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Behaviorally Conditioned Immunosuppression: Replication of a Recent Study

MALCOLM P. ROGERS, MD,* PETER REICH, MD,**
TERRY B. STROM, MD,† CHARLES B. CARPENTER, MD‡

INTRODUCTION

Ader and Cohen (1) have recently presented evidence suggesting that immunosuppression can be behaviorally conditioned. Their work developed out of an incidental observation made during a study of illness-induced taste aversion (2-4). Saccharin (conditioned stimulus) had been paired with cyclophosphamide (unconditioned stimulus), an agent that produces gastrointestinal upset. The unexpected mortality rate in some of the

animals subsequently exposed to saccharin suggested to the authors that saccharin had taken on some of the immunosuppressive properties of cyclophosphamide in addition to its gastrointestinal effects (5). The confirmation of their hypothesis that immunosuppression could thus be behaviorally conditioned was recently reported (1). We are now reporting on our replication of their work.

METHODS

The methods were essentially the same as those of Ader and Cohen (1). Male Sprague-Dowley rats approximately 3 months old and weighing between 250 and 300 g were obtained from Charles River Laboratories, Inc. They were all individually caged and maintained on a 12-hr light and dark cycle. For the first week after arrival the animals were given tap water and food *ad lib*. During the second week, the only change made was that the provision of water was limited to a 15-min interval occurring at approximately the same time each afternoon. The volume consumed was measured throughout. The animals were maintained throughout the remainder of the study on the daily 15-min drinking period.

The first day of the animals' third week in the laboratory, designated "Day 0," was the day of conditioning. On Day 0, 50 conditioned animals received a 0.1% sodium saccharide solution (SACCH), which was followed 30 min later by an intraperitoneal (IP) injection of cyclophosphamide (CY) at a dose of 50 mg/kg ranging in volumes from 0.5 to 0.8 cc. Twenty nonconditioned animals received water followed by IP CY, and 10 placebo animals received water followed by IP saline (SAL) in volumes equal to the CY injections.

*Assistant Director, Psychiatry Division, Peter Bent Brigham Hospital, and Instructor in Psychiatry, Harvard Medical School, Boston, Massachusetts.

**Director, Psychiatry Division, Peter Bent Brigham Hospital, and Associate Professor of Psychiatry, Harvard Medical School, Boston, Massachusetts.

†Associate in Medicine, Peter Bent Brigham Hospital, and Assistant Professor of Medicine, Harvard Medical School, Boston, Massachusetts.

‡Director, Immunology Laboratory, Renal Division, Peter Bent Brigham Hospital, Associate Professor of Medicine, Harvard Medical School, and Investigator, Howard Hughes Medical Institute.

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Address reprint requests to: Malcolm P. Rogers, M.D., Peter Bent Brigham Hospital, 721 Huntington Avenue, Boston, Massachusetts 02115.

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TABLE 1. Experimental Treatments

Group	Day 0		Subgroup	N	Day 3		Day 6	
	PO	IP			PO	IP	PO	IP
Conditioned (N = 50)	Saach.	CY	CS ₀	10	H ₂ O	Sal.	H ₂ O	—
			US	10	H ₂ O	CY	H ₂ O	—
	CS ₁	10	H ₂ O	Sal.	Sacch.	—		
	CS ₁	10	Sacch.	Sal.	H ₂ O	—		
	CS ₂	10	Sacch.	Sal.	Sacch.	—		
Nonconditioned (N = 20)	H ₂ O	CY	NC ₁	10	Sacch.	Sal.	Sacch.	—
			NC ₂	10	H ₂ O	—	H ₂ O	—
Placebo	H ₂ O	Sal.	P	10	H ₂ O	Sal.	H ₂ O	—

On Day 3, 30 min before their drinking period began, all animals were immunized with IP injections of antigen, 2 ml/kg of a 1% thrice-washed suspension of sheep red blood cells (SRBC) of approximately 3×10^8 cells/ml (Baltimore Biological, Baltimore, Md.). The further experimental interventions made on Days 3 and 6 are summarized in Table 1. The conditioned animals were subdivided into five separate groups: a group that received no further SACCH (CS₀), a group that received a second injection of CY (US), two groups that received one further SACCH, one on Day 3, and the other on Day 6 (CS₁), and a group that received two further SACCH exposures (CS₂). The nonconditioned animals were divided into two groups, one controlling for the effects of CY and SACCH alone (NC₁), and the other for the effects of CY and injections alone (NC₂). The placebo group, the only group that received no CY, also provided a control for the effect of injections alone.

On Day 9, all animals were killed by decapitation with a guillotine. Trunk blood was collected in heparinized tubes. These were centrifuged and the serum was collected and frozen for storage. The serum was heat inactivated at 57°C for 30 min prior to determinations of hemagglutinating antibody (Ab) titers by the hemagglutinating microtiter method (6).

RESULTS

Figure 1 shows that significant taste aversion occurred in the two CS₁ groups as indicated by decreased intake of fluid upon subsequent presentation of the con-

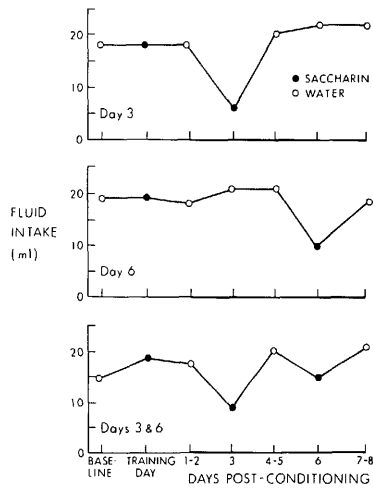


Fig. 1. The effect of conditioning on fluid intake. The mean intake of water is indicated by open circles (○). Intake of SACCH is indicated by closed circles (●). SACCH was given to all above groups on the day of conditioning, and then either on Day 3 (top graph) or Day 6 (middle graph) (CS₁ groups) or on both Days 3 and 6 (bottom graph) (CS₂ group).

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ditioned stimulus ($P < 0.001$). In the CS_2 group, SACCH consumption was significantly reduced on Day 3 ($P < 0.01$), but not on Day 6, indicating extinction of the taste aversion response *per se*.

Figure 2 summarizes the anti-SRBC antibody titers discerned in the various groups. The mean value for the placebo group, which received no CY, was 7.3. The mean value for the US group, which received two exposures to CY, was 3.1. Using a one-way variance analysis, and Duncan's Multiple Range test, the mean values of both groups were found to differ

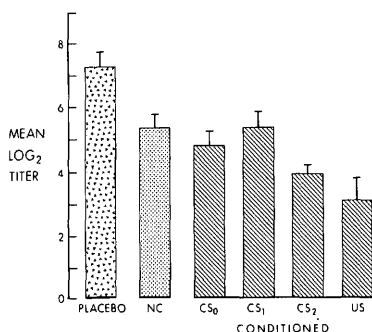


Fig. 2. The effect of conditioning on hemagglutination titers. The hemagglutination titers (means \pm SEM) are expressed as powers to the base 2. The titers were obtained 6 days after IP injection of antigen (SRBC). Placebo animals received IP saline instead of cyclophosphamide (CY). NC = nonconditioned animals provided with saccharin (SACCH) or water on Days 3 and 6. CS_0 = conditioned animals that did not receive SACCH following conditioning. CS_1 = conditioned animals given a subsequent exposure to SACCH on Days 3 or 6. CS_2 = conditioned animals exposed to SACCH on Days 3 and 6. US = unconditioned animals given a second IP injection of CY on Day 3.

significantly from those of each other groups ($P < 0.01$). The only exceptions to this were that the US group (3.1) did not differ significantly from the CS_2 group (3.9), and the degree of difference from the CS_0 group (4.8) was significant at a $P < 0.05$ rather than 0.01.

The mean antibody titer of the CS_2 group (3.9) was significantly lower than the mean titer of the control groups (5.1). This difference was analyzed using a one-tailed *t* test and was found to be significant ($P < 0.01$). The appropriate controls included the animals that had received only one CY injection and no exposures to SACCH after conditioning (CS_0) and those that were not conditioned (NC). In the analysis of the data, the two NC groups (antibody titers of 5.1 and 5.4) were treated as one NC control group of 20 animals; the two CS_1 groups (both with antibody titers of 5.3) were also treated as one group of 20 animals.

DISCUSSION

Our results essentially replicate the findings of Ader and Cohen (1). The saccharin exposures in conditioned animals have a clear immunosuppressive effect when two exposures occur. The major difference between our findings and those of Ader and Cohen is that we observed an increase in immunosuppression only after two exposures to the CS, while Ader and Cohen found significant changes after one exposure as well. Their results are summarized in Fig. 3.

Our use of the more sensitive hemagglutination microtiter method of determining antibody titers provided a more rigorous quantitation than the hemagglutinating method used by Ader and Cohen. This methodological departure

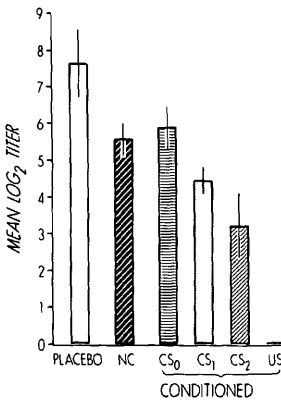


Fig. 3. Hemagglutination titer results of Ader and Cohen (1). Hemagglutination titers (means \pm SE) obtained 6 days after IP injection of antigen (SRBC). NC = nonconditioned animals provided with saccharin on Days 3 or 6. CS₀ = conditioned animals that did not receive saccharin following antigen treatment. CS₁ = conditioned animals given one exposure to saccharin on Days 3 or 6. CS₂ = conditioned animals exposed to saccharin on Days 3 and 6. US = conditioned animals injected with cyclophosphamide following treatment with antigen. (Ader and Cohen, *Psychosomatic Med*, Vol. 37, No. 4, July-Aug. 1975. Reproduced with permission of the authors.)

may account for the higher titers in the US animals found in our study, especially since the technique permits detection of low antibody titers.

Crucial issues concerning the mechanisms and nature of behaviorally conditioned immunosuppression remain unresolved. Since the production of anti-SRBC IgM antibody is not markedly effected by the influence of thymus derived (T) lymphocyte, i.e., helper or immunosuppressive, whereas IgG antibody production is modulated by regulatory T cells (7), it will be important to discern any possible differences in the generation of IgM and IgG anti-SRBC titers. Does behavior modification of immunity induce a selective lesion in the production of antibodies to antigen utilized during conditioning or does conditioning provoke a broad immunodepressed state? Is the conditioned state dependent on neuroendocrine pathways?

It is of interest that the conditioned immunosuppressive response is augmented by a second exposure to the saccharin solution, while the conditioned behavioral response (reduced drinking of saccharin solution) is weakened. The dissociation between the responses suggests that some internal response is triggered by the saccharin that is independent of the overt drinking behavior.

Further research in our laboratory will attempt to answer many of the interesting questions raised by the phenomenon of behaviorally conditioned immunosuppression.

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