioning of the immune system was initially demonstrated by Ader and Cohen [2]. Classical conditioning is the process by which an initially neutral stimulus termed the conditioned stimulus (CS), is paired with a nonneutral stimulus termed the unconditioned stimulus (UCS). When a UCS is presented it results in an unconditioned response (UCR). Following the initial pairing of the CS with the UCS, the CS itself acquires some of the reaction-eliciting potential of the UCS, resulting in a conditioned response (CR) that resembles the original UCR. In the studies of Ader and Cohen [2], presentation of saccharine drinking solutions (the CS) were paired with a constant immunosuppressive dose of cyclophosphamide (the UCS) with the resultant immunosuppression being the UCR. Following a single pairing of the CS and UCS, presentation of the saccharine drinking solution alone (CS) resulted in significant immunosuppression (CR). Furthermore, this conditioned immune response was shown to extinguish, (i.e., wane) following several presentations of the CS alone [9]. The types of immune responses shown to be conditionable are numerous and include humoral responses [1,31], cellular responses [21], graft versus host responses [9], alterations in rate of tumor growth

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TABLE 1
EXPERIMENTAL PROTOCOL

| Group (49–50 mice each) | Days After Conditioning (Test Day Relative to Immunization) 0 Conditioning Day | 15 (−4) Test 1 | 17 (−2) Test 2 | 19 (0) Test 3 | 21 (+2) Test 4 | 23 (+4) Test 5 | 25 (+6) Sample |
|---|---|---|---|---|---|---|---|
| Conditioned (C) | SAC + CY | $C_1$ | $C_2$ | $C_3$ | $C_4$ | $C_5$ | ALL |
| Placebo (P) | SAC + SAL | $P_1$ | $P_2$ | $P_3$ | $P_4$ | $P_5$ | ALL |
| Unconditioned (U) | SAC + CY | $U_1$ | $U_2$ | $U_3$ | $U_4$ | $U_5$ | ALL |
| Residual (R) | $H_2O$ + CY | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | ALL |

Subscripts: Identify subgroups (n=9–10) of separate animals given test day treatments.
$H_2O$=Presentation of drinking water.
SAC=Presentation of 0.7% saccharin solution.
SAL=Saline injection (IP).
CY=Cyclophosphamide injection (250 mg/kg IP).
Test day treatments: $C_x$=SAC + SAL; $U_x$=$H_2O$ + CY; $P_x$=SAC + SAL; $R_x$=$H_2O$ + SAL.

[13], and the severity of autoimmune diseases [3,20]. Interest in external factors associated with the immunological effects induced by exogenous compounds is increasing [22]. If human prescribed, accidental, or recreational exposure to immunosuppressants can result in similar conditioned immunosuppression, then it is possible that susceptibility to infection and/or carcinogenicity could be influenced by behavioral factors.

The unconditioned effects of immunosuppressive drugs are frequently dependent on the time of their administration relative to immunization. For example, the effect of cyclophosphamide (CY) on antibody production is quite different when the drug is given two to four days before immunization than when given at the time of immunization [19,30]. Here, we address the temporal relationship between stimuli presentation and immunization with respect to optimizing the conditioned response. Additionally, we explore the CS effects on both T-cell dependent and T-cell independent processes. Specifically, the present studies were designed to explore the temporal relationship between presentation of the CS (SAC) and immunization with respect to primary antibody responses in saccharine/cyclophosphamide conditioned Balb/c mice, and the relative efficacy of SRBC, a T-cell dependent antigen [31], and type III pneumococcal polysaccharide, a T-cell independent antigen [16], in demonstrating this CR.

## METHOD

### *Animals*

One hundred ninety-eight female Balb/c mice (Charles Rivers Canada, St. Constant, Quebec, Canada) were used as subjects. The mice were six weeks of age when received and were quarantined for two weeks upon arrival. The mice were individually identified by placing sequentially numbered metal tags (National Band & Tag Co., Newport, KY) on the nuchal region of each animal. Subsequently, animals were randomly assigned to four treatment groups (49–50 per group), and each of these groups was further subdivided into five subgroups (9–10 mice per subgroup). Table 1 summarizes the treatment groups and subgroups. Animals were singly housed under a 12 hour light-dark cycle (lights on at 0600), with controlled temperature and humidity of 25±2°C and 50±10% respectively.

### *Drugs*

Cyclophosphamide monohydrate (CY, Sigma Chemical Co., St. Louis, MO; 99% purity) was dissolved in sterile physiological saline at a concentration of 25 mg/ml and administered at a dose of 250 mg/kg by IP injection in a volume of 0.01 ml/g. The purity of cyclophosphamide was found to be 99% as determined by reversed phase HPLC/UV using a mobile phase of acetonitrile/water:30/70 and flow rate of 1.5 ml/min. Sterile pyrogen-free physiological saline (Lot No. 72110 Travenol Laboratories Inc., Deerfield, IL) served as vehicle control. Sodium saccharine (Fluka Chemical Corp., Ronkonkoma, NY) was dissolved in tap water at a concentration of 0.7% (w/v) and delivered via modified pipette drinking tubes. The dose of CY was chosen because it is known to produce maximal immune suppression and taste aversion in mice [22,24]. The concentration of saccharine was chosen based on reports which indicated lower concentrations were ineffective as a CS in taste aversion procedures [19]. SRBC's (The Brown Laboratory, Topeka, KA) were washed three times with sterile physiological saline and then diluted to a final volume of $2.24\times10^9$ cells/ml and injected IP at a volume of 0.2 ml/mouse. Polyvalent Pneumovax 23 (Merck Sharp & Dohme, West Point, PA) containing 50 μg capsular type polysaccharide per ml was diluted in sterile saline to a final volume of 2.5 μg/capsular type/ml and injected IP at a volume of 0.2 ml/mouse. The resultant dose, 0.5 μg S3, was previously shown to be the optimal immunizing dose in Balb/c mice based on kinetic analysis of the anti-S3 antibody response [27].

### *Conditioning Procedure*

Conditioning and test drug administration schedules are outlined in Table 1. All experimental manipulations were carried out between 1300–1430 hours. A gradual water deprivation schedule was employed for five days prior to conditioning, such that the animals were trained to fulfill their fluid

requirements for a 24 hour period during a single 20 min period of water availability. Food was available ad lib. Water was delivered in modified 10 ml plastic serological pipettes graduated in 0.1 ml increments and fitted with metal sipper tubes. On the conditioning day, group C (conditioned) received a 20 min exposure to a 0.7% saccharine drinking solution followed 30 minutes later by a single IP injection of CY (250 mg/kg). Group P (placebo) received the same saccharine exposure followed 30 min later by a saline injection. Group R (residual) received a 20 minute exposure to water followed by a CY injection. Group U (unconditioned) was treated similar to Group C. Following a two week recovery period, subgroups of these groups each received a second CS or UCS exposure on days −4, −2, 0, +2, and +4 relative to antigen administration (immunization). On these test days, behavioral drinking tests were given; Cx (conditioned) and Px (placebo) subgroups were reexposed to the saccharine solution for 20 minutes. Ux (unconditioned) and Rx (residual) subgroups received only water for 20 min. Thirty minutes later, Ux (unconditioned) subgroups received CY (250 mg/kg IP) while all other subgroups received saline IP. Drinking volume was monitored throughout the study.

*Immunization*

Mice were immunized with 0.5 μg/mouse IP of the T-cell independent immunogen Type III pneumococcal polysaccharide (S3) included as one of the compoments in Pneumovax and $4.5\times10^8$ SRBCs, a T-cell dependent immunogen given IP. The day of immunization was designated 'day 0' with conditioning thus being day −19. Testing for the presence of conditioned taste aversion responses (i.e., decreased SAC consumption) occurred on days −4, −2, 0, +2, and +4. Testing for conditioned immunosuppression occurred at bleeding (day +6). The time for assessing immune response (day +6) was chosen based on kinetic analysis of the primary anti-SRBC and anti-S3 antibody response which generally peaks around six days post-immunization [15, 16, 27]. Mice were bled between 0900–1100 from the orbital sinus while under light carbon dioxide anesthesia. Blood was collected in plastic microfuge tubes containing 0.2 ml Sure-Sep (General Diagnostics, Morris Plains, NJ), allowed to clot at 4°C for three hours, and centrifuged. The serum was transferred to a clean tube, and frozen (−70°C) until analysis.

*Immunological Assays*

*Radioimmunoassay for serum antibody to S3.* Serum antibodies to S3 were determined for all groups six days after immunization by a radioimmunoassay method developed by Schiffman and Austrian [26], conducted as described by Benson and Roberts [6] utilizing $^{14}C$ labeled Type III pneumococcal polysaccharide and standard antisera (Trustees Research Foundation, Brooklyn, NY). Each assay included control tubes containing aliquots of reference antisera for constructing standard curves as well as tubes containing labeled antigen only. All samples were assayed in duplicate and nanograms of antibody nitrogen per ml (ng AbN/ml) were calculated using the standard curve for that assay.

*Quantitation of antibody to SRBC by complement-mediated hemolysis.* Mouse antisheep red blood cell (SRBC) antibody was quantitated in serum obtained 6 days after immunization by a standard hemolysis assay [15] using the following modification: Serum samples were heat inactivated at 56°C for 30 minutes to destroy endogenous complement and diluted either 1:400 (groups C,R, and P) or 1:60 (group U). SRBCs used as target cells in the assay (The Brown Laboratory, Topeka, KA) were washed 4× in pyrogen free isotonic saline and resuspended in saline at a concentration of 2.5% v/v. Lyophilized guinea pig complement (Pell-Freeze, Rogers, AR) was rehydrated immediately before use, diluted 1:25 in saline, and kept at 4°C. Hemolysis assays were performed in 12×75 borosilicate glass tubes by sequential addition of 0.4 ml of 2.5% SRBC suspension, 0.4 ml of appropriate dilution of unknown or standard antiserum, and 0.4 ml of 1:25 guinea pig complement. Standard antisera consisted of a heat inactivated pool prepared by combining 25 μl from each of the immunized placebo (group P) animals. The standard curve consisted of serial 2-fold dilutions of standard antisera from 1:200 to 1:2097 assayed in the same manner as the unknown samples. Control tubes were identical to assay tubes except that 0.4 ml of 0.7% nonionic detergent (Nonidet P-40, Bethesda Research Laboratories, Rockville, MD) was used in place of serum to determine 100% lysis, and saline was used in place of serum to determine background lysis. All tubes were mixed and incubated at 37°C for 45 minutes prior to being centrifuged at 300×g for ten minutes at 4°C to pellet cellular debris and unlysed SRBC. Duplicate 250 μl samples of each supernatant were transferred to optical quality 96-well polystyrene assay plates (Nunc Immuno-Plate I, Vangard International, Inc., Neptune, NJ), and the optical density of each well was read on a MR 600 spectrophotometer (Dynateck Laboratories, Inc., Alexandria, VA) in the dual wavelength mode using a 570 nm sample filter and a 630 nm reference filter. Data were collected on an interfaced Apple 2e computer (Apple Computer Inc., Cupertino, CA) and analyzed using software developed in our laboratory which fits a 3° polynomial to the standard curve data plotted as % hemolysis vs. $\log_2$ dilution of standard. In order to avoid problems inherent with incremental (titer) data (e.g., to produce continuous data appropriate for parametric statistical analysis) the mean optical density resulting from duplicate determinations ($OD_i$), the background optical density (BKG) and the sample dilution factor were used to solve for the dilution of serum standard required to produce an equivalent optical density. Anti-SRBC hemolytic antibody in experimental samples is reported as % of standard. For these calculations:

$$\%\ \text{Hemolysis} = \frac{OD_i - BKG}{OD_{100\%\ lysis} - BKG} \times 100$$

$$\%\ \text{Standard} = \frac{\text{Dilution of Sample which produced } OD_i}{\text{Calculated dilution of Standard to produce } OD_i} \times 100.$$

*Statistical Analysis*

The overall significance of effects of treatments on immunological as well as behavioral parameters was determined by using a two-way analysis of variance (ANOVA) with the conditioning treatment and time of treatments serving as the fixed effects of interest [33]. If overall significance was evident ($p<0.05$) then one-tailed Fisher's (LSD) $t$-tests were used for individual comparisons [23]. Absorbance values resulting from lysis of SRBC were log transformed prior to ANOVA or $t$-test to insure homogeneity of variance. The nominal comparison-wise significance level was selected in advance to be 5%, but isolated significant results at this level were interpreted with caution.

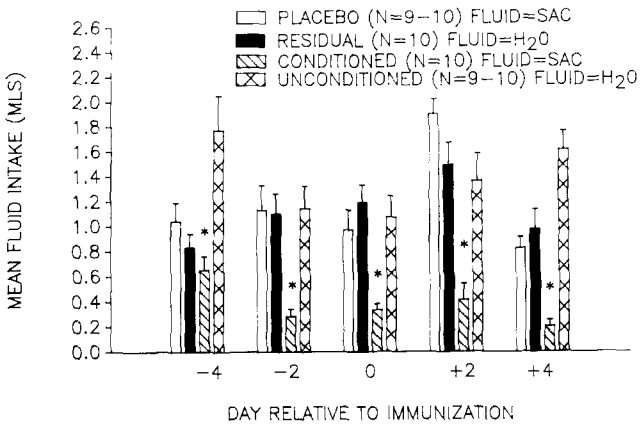


FIG. 1. Effects of cyclophosphamide administration on mean fluid consumption in ml on test days −4, −2, 0, +2, +4 relative to immunization for the conditioned, unconditioned, placebo and residual subgroups. All means given ±S.E. *Indicates significant difference from placebo group or from saccharine consumption on conditioning day as determined by Fishers (LSD) *t*-tests ($p<0.05$).

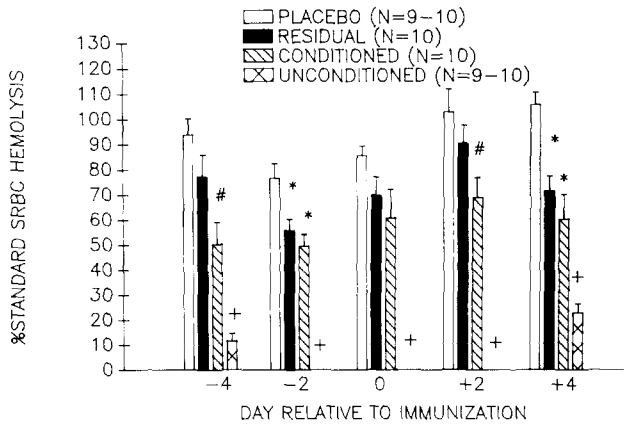


FIG. 3. Effects of conditioning on anti-SRBC antibody response as percent of standard hemolysis when administered drinking tests on days −4, −2, 0, +2, +4 relative to immunization. Significance as described in Fig. 2.

## RESULTS

### *Conditioned Effects on Drinking Behavior*

The drinking data for each treatment subgroup are presented in Fig. 1 (data from −4, −2, 0, +2, and +4 are presented here). Overall, significant treatment effects, $F(3,177)=38.86$, $p<0.01$, time effects, $F(4,177)=5.50$, $p<0.01$, and significant treatment-time interactions, $F(12,177)=5.29$, $p<0.01$, were evident. Conditioned animals consumed significantly less saccharine upon its second presentation than when initially presented on conditioning day, $F(1,148)=8.25$, $p<0.01$. Under the test conditions, the data indicate that conditioned animals drank significantly less saccharine upon its second presentation than the placebo group in which saccharine presentation was never paired with CY. General water consumption in the residual and unconditioned groups is also shown and did not differ significantly between groups.

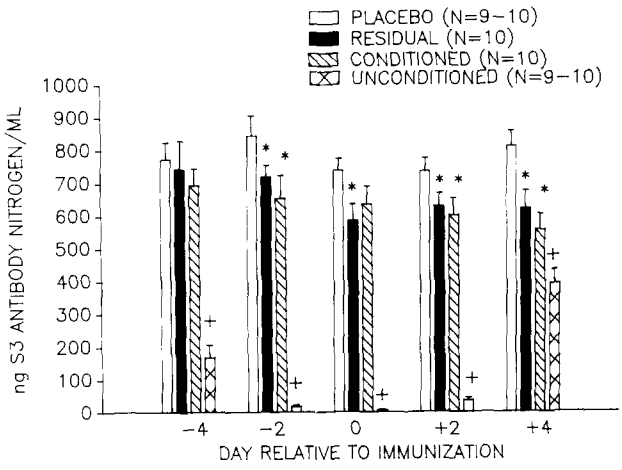


FIG. 2. Effect of conditioning on anti-S3 antibody response in ng of antibody nitrogen per ml serum when administered drinking tests on days −4, −2, 0, +2, +4 relative to immunization. Subgroups as described in Fig. 1. All means±S.E. *Indicates significant difference from placebo groups. #Indicates significant difference from residual and placebo groups. +Indicates significant difference from residual, placebo and conditioned groups.

### *Effects of Conditioning on Anti-S3 Antibody Response*

The temporal relationship between CS or UCS presentation and immunization on anti-S3 antibody response is presented in Fig. 2. Significant treatment effects, $F(3,177)=182.40$, $p<0.01$, time effects, $F(4,177)=4.43$, $p<0.01$, and significant treatment-time interactions, $F(12,177)=3.84$, $p<0.05$, were evident. CY injection consistently suppressed antibody response as compared to the other subgroups from any test day. The residual effects of the initial CY injection were manifest as significantly lowered antibody levels in the residual subgroups receiving treatments on days −2, 0, +2, and +4 relative to immunization as compared to the corresponding placebo subgroup. The conditioned group did not have significantly lowered antibody levels as compared to the residual control group on any reexposure days. The effect of the second CY injection was dependent on the temporal relationship of CY treatment relative to immunization. Antibody levels were maximally suppressed when CY was administered on days −2, 0 or +2 relative to immunization.

### *Effects of Conditioning on SRBC Antibody Response*

The temporal relationship between CS or UCS presentation and immunization on the subsequent anti-SRBC antibody response is illustrated in Fig. 3. Overall, significant treatment effects, $F(3,177)=160.75$, $p<0.001$, and significant time effects, $F(4,177)=5.60$, $p<0.01$, were found. No significant treatment-time interaction occurred, $F(12,177)=1.69$. It is evident that CY injection at any of these times relative to immunization resulted in suppressed antibody responses as compared to any of the other corresponding subgroups. Similar to the S3 data, the maximum effect of CY in suppressing this anti-SRBC antibody response occurs when given on days −2, 0, and +2 relative to immunization. However, in contrast to the anti-S3 antibody response, the presentation of the CS (SAC) also resulted in significantly sup-

pressed anti-SRBC antibody response, as compared to the corresponding residual subgroup, only when presented on days −4 or +2 relative to immunization. The residual effects of the initial CY injection were evident only in those subgroups receiving drinking tests on days −2 and +4 relative to immunization.

## DISCUSSION

The intent of these experiments was to determine some conditions that would aid in optimizing production of conditioned immunosuppression. The relationship between CS presentation and immunization on the subsequent antibody response was studied because the unconditioned effects of CY on immune function are dependent upon the time of administration relative to immunization as well as upon route, dose and species [6, 19, 30]. We have demonstrated that cyclophosphamide-saccharine pairing can result in both conditioned taste aversion and a small but significant suppression of antibody responses when saccharine is subsequently presented. Furthermore, this conditioned suppression of antibody response appears to be relatively specific for T-cell dependent processes since conditioning was evident only for the T-cell dependent anti-SRBC antibody response and not the T-cell independent anti-S3 antibody response. These experiments extend the findings of others [1–5, 8–12, 22, 24, 25], and are in agreement with some reports indicating the T-cell dependent conditioning specificity of antibody responses [31] and of lymphocyte proliferative responses [24]. However, these results appear to be in conflict with one report by Cohen *et al.* [10] indicating conditioned suppression of a different T-cell independent antibody response in mice. It is possible that the differences between our results and those of Cohen *et al.* [10] are due to sex and strain differences in the experimental subjects, the type of T-cell independent antigen used, or differences in the method of evaluating the antibody response (radioimmunoassay vs. passive hemagglutination titer). At any rate, our data and that of others [24,31] do not support the general hypotheses that conditioning affects both T-cell dependent and T-cell independent antibody responses.

The unconditioned effects of CY on immune function are well characterized [19,30]. The administration of CY produces depression of antibody response, and depletion of B-lymphocytes from lymph follicles and nodes [30]. One injection of CY reversibly depresses humoral antibody synthesis and release for approximately seven days, after which antibody levels begin to recover [31].

Residual effects of CY exposure on conditioning day were apparent as overall antibody levels in the residual group were significantly suppressed compared to the placebo group which never received CY. The residual effect of CY was more pronounced for the anti-S3 response than for the anti-SRBC response. We chose a two week recovery period between the initial CY-SAC pairing and reexposure to SAC in order to minimize the residual effects of CY and more clearly demonstrate a conditioning response. Others have used shorter recovery periods in which the residual effects of CY were more pronounced resulting in a conditioned immunosuppression of equal magnitude to the unconditioned effects of CY [22]. This observation suggests that the presence of residual CY effects may be important to the expression of conditioned immunosuppression. In this respect it has been hypothesized that conditioning serves to augment the immunosuppressive effects of CY [11]. Our findings suggest at least two factors which are important for the detection of conditioned suppression of antibody responses. These factors are: (1) the recovery time between the initial pairing of CY and SAC and reexposure to SAC, and (2) the time at which reexposure to SAC occurs relative to immunization.

The relationship between conditioned taste aversion and conditioned immune suppression is not well understood. The noxious effects of CY become associated with the gustatory stimuli produced by saccharine, resulting in a decreased consumption of saccharine upon subsequent presentation. Similarly, the immunosuppressive effects of CY become associated with saccharine, evidenced by immunosuppression by SAC alone on subsequent presentations. Some controversy exists as to whether conditioned taste aversion and conditioned immunosuppression are intimately linked or whether they can become dissociated. Some reports suggest that conditioned immunosuppression occurs in the absence of taste aversion [21,25] while others intimate that the two are intimately linked [17, 18, 31]. Still others have suggested that the "stress" induced by taste aversion alone is sufficient to produce immunosuppression [17,18]. We report that the conditioned taste aversion induced by a CY-SAC pairing is a good indicator that the two became associated. However, all of the subgroups demonstrated conditioned taste aversion whereas only the subgroups which received SAC on days −4 or +2 relative to immunization showed significant depression of antibody response. This suggests that the two phenomena can be dissociated and that their association may depend upon the time interval between reexposure to the CS and immunization. Therefore the timing of both behavioral and immunological factors become important in conditioning antibody responses.

The mechanisms involved in conditioned immunosuppression are poorly understood. However, from our results and results of others [24,31] it is becoming apparent that, for conditoned antibody suppression, T-cell dependent mechanisms may be involved, although some controversy on this point exists. Identification of some T-cell dependent regulatory processes (e.g., neuronal and/or hormonal processes) may prove useful in delineating the mechanisms involved between the central nervous system and immune system [13,14]. A discussion on possible mechanisms underlying conditioned immunosuppression should address the hypothesis that "stress-induced" activation of the pituitary-adrenal axis accompanied by increased corticosterone concentrations is responsible for the resulting immunosuppression [1, 24, 31]. This suggestion has been disputed by the observation that lithium chloride (a nonimmunosuppressive agent) when substituted for CY, conditioned the adrenal response, but not the suppression of antibody response [1, 5, 20]. Lithium chloride alone, as well as conditioned taste aversion itself, have been reported to produce immunosuppression, but only of the delayed type hypersensitivity immune reactions rather than antibody responses [17,18]. These findings suggest that the mechanisms by which conditioning can suppress various components of the immune system may be different [24].

It is becoming evident that environmental and behavioral factors may be associated with immunologic dysfunction and disease [22,28]. Evidence indicating that conditioned immunosuppression can modify responses in various animal models of disease ranging from arthritis to systemic lupus erythrematosus has been reported [3,20]. The psycho-social factors associated with human disease-states and their importance should not be underestimated. Research into the connections between the brain and various

aspects of the immune system may ultimately help optimize drug therapy in autoimmune disease [3,20], and increase the understanding of how behavioral factors may predispose organisms to opportunistic infection [28].

In conclusion the behavioral conditioning of T-cell dependent anti-SRBC antibody responses were demonstrated while T-cell independent anti-S3 antibody responses were not. When the CS was presented either before or after immunization conditioned suppression of the resulting antibody response was observed. It has been suggested that immunization (antigen) serves to reveal rather than to initiate the CNS mediated immunological effects of conditioning [4]. Further investigations of this phenomenon and the mechanisms involved should focus on both behavioral and immunological variables. Pharmacological and hormonal manipulations of specific common neuronal systems and T-cell dependent mechanisms may prove useful in elucidating mechanisms connecting the central nervous and immune systems.

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