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Enhancement of Pavlovian conditioned immunosuppression in rats

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Abstract. The goal of this study was to define conditions under which conditioned immunosuppression may be observed reliably. In three experiments, rats were exposed to a gustatory conditioned stimulus (CS) paired with cyclophosphamide (US), which induces immunosuppression and malaise. In Experiment 1, a single pairing of the CS with low, medium, or high doses of cyclophosphamide in separate groups produced no reliable conditioned immunosuppression even though conditioned taste aversion was observed in groups trained with high and medium doses of CY. Experiment 2 replicated the lack of effect following a single pairing of the CS with the medium dose of cyclophosphamide but demonstrated that three pairings are sufficient to induce conditioned immunosuppression. Experiment 3 demonstrated that significant immunosuppression is observable following a single CS–US pairing if the CS is presented in compound with a previously nonreinforced CS during training, an effect reminiscent of supernormal conditioning. These findings indicate that conditioned immunosuppression effects can be enhanced in magnitude through the use of certain procedural techniques.

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INTRODUCTION

Over 30 years of research has established that immune responses can be modulated by Pavlovian conditioning procedures. In these experiments, an immunologically neutral conditioned stimulus (CS) is paired with an immunologically active unconditioned stimulus (US) and thereby comes to control a conditioned response (CR) that is similar to the unconditioned response controlled by the US. Initially, this phenomenon was studied using immunosuppressive drug USs, such as cyclophosphamide (CY) or cyclosporin A, paired with gustatory CSs, in what has been called the "conditioned inmunosupression paradigm" (Ader and Cohen 2001). In this paradigm, the conditioned effects consist of a decreased antibody response to certain antigens (typically, sheep red blood cells or SRBC) in the presence of the gustatory CS. In subsequent studies using similar CSs and USs, other conditioned immunological responses have been observed, such as decreased graft-versus-host responses (Bovbjerg et al. 1984) and reduction in natural killer cell activity (O'Reilly and Exon 1986). More recently, researchers have become interested in the possibility of conditioned immunoenhancement through the use of immune system-potentiating drugs (Solvason et al. 1991) and antigen USs (Ader et al. 1993, Alvarez-Borda et al. 1995, Gorczynski et al. 1982, Madden et al. 2001).

The finding that the immune system is conditionable has stimulated a respectable volume of research, contributing greatly to the development of an emergent discipline known as psychoneuroimmunology. The corpus of the empirical data and its clinical implications have been reviewed periodically by Ader, Cohen, and their colleagues (Ader 1981, 2003, Ader and Cohen 1991, 2001, Cohen et al. 1994) as well as others (e.g., Dunn 1989, Exton et al. 2000, Hucklebridge 2002, Kusnecov et al. 1989, Stockhorst and Klosterhalfen 2005). There is considerable agreement among the reviewers as to the reality of the conditioned immunomodulation phenomenon, but there is also some concern with respect to the reliability and size of the effects. For instance, Ader (2003) discussed some of the failures to replicate the finding of conditioned immunoenhancement, and concluded that these studies may have failed to detect what is often a small effect. Support for this idea comes from the finding that conditioned immunomodulation sometimes is detectable only when the CS is accompanied by a subthreshold "booster" dose of the antigen US (Ader et al. 1993).

Small and sometimes inconsistent conditioned immunosuppression effects have been observed as well. The simplest type of conditioned immunosuppression experiment measures humoral immunity after pairing a saccharin solution with CY. The experimental design typically consists of three phases. In the conditioning phase, a conditioned group of rats is exposed to a single pairing of a saccharin solution (CS) and an IP injection of CY (US). In the testing phase, which occurs several days later, conditioning is evaluated by exposing the rats to the CS in conjunction with an immunological challenge consisting of antigen inoculation (SRBC). In the third phase, immune assays are conducted to quantify the immune reaction to the antigen. Antibody titers in the conditioned group are compared to those in several control groups, which can be classified into two categories: the placebo group and the nonconditioned groups. In the placebo group, the animals receive a saline injection instead of CY and are expected to exhibit no immunosuppression at the time of testing (neither conditioned, due to the CS, nor unconditioned, due to residual effects of CY). In the nonconditioned groups, the animals receive the same dose of CY as does the conditioned group, but no CS-US pairings occur. This can be done in any of several ways, including presenting the US but not the CS during the conditioning phase (US only group); omitting the CS during testing in a "conditioned group" (CSo group); and/or presenting the CS and the US in an explicitly unpaired fashion (unpaired group). The nonconditioned groups are expected to exhibit no immunosuppression due to conditioning and little, if any, immunosuppression due to the residual effects of CY. Thus, to probe the effects of conditioning, it is necessary to demonstrate that the conditioned group is more immunosuppressed than is any of the nonconditioned groups. Since CY also produces malaise, the effectiveness of the CS/US pairings can be further confirmed by observing a decreased consumption of saccharine in the conditioned group as compared to the control groups (i.e., conditioned taste aversion).

There are a few studies on humoral conditioned immunosuppression similar to the one described above. For instance, in the first report by Ader and Cohen (1975), the results were in the expected direction; namely, the paired group exhibited the lowest

antibody titers, the placebo group exhibited the highest antibody titers, and the nonconditioned groups were intermediate. Subsequently Ader and coauthors (1982) reported similar findings in a study that included conditioned, placebo, and CSo groups. Similar experiments were conducted by Rogers and others (1976) and Wayner and others (1978) and, although the data were in the same direction as in previous studies, the effects were modest.

Collectively, these experiments on humoral immunosuppression support the idea that immune responses can be conditioned, since they found approximately the same pattern of results. However, taken individually, none of these experiments provides conclusive evidence of a robust conditioned immunosuppressive response. Furthermore, the results of some of these studies suggested that in order to see conditioning, the experimental protocol must include at least two test trials with the CS.

An additional source of uncertainty comes from a few studies that report opposite conditioned effects; i.e., immunoenhancement instead of immunosuppression following training with an immunosuppressive drug (e.g., Krank and McQueen 1988, McQueen and Siegel 1989).

The present set of experiments was designed to explore the possibility of obtaining more reliable and robust conditioned immunosuppression effects using a classic experimental procedure in which rats are exposed to a gustatory CS and cyclophosphamide US. Experiment 1 was designed to reproduce the findings of Ader and Cohen (1975) using saccharin and three different intensities (low, medium and high doses) of CY. Having obtained no evidence of conditioned immunosuppression in this experiment despite of robust conditioned taste aversion, we next employed two further enhancement strategies. In Experiment 2, a reliable conditioned immunosuppression effect was observed after increasing the number of saccharin-CY pairings from one to three. In Experiment 3, reliable conditioned immunosuppression was obtained following a single saccharin-CY pairing when saccharin was presented in compound with lemon juice, which had been trained to signal the absence of CY in a nonreinforced pre-exposure phase. This later strategy resembles the so called "supernormal conditioning" procedure, which refers to the enhancement of the learning that accrues to a given CS, A, after being compounded with an inhibitory CS, B, during the conditioning trials. The rationale of this procedure is that given that CS B by itself developed a negative association with the US and that the AB compound is followed by the US, the amount of association developed by A should be greater than normal in order to counteract the negative expectation created by the presence of B.

METHODS

Animals and apparatus

One hundred eighty-two male albino rats (purchased from Pontificia Universidad Católica de Chile breeding colony, Santiago, Chile) weighing 200–350 g, were housed in individual cages with free access to food. Mounted on each cage was a holder that could support a 100-ml plastic syringe adapted with a glass-drinking spout at the bottom. All fluids were delivered in these syringes. All experimental treatments took place in the individual home cages. Different animals were used for each experiment (n=94 for Experiment 1, n=44 for Experiment 2, and n=44 for Experiment 3). Rats in experiments 1 and 2 had restricted access to water at some stages of the experiment whereas rats in Experiment 3 were kept on an ad libitum regimen of water throughout the experiment.

All experimental protocols were approved by Universidad de Talca Ethics Committee and CONYCIT (*Comisión Nacional de Investigación Científica y Tecnológica de Chile*).

Procedures

CONDITIONING PROTOCOL OF EXPERIMENT 1

Experiment 1 was designed to evaluate conditioned immunosuppression after training separate groups of animals with three different doses of CY (low, medium and high). The experiment was conducted in two replications, each including 47 animals. The two replications were identical except as noted below. One week after arrival, the rats were adapted over a 7-day period to 15 minutes of access to water per day. After adaptation, the rats in each replication were matched into the 7 groups based on their water intake and body weight.

Table I outlines the experimental treatments for each group. On the day following the last day of adaptation (day 1), animals in the groups unpaired-high, unpaired-medium and unpaired-low were exposed to a novel

Table I

Design of Experiment 1

Group	Day 1	Day 2 Conditioning	Day 5 (rep. 1) or Day 12 (rep. 2) Immunization	Day 11 (rep. 1) or Day 18 (rep.2) Immune assay
Paired High (*n*=14)	H_2O	SAC + CY_{75}	SAC + SRBC	Blood sample
Unpaired High (*n*=12)	SAC	H_2O + CY_{75}	SAC + SRBC	Blood sample
Paired Medium (*n*=14)	H_2O	SAC + CY_{50}	SAC + SRBC	Blood sample
Unpaired Medium (*n*=12)	SAC	H_2O + CY_{50}	SAC + SRBC	Blood sample
Paired low (*n*=14)	H_2O	SAC + CY_{25}	SAC + SRBC	Blood sample
Unpaired low (*n*=14)	SAC	H_2O + CY_{25}	SAC + SRBC	Blood sample
Vehicle (*n*=14)	H_2O	SAC+ saline	SAC + SRBC	Blood sample

taste by replacing the plain water with a 0.1% sodium saccharin (SAC) solution during the 15-min drinking period. On this day, all other groups received the regular 15-min access to plain water. The next day (day 2) was the conditioning day. Animals in groups unpaired-high, unpaired-medium and unpaired-low received plain water, and all other groups received flavored water. Thirty minutes after the drinking period, animals in the vehicle group received an intraperitoneal (i.p.) injection of 1 ml of physiological saline, and all other groups received an i.p. injection of CY. The dose of CY was of 75 mg/kg in groups paired-high and unpaired-high, of 50 mg/kg in groups paired-medium and unpaired-medium, and of 25 mg/kg in groups paired-low and unpaired-low.

Thereafter all animals were returned to daily 15-min access to plain water, except on the test day. The test was conducted on day 5 (replication 1) or day 12 (replication 2) and consisted of the replacement of plain water with the saccharin solution in all groups. Immediately after finishing the 15-minute drinking period, all animals were immunized with i.p. injections of 2 ml/kg of a 1% thrice-washed suspension of SRBC. Seven days after immunization (day 11 for replication 1 and day 18 for replication 2), blood was drawn by cardiac puncture performed in anesthetized animals. The blood was centrifuged and the serum was collected and inactivated at 57°C for 30 min prior to determination of antibody titers by the hemagglutinating microtiter method described by Ader and Cohen (1975).

Table II

Design of Experiment 2

Group	Day 1	Day 3 Trial 1	Day 6	Day 8 Trial 2	Day 11	Day 13 Trial 3	Day 24 Imunization	Day 30 Immune assay
Paired 1 trial (*n*=11)	H_2O	H_2O	H_2O	H_2O	H_2O	SAC + CY_{50}	SAC + SRBC	Blood sample
Unpaired 1 trial (*n*=11)	H_2O	H_2O	H_2O	H_2O	SAC	H_2O + CY_{50}	SAC + SRBC	Blood sample
Paired 3 trials (*n*=11)	H_2O	SAC + CY_{50}	H_2O	SAC + CY_{50}	H_2O	SAC + CY_{50}	SAC + SRBC	Blood sample
Unpaired 3 trials (*n*=11)	SAC	H_2O + CY_{50}	SAC	H_2O + CY_{50}	SAC	H_2O + CY_{50}	SAC + SRBC	Blood sample

Table III

Design of Experiment 3

Group	Day 1 (P.M.) Preexp.1	Day 2 (A.M.) Preexp.2	Day 2 (P.M.) Preexp.3	Day 3 (A.M.) Preexp.4	Day 3 (P.M.) Conditioning	Day 7 (P.M.) Immunization	Day 13 (P.M.) Immune assay
Supercond. (*n*=11)	Lemon	Lemon	Lemon	Lemon	Lemon/SAC + CY_{50}	SAC + SRBC	Blood sample
Paired (*n*=10)	Lemon	Lemon	Lemon	Lemon	SAC + CY_{50}	SAC + SRBC	Blood sample
Unpaired (*n*=12)	Lemon	Lemon	SAC	Lemon	Lemon + CY_{50}	SAC + SRBC	Blood sample
US (*n*=11)	H_2O	H_2O	H_2O	H_2O	H_2O + CY_{50}	SAC + SRBC	Blood sample

CONDITIONING PROTOCOL OF EXPERIMENT 2

Experiment 2 was conducted to test whether the conditioned immunosuppression effect can be augmented by increasing the number of CS–US pairings. The conditioning and test protocols were similar to those of the second replication of Experiment 1, except that only a medium dose of CY was used (50 mg/kg). Table II outlines the different treatments. Animals in groups paired-one trial and unpaired-one trial received a single dose of CY (day 13), whereas animals in groups paired-three trials and unpaired-three trials received three doses of CY (days 3, 8 and 13). Animals in groups paired-one trial and paired-three trials were exposed to the saccharin solution 30 minutes before being injected with CY, whereas animals in groups unpaired-one trial and unpaired-three trials were exposed to saccharin two days before the injection of CY. Similar to replication 2 of Experiment 1, immunization occurred 10 days after the last day of conditioning and blood samples were drawn 7 days after immunization.

CONDITIONING PROTOCOL OF EXPERIMENT 3

Experiment 3 was conducted to test whether the conditioned immunosuppression effect can be augmented by presenting a target CS, A, in compound with another CS, B, that previously had been trained to signal the absence of the US. The rationale was that animals should learn that taste B was not followed by any consequence during the pre-exposure phase, and as a result, taste A might acquire more associative strength when presented in compound with taste B as compared to when presented alone. That is, similar to the supernormal conditioning phenomenon, taste A might be assigned greater than normal significance as a function of its correctly signaling an outcome (US delivery) different from that signaled by taste B (no US delivery). The major features of this experiment are outlined in Table III. The animals were divided into 4 groups that were treated differentially in two experimental phases, the pre-exposure phase and the conditioning phase. Group superconditioning was exposed to taste B during the pre-exposure phase and to the AB compound followed by CY during the conditioning phase. Group Paired also was exposed to taste B during the pre-exposition phase, but during conditioning A was presented in isolation (i.e., not as part of an AB compound) and paired with CY. It was expected that the pre-exposure to taste B would not have any influence over conditioning to the qualitatively different taste A in this group. Two nonconditioned control groups were included in the design. Animals in the unpaired control group were exposed to taste B during all trials of the pre-exposure and conditioning phases, except for pre-exposure trial number 3, in which they were exposed to taste A approximately 24 hours before the administration of CY. This arrangement constituted an unpaired presentation of taste A and CY, such that no conditioning should occur to taste A. Animals in the US control group were exposed to no flavor at any point in the experiment, but received a CY injection during the conditioning phase. This new control group was included to have a measure of the unconditioned residual effect of CY administration upon the immune

response, without the influence of exposure to the conditioned stimuli.

The pre-exposure and conditioning phases lasted three days, each of which was divided into morning and evening sessions (at about 8:00 A.M. and 8:00 P.M. respectively). The four pre-exposure sessions occurred in the afternoon session of day 1, in the morning and afternoon session of day 2, and in the morning session of day 3. Conditioning took place in the afternoon session of day 3, where all animals received a 50 mg/kg of CY. Unlike experiments 1 and 2, in this experiment the animals were exposed to the flavors by forcing them to drink 1 ml of fluid by pipette. Immunization occurred 4 days after the last day of conditioning (day 7). During immunization, all animals were exposed to the target taste A. Blood samples were drawn 7 days after immunization (day 13).

Scoring and statistical analysis

In Experiments 1 and 2, conditioned taste aversion (CTA) was assessed through a preference ratio calculated for each animal by dividing their water intake during the test by the mean water intake on the two immediately preceding days. A preference ratio of less than 1 suggests that an aversion was developed to the taste, whereas a value equal to or higher than 1 indicates the absence of aversion. In Experiment 3, CTA was not examined. Conditioned immunomodulation was assessed through the antibody titers recorded as log2 reciprocals of the end point dilutions in Experiments 1–3.

One-way ANOVAs were conducted to test the main effect of group in each experiment. Planned comparisons using the Fisher PLSD technique were employed in order to test for relevant differences among specific groups. All statistical differences were considered significant at the 0.05 level. Effect sizes were reported as partial eta squared (Cohen 1973).

RESULTS

Experiment 1: Effects of pairing a saccharin solution with three different doses of CY

Table IV presents the preference ratios for the seven groups in the training and test trials. Because equivalent results were obtained between replications, the data were collapsed across the two replications. As can be seen in the table, there were no major differences between the groups in the first exposure to the saccharin solution. This result was confirmed by an ANOVA in which no significant main effect of group was noted in the conditioning session ($F_{6,87}$=1.583, P=0.162, partial η^2=0.098). Group differences arose during the testing day and were confirmed by a significant main effect of group ($F_{6,87}$=4.718, P<0.001, partial η^2=0.245). As can be seen in the table, the unpaired (high, medium, and low) and vehicle groups did not show evidence of taste aversion, while groups paired-high and paired-medium showed a large decrement in their preference ratios. Contrary to expectations, the paired-low group did not show evidence of CTA. Planned contrasts confirmed the reliability of these observations since group paired-high differed significantly from groups unpaired high and vehicle (Ps=0.011 and 0.051; partial η^2s=0.072 and 0.043, respectively), and group paired-medium differed significantly from groups unpaired-medium and vehicle (Ps=0.001 and 0.005; partial η^2s=0.138 and 0.088, respectively). Finally, group paired-low did not differ significantly from group unpaired-low or from group vehicle (Ps=0.427 and 0.791; partial η^2s=0.007 and 0.001, respectively). These results indicate that there was an association between saccharine and CY at least in groups paired-high and paired-medium.

Figure 1 presents the mean antibody titers in the 7 groups. As expected, group vehicle showed the highest immune response, followed by the two groups that were trained with a low dose of CY, then by the two groups trained with an intermediate dose, and finally by the two groups trained with the high dose. Since all

Table IV

Mean preferences ratios and standard error of the mean (SEM) in the training and test trials of Experiment 1

Group	Training trial Mean (SEM)	Test trial Mean (SEM)
Paired high	1.12 (0.08)	0.70 (0.15)
Unpaired high	1.36 (0.13)	1.21 (0.10)
Paired medium	1.31 (0.23)	0.53 (0.15)
Unpaired medium	1.07 (0.12)	1.26 (0.11)
Paired low	1.56 (0.12)	1.12 (0.14)
Unpaired low	1.50 (0.07)	1.27 (0.12)
Vehicle	1.32 (0.18)	1.07 (0.15)

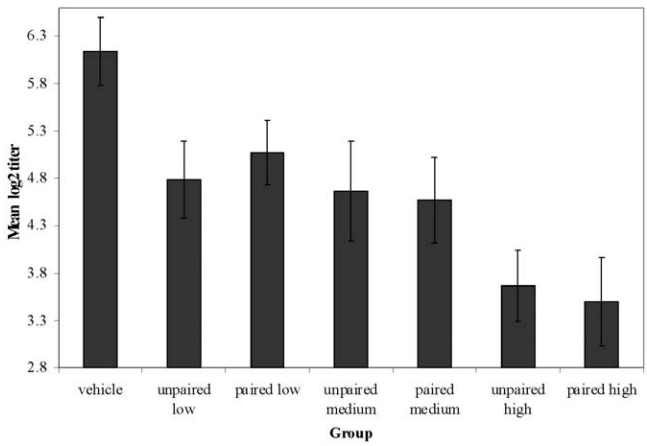

Fig. 1. Mean hemagglutination titers (± standard error of the mean) in Experiment 1

groups that were exposed to CY exhibited some degree of immunosuppression, the critical comparisons to demonstrate conditioning are between the respective paired and unpaired groups. As the figure shows, these differences were very minor. The data were explored with a 2 (replication) × 7 (group: paired-high; unpaired-high; paired-medium; unpaired-medium; paired-low; unpaired-low; vehicle) ANOVA, which indicated a reliable main effect of replication ($F_{1,80}$=5.913, P=0.017, partial η^2=0.069) and group ($F_{6,80}$=5.206, P<0.001, partial η^2=0.281), but no reliable interaction between group and replication ($F_{6,80}$=1.791, P=0.111, partial η^2=0.118). The main effect of replication is due to the fact that the antibody titers of replication 1 were lower than those in replication 2, which is explained by the difference in the interval between CY administration and immunization in the two replications (4 *versus* 10 days). Since there was no interaction between group and replication, no further analysis was pursued with the later variable and the data are presented collapsed across replications in the figure.

The main effect of group was explored further through nine planned contrasts. The first three contrasts indicate that there was no reliable difference between groups paired-high and unpaired-high (P=0.784, partial η^2=0.001); between groups paired-medium and unpaired-medium (P=0.876, partial η^2=0.000); or between groups paired-low and unpaired-low (P=0.626, partial η^2=0.003). On the other hand, the mean antibody titers of group vehicle was significantly greater than that of every other group (Ps<0.07, partial η^2s>0.077), indicating a residual effect of CY. Thus, contrary to what was observed with CTA, there was no evidence of conditioned immunomodulation in this experiment.

Experiment 2: Effect of increasing the number of CS–US pairings

Evidence of CTA was again found in this experiment. Table V presents the mean preference ratios for all groups in the testing trials. As expected, there were no major differences between the groups on the first trial, as confirmed by an ANOVA indicating no reliable effect of group in this trial ($F_{3,40}$=0.789, P=0.507, partial η^2=0.056). Group differences arose during the second testing day ($F_{3,40}$=10.325, P<0.001, partial η^2=0.436). As can be seen in the table, group paired-three trials showed an important decrement of water intake in trial 2 and the lowest preference ratios among the groups. Planned comparisons confirm the reliability of this CTA, indicating that group paired-three trials presented a preference ratio significantly lower than that of group unpaired-three trials (P<0.001, partial η^2=0.383) and that groups paired-one trial and unpaired-one trial did not differ significantly (P=0.988, partial η^2=0.000). Reliable differences between groups were again found during trial 3 ($F_{3,40}$=6.526, P=0.001, partial η^2=0.329), with planned

Table V

Mean preferences ratios and standard error of the mean (SEM) in the four testing trials of Experiment 2

Group	Trial 1 Mean (SEM)	Trial 2 Mean (SEM)	Trial 3 Mean (SEM)	TEST Mean (SEM)
Paired 1 trial	1.03 (0.28)	0.85 (0.08)	0.73 (0.42)	0.60 (0.13)
Unpaired 1 trial	0.64 (0.12)	0.86 (0.11)	1.23 (0.11)	1.22 (0.14)
Paired 3 trials	0.75 (0.19)	0.44 (0.10)	0.36 (0.14)	0.16 (0.09)
Unpaired 3 trials	0.82 (0.07)	1.61 (0.15)	1.92 (0.26)	1.87 (0.25)

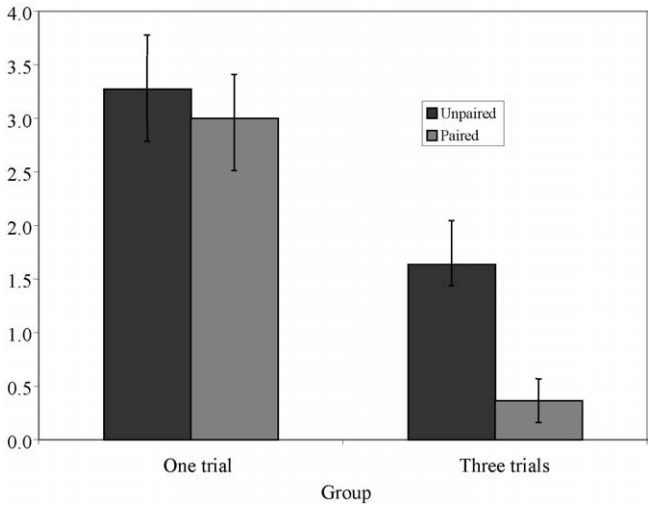

Fig. 2. Mean hemagglutination titers (± standard error of the mean) in Experiment 2

tests indicating a reliable difference between groups paired-three trials and unpaired-three trials (P<0.001, partial η^2=0.303) and no reliable difference between groups paired-one trial and unpaired-one trial (P=0.187, partial η^2=0.043). In trial 4, which tested learning after three CS–US parings in paired-three trials group and learning after a single paring in the paired one-trial group, the ANOVA indicated reliable differences among the groups ($F_{3,40}$=20.954, P<0.001, partial η^2=0.611). In this last trial, planned comparisons showed that the two conditioned groups (paired-three trials and paired-one trial) differed significantly from their respective unconditioned control groups (unpaired-three trials and unpaired-one trial; Ps=0.000 and 0.010; partial η^2s=0.580 and 0.153, respectively). In summary, CTA was apparent in the two conditioned groups and seems to be stronger as the number of CS-US pairings increases.

Figure 2 depicts the results of the immune assays for the four groups. As can be seen, there were clear differences among the groups that were confirmed by a reliable main effect of group ($F_{3,40}$=10.271, P<0.001, partial η^2=0.435). As expected, the number of CY innoculations had a major impact on the immune response, as confirmed by planned contrasts comparing the two groups that received a single dose of CY (i.e., paired-one trial and unpaired-one trial) with the groups that received three doses (i.e., paired-three trials and unpaired-three trials; P<0.001, partial η^2=0.611). The possibility of conditioning was examined by planned contrasts comparing each paired group with its respective unpaired group. This latter analysis revealed a reliable conditioning effect in group paired-three trials, whose mean immune response was significantly lower than the unpaired-three trials group (P=0.038, partial η^2=0.103). Groups paired one-trial and unpaired one-trial did not differ reliably (P=0.648, partial η^2=0.005).

In summary, Experiment 2, like Experiment 1, showed an important dose-dependent residual effect of CY and no reliable conditioned immunosuppressive effects after training with a single dose of CY. However, this experiment showed that three CS–US pairings may be more effective in producing reliable conditioned immunosuppression effects.

Experiment 3: Effects of training a target CS in compound with a pre-exposed CS

Figure 3 presents the mean antibody titers for all groups in this experiment. The highest mean was exhibited by the US control group, followed by the unpaired group, the paired group and, finally, the superconditioning group. Given that the mean antibody titers of the superconditioning group was lower than that of the paired group, it seems that the superconditioning procedure was effective in enhancing learning of a conditioned immunosuppressive response to the target taste. Although the simple main effect of group was not reliable ($F_{3,40}$=2.004, P=0.129, partial η^2=0.131), planned comparisons showed a reliable difference between group superconditioning and the pooled control groups (P=0.037, partial η^2=0.104), whereas group paired, as in all the preceding experiments, did not differ reliably from the control groups (P=0.162, partial η^2=0.048). The differences between

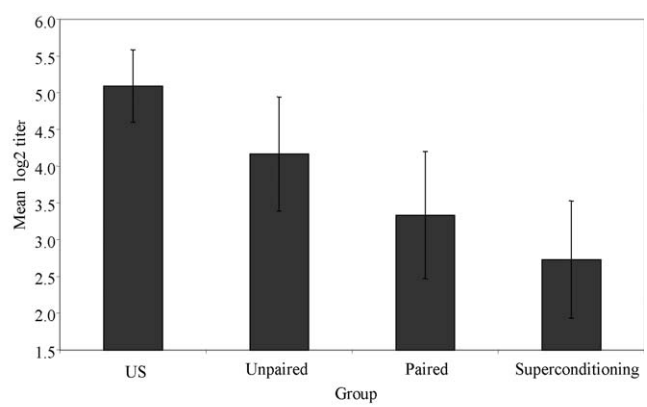

Fig. 3. Mean hemagglutination titers (± standard error of the mean) in Experiment 3

groups superconditioning and paired, and between both control groups, were not reliable according to planned comparisons (*Ps*=0.567 and 0.362; partial η^2s=0.08 and 0.021, respectively). Thus, although conditioning a target CS in compound with a pre-exposed CS did not produce an immunosuppressive response greater than that observed by normal CS/US pairings, it was large enough to support a reliable conditioned immunosuppression effect whereas the paired procedure was not.

DISCUSSION

Taken together, Experiments 1–3 suggest that the conditioning procedure based upon a single pairing of a gustatory CS with CY is effective in producing robust CTA but does not lead to a comparable conditioned immunosuppression. In all three experiments, animals that received a single pairing of CY with saccharin presented an immune response that was not significantly lower than that of the animals that received the same stimuli in an unpaired fashion.

It should be pointed out that in the present experiments the immunosuppressive response was tested using a single presentation of the gustatory CS (i.e., a single test trial), whereas previous research showing evidence of conditioned immunosuppression (e.g., Ader and Cohen 1975, Ader et al. 1982, Rogers et al. 1976) used a slightly different conditioning procedure, in which two test trials were included. Therefore, it is possible that the negative results reported here may be the result of a small-sized effect that could be enhanced by the use of several test trials. The results of the present experiments suggest other factors that could enhance the conditioned immunosuppression effect.

The results from Experiment 1 indicate that manipulating the salience of the US (i.e., the CY dose) may not be an effective method to uncover potential conditioned effects in this paradigm. In Experiment 2, rather than increasing the salience of the US, we increased the number of CS–US trials. This was, in fact, a much more effective method that has been used by others to demonstrate the clinical impact of conditioning. For instance, Ader and Cohen (1982) reported that three saccharin/CY pairings delayed the development of lupus in mice. Of course, increasing the number of conditioning trials implies prolonged exposure to the immunosuppressive drug and its chronic effects upon the immune response, which may be less than ideal.

Experiment 3 demonstrated that it is possible to enhance conditioned immunosuppression without appealing to several doses of the drug by means of a procedure that resembles supernormal conditioning. In the standard supernormal conditioning procedure, learning to a target stimulus is enhanced when it is reinforced in compound with an inhibitory stimulus (Rescorla 1971, 2004, Wagner 1971, Williams and McDevitt 2002). Considering that the training of a conditioned inhibitor normally requires several presentations of the US, and that one of our goals was to avoid the chronic effects of repeated drug administration, we attempted to obtain a superconditioning-like effect without a conditioned inhibitor. Hence, we trained a CS to signal the absence of reinforcement by pre-exposing it without any consequence before conditioning. Then we presented a compound of this pre-exposed CS and a target CS, followed by CY. This procedure was sufficient to obtain a significant conditioned response that was not seen with the regular CS/US pairing.

A common explanation of supernormal conditioning is that, during compound conditioning, the inhibitory stimulus creates an expectation of non-reinforcement that is violated when reinforcement is presented, augmenting excitatory conditioning (e.g., Rescorla and Wagner 1972). Although the presentation of an inhibitory stimulus is the most common way to produce supernormal conditioning to a target CS, there are other ways to obtain the effect. For example, Dickinson (1977) found that fear conditioning to a target CS is enhanced when it is reinforced (i.e., paired with shock) in compound with a stimulus previously paired with food. An interpretation of this result is that the presence of the CS previously paired with food during compound conditioning generates an expectation of an appetitive outcome, which is violated when the shock, an aversive outcome, is presented. In this case, what seems to be the critical condition to produce supernormal conditioning is the presentation of a US that is contrary to the expectations of reinforcement based on previous learning. It seems that more conditioning occurs whenever reinforcement is particularly surprising for the organism. The results of Experiment 3 are consistent with this view.

It should be noticed that compounding a target CS with a pre-exposed stimulus does not always lead to an augmentation of conditioning. For instance, Navarro and others (1989) compared fear conditioning to a target stimulus after training it either in compound with a pre-exposed CS (supernormal condition) or with

a novel CS (normal condition) and found no differences between the two conditions. The results of our Experiment 3 are similar to those reported by Navarro and others (1989) in that they cannot be pointed as direct evidence of supernormal conditioning, because there is no reliable difference between the supernormal and normal conditions. However, our results provide indirect evidence of enhancement since the animals trained in the supernormal condition differed significantly from animals in the non-conditioned control conditions, whereas the animals in the normal condition did not.

CONCLUSIONS

In conclusion, the results of the present set of experiments provide further evidence that the immune system can be associatively conditioned by Pavlovian procedures. The small size of the effect has been a recurrent finding in this area of research, and here it is shown that it can be enhanced. Further studies may explore other phenomena of Pavlovian conditioning that may influence the strength of the conditioned response. Examples of this are the so-called "timing effects", in which the temporal relationship among stimuli appears to play a role in the strength of conditioning. Among the many temporal phenomena that have been described, one of the simplest is the observation that there is an optimal CS–US interval. This phenomenon has been demonstrated in virtually all Pavlovian conditioning preparations (see: Vogel et al. 2006) but has not been examined in conditioned immunomodulation procedures.

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