Behaviorally Conditioned Immunosuppression

ROBERT ADER, PHD AND NICHOLAS COHEN, PHD

An illness-induced taste aversion was conditioned in rats by pairing saccharin with cyclophosphamide, an immunosuppressive agent. Three days after conditioning, all animals were injected with sheep erythrocytes. Hemagglutinating antibody titers measured 6 days after antigen administration were high in placebo-treated rats. High titers were also observed in nonconditioned animals and in conditioned animals that were not subsequently exposed to saccharin. No agglutinating antibody was detected in conditioned animals treated with cyclophosphamide at the time of antigen administration. Conditioned animals exposed to saccharin at the time of or following the injection of antigen were significantly immunosuppressed. An illness-induced taste aversion was also conditioned using LiCl, a nonimmunosuppressive agent. In this instance, however, there was no attenuation of hemagglutinating antibody titers in response to injection with antigen.

INTRODUCTION

The hypothesis that immunosuppression might be behaviorally conditioned was invoked to explain certain incidental observations made in a study of illness-induced taste aversion (1). In the illness-induced taste aversion paradigm (2–4) an animal is given a distinctively flavored drinking solution such as saccharin, which is followed by a toxic agent capable of eliciting temporary gastrointestinal upset. Lithium chloride, apomorphine, and cyclophosphamide are but a few of the toxins that are effective in inducing a taste aversion after a single trial in which the toxin (the unconditioned stimulus or US) is paired with a novel drinking solution (the conditioned stimulus or CS). By pairing different volumes of a preferred saccharin solution with a single intraperitoneal (ip) injection of 50 mg/kg cyclophosphamide (CY), rats acquired an aversion to the saccharin solution; the magnitude of the reduction in saccharin intake and the resistance to extinction of this aversion were directly related to the volume of saccharin consumed on the day of conditioning. It was also observed that some of the cyclophosphamide-treated animals died and that mortality rate tended to vary directly with the volume of saccharin originally consumed.

In order to account for this observation, it was hypothesized that the pairing of a neutral stimulus (saccharin) with cyclophosphamide, an immunosuppressive agent (5), resulted in the conditioning of immunosuppression. If the conditioned animals that were exposed to saccharin every 2 days over a period of 2 months responded to this conditioned stimulus by becoming immunologically impaired,

From the Departments of Psychiatry and Microbiology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642.

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Address reprint requests to: Dr. Robert Ader, Department of Psychiatry, University of Rochester Medical Center, 300 Crittenden Blvd., Rochester, New York 14642.

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they would have been more vulnerable to the superimposition of latent pathogens that may have existed in the environment.

We report here our initial documentation of behaviorally conditioned immunosuppression.

METHODS

Ninety-six male Charles River (CD) rats, approximately 3 months old, were individually caged under a 12 hr light-dark cycle (light from 5 AM to 5 PM) and provided with food and water ad libitum. During a period of adaptation the daily provision of tap water was slowly reduced until all animals were provided with and consumed their total daily allotment during a single 15 min period (between 9 and 10 AM). This regimen was maintained throughout the experiment. The first 5 days under this regimen provided data on the baseline intake of water under these conditions.

On the day of conditioning (Day 0), animals were randomly distributed into conditioned, nonconditioned, and placebo groups. Conditioned animals received a 0.1% saccharin chloride solution of tap water during their 15 min drinking period and 30 min later were given ip injections of CY (50 mg/kg in a volume of 1.5 ml/kg).[1] Nonconditioned animals were, as usual, provided with plain tap water and 30 min after drinking were similarly injected with CY. Placebo animals received plain water and ip injections of an equal volume of vehicle (distilled water). On the following two days all animals were provided with plain water during their 15 min drinking period.

Three days after conditioning all animals were injected ip with antigen, 2 ml/kg of a 1% thrice washed suspension of sheep red blood cells (SRBC; approximately 3×10^8 cells/ml). Thirty minutes later randomly selected subgroups of conditioned and nonconditioned animals were provided with saccharin or plain water and/or received ip injections of CY or saline according to the treatment schedule outlined in Table 1.

One group of conditioned animals received a single drinking bottle containing the saccharin solution and drinking was followed by a saline injection; these animals constituted an experimental group. Two additional groups of conditioned animals received plain water; one of these groups was subsequently injected with CY (in order to define the unconditioned response produced by the immunosup-

[1]Cyclophosphamide was generoulsy supplied by the Mead Johnson Research Center, Evansville, Indiana.

TABLE 1. Experimental Treatments

Group	Day 0		Subgroup	N	Day 3		Day 6	
	Drnk. Soln.	Inj.			Drnk. Soln.	Inj.	Drnk. Soln.	Inj.
Conditioned								
(N= 67)	Saach.	CY	CS_1	11	Sacch	Sal	H_2O	—
				9	H_2O	—	Sacch	Sal
			CS_0	10	H_2O	Sal	H_2O	—
				9	H_2O	—	H_2O	Sal
			US	10	H_2O	CY	H_2O	—
				9	H_2O	—	H_2O	CY
			CS_2	9	Sacch	Sal	Saach	—
Nonconditioned								
(N=19)	H_2O	CY	NC	10	Sacch	Sal	H_2O	—
				9	H_2O	—	Sacch	Sal
Placebo								
(N=10)	H_2O	Placebo	P	10	H_2O	—	H_2O	—

pressive drug) while the second received saline (as a control for taste aversion conditioning, per se). Following antigen administration a nonconditioned group was provided with saccharin and injected with saline. These animals provided a control for the effects of saccharin consumption and the ip injections. Placebo animals remained unmanipulated and received plain water during the 15 min drinking period. On Day 6 of the experiment, conditioned and nonconditioned animals that had received antigen but had not been manipulated on Day 3 were first treated as described for Day 3, i.e., one conditioned group received the saccharin drinking solution, one conditioned group received water and CY, and one conditioned group received neither saccharin nor CY; a nonconditioned group also received saccharin. In addition, there was one experimental sample of conditioned animals that was provided with saccharin on Days 3 *and* 6. All animals remained unmanipulated on Days 7 and 8. Throughout this period the volume of plain water or saccharin consumed was measured daily.

On Day 9 (6 days after injection with SRBC), all animals were sacrificed. Trunk blood was collected in heparinized tubes for subsequent analysis of plasma corticosterone (8) and in nonheparinized tubes for the collection of sera to be used in the hemagglutinating antibody assay. Serum from each rat was heat inactivated (56°C for 30 min) and divided into aliquots some of which were stored at -70°C and others of which were refrigerated and assayed for hemagglutinating antibody activity within 24 hr of collection. Antibody titrations were performed according to standard procedures in microtiter trays and hemagglutination was assessed under the microscope. Titers were recorded as reciprocals of the endpoint dilutions expressed as powers of the base2.

The provision of plain water or saccharin and the injections of CY or placebo were conducted from coded data sheets. Similarly, antibody titrations and plasma corticosterone determinations were conducted without knowledge of the group to which an animal belonged.

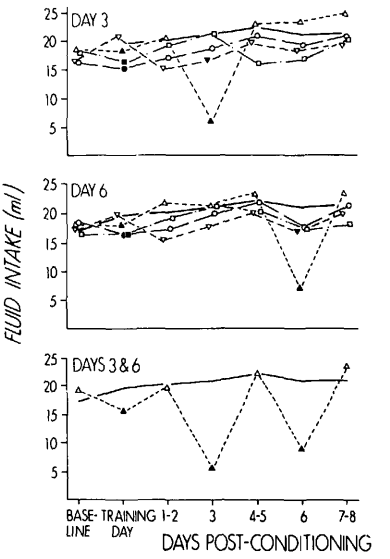

Fig. 1. Mean intake of plain water (open symbols) and saccharin (filled symbols) for placebo (———) and nonconditioned (▼) animals, and conditioned animals that received saccharin (Δ), cyclophosphamide (□), or neither (○) on Day 3, Day 6, or Days 3 and 6. As a point of reference, the placebo-treated animals are shown in each panel.

RESULTS AND DISCUSSION

Cyclophosphamide treatment administered 30 min after the ingestion of a novel saccharin drinking solution resulted in an aversion to the saccharin solution (Fig 1). Conditioned animals provided with saccharin on Day 3, on Day 6, or on Days 3 and 6 showed a reduced intake of the distinctively flavored solution on those days.

With regard to antibody responses, the following pattern of results was predicted. Sera from placebo-treated animals were expected to be relatively high titered. Nonconditioned animals, although subsequently presented with a saccharin drink-

ing solution, were also expected to show high antibody levels. However, it was anticipated that the titers of sera from nonconditioned animals might be somewhat lower than those of placebo animals as a result of the CY administered 3 days before injection with SRBS (6,7). Sera from conditioned animals that were given antigen but never again exposed to either saccharin or CY were expected to have antibody titers equivalent to those of unconditioned animals. Conditioned animals that were given a second injection of CY, an unconditioned stimulus for immunosuppression, were expected to show a minimum antibody response to SRBC. The critical groups for testing the hypothesis that immunosuppression can be behaviorally conditioned were the conditioned animals that were given one or two exposures to saccharin, the conditioned stimulus, following exposure to SRBC. Evidence in support of the hypothesis would be provided by an attenuation of the antibody response in these animals.

Antibody titers from the several groups are shown in Fig. 2. Conditioned animals exposed to saccharin on Day 3 or Day 6 did not differ and were combined to form a single conditioned group (group CS) that received only one exposure to the conditioned stimulus, saccharin. Similarly, the conditioned animals that remained unmanipulated (group CS_0), the conditioned groups treated with CY on Day 3 or 6 (group US), and the nonconditioned animals given saccharin on Day 3 or 6 (group NC) were combined into single groups.

The results were as we had predicted. Placebo-treated animals showed the highest antibody titers. Conditioned animals that received neither saccharin nor CY and nonconditioned animals that were subsequently exposed to saccharin after antigen

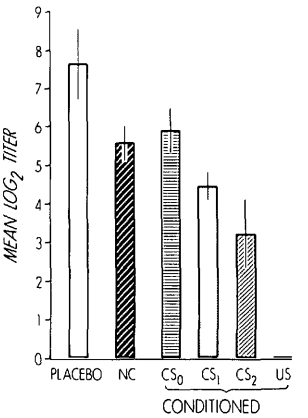

Fig. 2. Hemagglutination titers (means ± SE) obtained 6 days after ip injection of antigen (SRBC). NC = nonconditioned animals provided with saccharin on Day 3 or Day 6; CS_0 = conditioned animals that did not receive saccharin following antigen treatment; CS1 = conditioned animals given one exposure to saccharin on Day 3 or Day 6; CS2 = conditioned animals exposed to saccharin on Days 3 and 6; US = conditioned animals injected with cyclophosphamide following treatment with antigen.

treatment showed similar hemagglutination titers that were also relatively high, although significantly lower than the titers of immune sera from placebo animals in the case of both unconditioned ($t = 2.07, P < 0.05$) and conditioned ($t = 1.71, P < 0.10$) animals.[2] As expected, the hemagglutination tests revealed that administration of CY after SRBC caused complete

[2]The significance levels reported in the text are based on two-tailed *t*-tests. Based on the specific differences that were predicted, however, it would be appropriate to report one-tailed probabilities and the reader may wish to interpret the results in this light.

immunosuppression. Conditioned animals that experience a single exposure to saccharin following antigen treatment (group CS_1) showed an antibody response that was significantly lower than that of placebo as well as nonconditioned animals ($t = 1.96, P < 0.05$) and conditioned animals that were not exposed to saccharin ($t = 2.14, P < 0.05$). The conditioned animals that experience two exposures to saccharin also showed an attentuated antibody response that was significantly below all other groups with the exception of the conditioned animals that received only one exposure to the conditioned stimulus.

Relative to placebo-treated animals, the reduction in hemagglutinating antibody titers shown by nonconditioned animals (group NC) and conditioned animals that were not given either saccharin or CY after antigen treatment (group CS_0) is most simply explained as resulting from some residual effect of CY administered on the day of conditioning (3 days prior to injection with SRBC) (9). These groups, then, become the relevant control condition against which to assess the antibody responses of the conditioned animals exposed to saccharin following antigen treatment. This latter condition did not result in complete suppression of the immune response, but conditioned animals exposed to saccharin did show a significant attentuation of the antibody response relative to these control groups. The attentuation would not appear to have resulted from saccharin, per se, since a comparable exposure to saccharin in association with and following antigen treatment was experienced by the nonconditioned animals for whom saccharin was not a conditioned stimulus. Also, behavioral conditioning, per se, did not result in antibody titers that differed from those of nonconditioned animals. The results, then, support the notion that the association of saccharin with CY enabled saccharin to elicit a conditioned immunosuppressive response.

The present study yielded little additional data that would be of direct importance in suggesting an explanation for this phenomenon. There were no differences among the several groups in body weight measured prior to the adaptation period, on the day before conditioning, or at the time that animals were sacrificed. Also, in conditioned animals exposed to saccharin there were nonsignificant correlations ranging from -0.34 to 0.16 between hemagglutination titer and volume of saccharin consumed. The correlation between plasma corticosterone level sampled at the time that animals were sacrificed and antibody titer was virtually zero, and there were no group differences in steroid levels at this time.

Consistent with the known immunosuppressive properties of adrenocortical steroids and despite the failure to observe differences in plasma corticosterone levels *at the time of sacrifice*, it could be postulated that the attentuated antibody response observed in conditioned animals is a reflection of a nonspecific "stress" response to the conditioning procedures, or, perhaps, of a behaviorally conditioned elevation in steroid level in response to saccharin. Further support for such an explanation might be derived from the relationship between immune processes and physical and socioenvironmental "stress" or emotional responses (11–19) which, presumably, act through the hypothalamus, and from the several studies (e.g., 20,21) that suggest that hypothalamic lesions may influence some immune responses.

In order to evaluate the possibility that

an elevation in adrenocortical steroids was responsible for the attentuation of antibody titers in conditioned animals, a second study used lithium chloride instead of cyclophosphamide as the US in inducing a taste aversion. Whereas lithium chloride also produces noxious gastrointestinal effects, it is not immunosuppressive. In this study, antigen was injected 5 days after conditioning, and the population of conditioned animals that was subsequently provided with the saccharin drinking solution (Group CS, $N = 10$) was exposed to the CS three times: at the time of injection with SRBC, and 2 and 4 days later. As in the first experiment, all animals were sacrificed 6 days after treatment with antigen.

The association of LiCl with saccharin was effective in inducing an aversion to the saccharin solution. Conditioned animals showed a 66% reduction in consumption of the saccharin solution on the initial test day relative to the intake measured on the day of conditioning. This corresponds closely to the 61%–68% reductions shown by animals conditioned with cyclophosphamide. Antibody titers for the conditioned animals and for the several control groups are shown in Fig. 3. As indicated by the high titers found in animals injected with LiCl at the time of injection with SRBC, LiCl is not an unconditioned stimulus for suppression of the immune response. Although conditioning was effective in inducing an avoidance of the CS solution, antibody titers were similar in all groups.

It is not unreasonable to assume that an elevation in steroid levels might accompany the conditioning of a taste aversion. Nevertheless, the present data provide no support for the hypothesis that such an elevation in steroid levels could have been solely responsible for the attentuated immune response that was observed when conditioned animals were exposed to a CS previously associated with the administration of an immunosuppressive agent. The probability of an interaction between the magnitude and /or duration of an elevation in steroid level and the residual effects of cyclophosphamide, however, remains as a viable hypothesis.

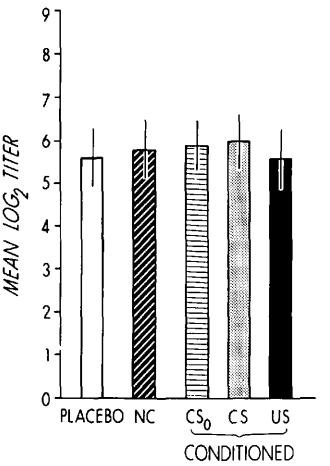

Fig. 3. Hemagglutination titers (means ± SE) obtained 6 days after ip injection of SRBC in animals conditioned with LiCl as the US. NC = nonconditioned animals; CS_0 = conditioned animals that did not receive saccharin following antigen treatment; CS = conditioned animals given three exposures to saccharin; US = conditioned animals injected with LiCl following treatment with antigen.

The present results suggest, again, that there may be an intimate and virtually unexplored relationship between the central nervous system and immunologic processes and that the application of behavioral conditioning techniques provides a means

for studying this relationship in the intact animal. Confirmation of the capacity of behavioral conditioning procedures to suppress (or elicit) immune responses would raise innumerable issues regarding the normal operation and modifiability of the immune system in particular and the mediation of individual differences in the body's natural armamentarium for adaptation and survival in general. Such data also suggest a mechanism that may be involved in the complex pathogenesis of psychosomatic disease and bear eloquent witness to the principle of a very basic integration of biologic and psychologic function.

SUMMARY

The present study was designed to examine the possibility that behavioral conditioning techniques could be used to modify immune processes.

An illness-induced taste aversion was conditioned in rats by pairing saccharin (CS) with cyclophosphamide (CY), an immunosuppressive agent (US). Three days after conditioning, animals received ip injections of SRBC; 30 min later, subgroups of conditioned animals were (a) supplied with the CS solution, (b) provided with water but injected with the US, or (c) given neither CS nor US. A nonconditioned group was provided with the saccharin drinking solution, and a placebo group was injected with antigen but was otherwise unmanipulated.

The association of saccharin and CY was effective in inducing an aversion to the CS when it was presented 3 days after conditioning (at the time of antigen administration). Hemagglutinating antibody titers measured 6 days after injection of SRBC were high in placebo-treated rats. Relatively high titers were also observed in nonconditioned animals and in conditioned animals that were not subsequently exposed to the CS. No agglutinating antibody was detected in conditioned animals treated with CY at the time of antigen administration. In contrast, conditioned animals exposed to the CS when injected with SRBC (and /or 3 days later in additional samples of conditioned animals) were significantly immunosuppressed.

Similar procedures were used in a second experiment in which LiCl, a nonimmunosuppressive agent, was used as the US. While LiCl was effective in inducing a taste aversion, conditioned animals showed no attentuation of hemagglutinating antibody titers.

The results are interpreted as providing evidence for behaviorally conditioned immunosuppression. Further, it is suggested that this phenomenon is not mediated directly by nonspecific elevations in adrenocortical steroids that may be presumed to accompany an illness-induced taste aversion.

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