Immunological Specificity of Classically Conditioned Immunoenhancement

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Abstract: Mice were conditioned to respond immunologically to either sheep red blood cells (SRBC) or chicken red blood cells (CRBC) using an altered taste aversion conditioning paradigm. Two groups of 11 mice each were provided with regular tap water and an additional two groups of the same size were provided with water containing saccharin and lithium chloride (conditioned stimulus). Each group was injected with either SRBC antigen or CRBC antigen on day one and injected with the opposite antigen 36 hours later without the conditioned stimulus. These two experimental groups were then compared to their respective control group which was treated identically to the corresponding experimental group without the conditioning paradigm. On day 14, the serum from each mouse was taken, and the antibody titer against each antigen was measured by a hemagglutination assay. Mice conditioned to SRBC demonstrated an average increase in anti-SRBC titer of 100%, while the titers of mice conditioned with CRBC showed no significant difference. These results indicate that immunoconditioning occurs only with certain types of antigens. Also, the antibody response to the conditioned antigen does not seem to have an effect on the titer of another antigen simultaneously injected without the conditioned stimulus. Therefore, it is apparent that the psychoneuroimmunological conditioning mechanism behind immunoenhancement differed for the SRBC response as compared to the CRBC response.

Since the mid-1920s, experimental conditioning of the immune system has been conducted in the manner described by Pavlov in his experiments with classical conditioning of salivation in dogs. Most of this research took place in the Soviet Union prior to the 1970s (Brittain and Weiner, 1985). In 1975, Ader and Cohen, of the United Stated, discovered that the immune system could be conditioned to be suppressed. The original experiment was intended to explore the notion of taste aversion. Rats were given saccharin (conditioned stimulus) along with cyclophosphamide (unconditioned stimulus), which causes gastric pain and immunosuppression, in order to condition the rats to dislike the normally favored taste of saccharin. Indeed, taste aversion was demonstrated, but a curious thing occurred. The conditioned animals began to die, while the control rats remained alive and healthy. Ader and Cohen then measured the immunological state of the conditioned rats and found that not only had their taste preference been conditioned, but their immune systems had also been conditioned to be suppressed (Ader and Cohen, 1975).

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This discovery was followed by an abundance of research in the conditioning of the immune system by way of the central nervous system (e.g., Jenkins, 1983; Kusnecov et al., 1983; Bovbjerg et al, 1984; MacQueen and Siegel, 1989). The new area of research was soon named psychoneuroimmunology. The main focus of this research has been on the conditioning of immunosuppression which has great promise in the area of host rejections in transplant surgeries. On the other hand, a good deal of research has shown that immunoenhancement may be conditioned as well (Ader and Cohen, 1985; 1991).

In this experiment, mice were injected with sheep red blood cells to determine whether or not conditioned immunoenhancement could be demonstrated. From previous research, the response to the sheep red blood cells was expected to show a greater antibody response when the antigen injection was given under a classical conditioning paradigm than when the sheep red blood cells were injected without conditioning (Jenkins et al., 1983).

A conditioned stimulus of drinking water supplemented with saccharin coupled with a lithium chloride injection, which provided a sweet taste followed by gastrointestinal upset, was utilized because of its previously determined effectiveness in other classical conditioning paradigms involving immunoenhancement (Jenkins et al., 1983). As with all classical conditioning stimuli, this stimulus was created with the intent of emulating a natural circumstance, such as eating a foul substance might be followed by the need to produce a specific type of antibody.

Additionally, it was hypothesized that an animal, when injected after the conditioned stimulus had subsided with a similar antigen--chicken red blood cells-- would show an equal amount of antibody response against chicken red blood cells as would an animal given both antigens without any type of conditioning paradigm. This hypothesis may help clarify the mechanism behind immunoconditioning by showing that the immune response produced occurs specifically with the type of antigen introduced rather than enhancing the entire immune system.

MATERIALS AND METHODS

In this experiment, Charles River CD1 mice were caged in same-sex groups of about four individuals each under a twelve hour light-dark cycle. Regular grade Purina rodent chow was provided *ad libitum.* Water was given at approximately the same time every day for fifteen minutes in order to train the mice to drink in a short period of time.

The mice were randomly assigned into four groups of eleven and acclimated to one another for at least two weeks. The first group received a ten percent solution of Sprinkle Sweet during their drinking period followed by an IP injection of lithium chloride (128 mg/kg in a volume of 20 mL/kg) and sheep red blood cells (SRBC) [2 mL/kg of 1% SRBC (Sigma R-3378) in PBS pH 7.2 (5 mM Na_2PO_4, 3 mM KPO_4, 148 mM NaCl)]. After thirty-six hours, the mice were given an IP injection of chicken red blood cells (CRBC) [2 mL/kg of 1% CRBC (Sigma R-0504) in PBS pH 7.2]. On the seventh and ninth day of the experimental period, the mice were given only the saccharin water followed by the lithium chloride injection. The second group was treated the same with the exception that the two types of antigen were reversed. The third group was given the sheep red blood cells and then the chicken red blood cells after thirty-six hours, but their drinking water remained unaltered and no lithium chloride injection followed. The fourth group was treated like the third with the exception that

the antigen order was once again reversed.

On the fourteenth day, all forty-four mice were killed via the cervical break method. The thorax of each mouse was cut horizontally and blood was collected in nonheparinized 50 mL capillary tubes at the posterior, dorsal area of the thorax. An average of seven tubes of blood was taken from each animal. The capillary tubes were then spun on a clinical centrifuge at the "blood" speed. The tubes were broken just below the serum phase and blown out with a capillary bulb into microcentrifuge tubes, which were placed in storage at four degrees centigrade. A chart of these procedures can be found in Table 1.

Table 1. Experimental procedures for the treatments for each of the groups with and without the conditioning paradigm. Groups 1 and 2 demonstrate the conditioning paradigm, while Groups 3 and 4 are control groups and do not feature the conditioning paradigm. Sac: Saccharin; LiCl: Lithium Chloride; SRBC: sheep red blood cells; CRBC: chicken red blood cells; Ab?: mice were sacrificed and the serum collected followed by the hemagglutination assay to measure the antibody titer against each antigen.

Group	N	Day 1	36 hours	Days 7 and 9	Day 14
1	11	Sac/LiCl-SRBC	CRBC	Sac/LiCl	Ab?
2	11	Sac/LiCl-CRBC	SRBC	Sac/LiCl	Ab?
3	11	Tap H_2O-SRBC	CRBC	Tap H_2O	Ab?
4	11	Tap H_2O-CRBC	SRBC	Tap H_2O	Ab?

A second mini-experiment was performed by using two groups of ten. One group was given the saccharin/lithium chloride stimulus without any initial antigen injection. Then, thirty-six hours later this group was given a sheep red blood cell injection as described above. On days seven and nine, the mice were once again given the saccharin/lithium chloride stimulus. The second group was only given the sheep red blood cell injection at the same time as the first. Blood was collected, separated, and stored as above.

A few days after the samples were collected, antibody titers were measured by a hemagglutination assay. The assay was derived from outside sources (Garvey et al., 1977) The samples were first diluted using one part serum and four parts PBS, pH 7.2. Microtiter plates were filled with 0.3% SRBC and 0.4% CRBC in alternating rows. Then, the diluted samples were coded, mixed up, and placed in a microcentrifuge rack at random. Using the diluted serum samples, serial dilutions of each individual mouse were then performed across the microtiter plates lengthwise noting which tube went into which row. The plates were then gently shaken and allowed to stand for 70 minutes. After the standing time, the plates were read by recording the last well containing detectable agglutination, otherwise known as the endpoint. Sources containing photographs of these endpoints were consulted prior to reading the plates (Garvey et al., 1977; Roitt et al., 1985). The serial dilutions caused the titers to increase exponentially. In order to convert from this exponential scale to a linear, interval scale to satisfy conditions for analysis of variance, titers were recorded as reciprocals

of the endpoint dilutions expressed as powers of the $base_2$ (Ader and Cohen, 1975). These results were finally decoded and interpreted using SYSTAT statistics software.

A MANOVA was first performed on all groups to detect any significant variance among the groups. This statistical technique should have revealed any interaction that may have been occurring among any of the groups. Next, Tukey HSD pairwise comparisons were utilized to give actual differences between any two groups. An independent t-test was used to determine the significance of the differences between the two groups in the mini-experiment.

RESULTS

The overall immune response to sheep red blood cells was greater than the response to chicken red blood cells (Table 2 and Appendix). The MANOVA showed that effects of different treatments were significant when anti-SRBC titers were measured ($p = 0.005$), but not for anti-CRBC titers ($p = 0.345$). A one-way ANOVA revealed significant differences between the anti-SRBC responses of treatment groups ($p = 0.029$).

Table 2. Average antibody titers (mean + or - SD) for four experimentally conditioned groups of laboratory mice. Treatments as in Table 1.

Treatment	N	Mean Titer	
		SRBC	CRBC
1	11	10.140 (1.079)	8.958 (0.674)
2	11	9.504 (0.751)	8.958 (1.027)
3	11	9.140 (0.874)	8.776 (1.036)
4	11	10.140 (0.874)	9.322 (1.000)

All post-hoc comparisons among groups shown by using Tukey HSD pairwise comparisons are given in Table 3. As was expected, conditioning to SRBC was evident, although conditioning to CRBC was not observed. Unexpectedly, the anti-SRBC titers in the control groups showed a noticeable difference. The mini-experiment showed that the 36 hour delay was long enough to avoid any delayed conditioning of the anti-SRBC response (t-test, $p = 0.72$).

Table 3. Results of Tukey HSD pairwise comparisons between mean anti-SRBC values of each group defined in Table 1 and 2.

	Group 1	Group 2	Group 3	Group 4
Group 1	1.000			
Group 2	0.361	1.000		
Group 3	0.060	0.781	1.000	
Group 4	1.000	0.361	0.060	1.000

DISCUSSION

Data gathered in this experiment seem to have very little variance at first glance. Out of the eighty-eight individual samples, the converted titers only show a range of five. With serial dilutions, the dilutions progressed along exponentially from a starting dilution of 1:5 (e.g., 1:10, 1:20, 1:40....1:20, 480). The conversion gives the reciprocal of the titration values expressed as a power of 2. In other words, the mean concentration differences of the conditioned SRBC group and nonconditioned SRBC group were actually around 1/724 and 1/1176. The ratio of the reciprocal of these main concentrations is 1.6. Therefore, the anti-SRBC titers in the conditioned group with SRBC given first was actually 60% greater than the nonconditioned group with the SRBC given first.

Therefore, results obtained in this experiment seem to support previous research by indicating that the antibody response to sheep red blood cells can be conditioned. In past experiments, conditioning was done using only one antigen on one animal. Furthermore, most of this research has been done by actually conditioning the suppression of the immune system (Ader and Cohen, 1985; Brittain and Weiner, 1985; Dunn, 1988). The evidence in this experiment seems to indicate that conditioning to SRBC can be elicited without simultaneously causing the elevation of an antibody titer to an antigen injected without conditioning.

On the other hand, the antibody response to chicken red blood cells seems to lack this ability in the reverse circumstance. The immunological pathway that responds to chicken red blood cells may not be subject to conditioning, whereas the response to sheep red blood cells does show this phenomenon. Previous research has indicated that only T-cell dependent antigens, like red blood cells, may be able to elicit conditioned responses (Wayner et al., 1978). The reason for this kind of specific conditionability may have to do with the T-cell's role in the neuroendocrine system. T-cells are believed to not only produce chemical messengers, but also react to them with a much greater sensitivity than the rest of the immune system. Some of these chemical messengers may even be neurotransmitters, coming directly from the nervous system, and communicate via T-cell's beta adrenergic receptors (Rosman and Carlson, 1991). The presence of these receptors has even been

shown to have an influence in Pavlovian conditioning of the immune system (Lysle et al., 1991). Therefore, the lack of conditioning found in the response to chicken red blood cells may be due to of the T-cell. Unfortunately, data on mechanism by which antibodies are produced towards chicken red blood cells was not available. Another possibility may be that the sheep red blood cell response is masking chicken red blood cell response. Further data, collected by conditioning the chicken antigen alone, may clarify whether or not masking effect was occurring.

The control groups in this experiment surprisingly showed a distinct difference between each other with respect to anti-SRBC response. In fact, this difference was equally as great as the difference found between the conditioned and nonconditioned groups. This difference was most likely caused by the reverse order of the SRBC and the CRBC. Since the SRBC were given first in Group 3 (nonconditioned control group), the antibody titer against this antigen had probably already began to naturally decline from peak antibody levels. In Group 4 (nonconditioned control group), the SRBC were given 36 hours later which caused the fourteenth day to be closer to the peak time for antibodies against this antigen. As seen in Table 2, this point does not hold true for the CRBC but the statistical analysis does not indicate that these differences are significant. Therefore, the lag time effect described along with chance elevation in general titer levels in Group 4 was probably responsible for the difference in anti-SRBC between the two control groups.

In the mini-experiment, the thirty-six hour delay appeared to be enough to prevent any coupling with the conditioned stimulus. Therefore, studies using this type of design seem not to need a delay time any longer than thirty-six hours.

This experiment seemed to demonstrate that the mechanism of immunoconditioning may be narrowed at least to the extent of the involvement of T-cell interaction. In addition to the needed data described above, future research may also use a known T-cell dependent antigen such as ovalabumin and a known T-cell independent antigen such as *Brucellus abortus* with sheep red blood cells in order to better clarify the involvement of T-cells in immunoconditioning.

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APPENDIX

Table 4. Antibody response to Sac/LiCl immunoconditioning in laboratory mice. Treatments: 1 - Sac/LiCl, SRBC first, CRBC second; 2 - Sac/LiCl, CRBC first. SRBC second; 3 - No Sac/LiCl, SRBC first, CRBC second; 4 - No Sac/LiCl, CRBC first, SRBC second. Antibody titers were determined using SRBC and CRBC and are reported as log base$_2$ rounded to the nearest whole number.

Animal	Treatment	Antibody Titer	
		SRBC	CRBC
1	1	11	8
2	1	11	9
3	1	9	8
4	1	9	8
5	1	9	9
6	1	9	9
7	1	12	10
8	1	9	9
9	1	9	8
10	1	10	8
11	1	10	9
12	2	9	8
13	2	9	8
14	2	9	8
15	2	8	8
16	2	10	10
17	2	9	9
18	2	10	10
19	2	10	10
20	2	9	8
21	2	8	7
22	2	10	9
23	3	11	10
24	3	9	8
25	3	9	9
26	3	9	9
27	3	8	7
28	3	8	9
29	3	8	7
30	3	8	8
31	3	9	8
32	3	9	8

Table 4. continued.

Animal	Treatment	Antibody Titer	
		SRBC	CRBC
33	3	9	10
34	4	9	9
35	4	10	9
36	4	11	10
37	4	9	8
38	4	10	9
39	4	10	9
40	4	11	8
41	4	11	11
42	4	9	8
43	4	9	9
44	4	9	8