A Simple, Single, Trial-Learning Paradigm for Conditioned Increase in Natural Killer Cell Activity (43347)

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Abstract. **A change in natural killer (NK) cell activity can be conditioned with one trial learning when conditioned stimulus (CS) precedes the unconditioned stimulus (US). To avoid the problems associated with two reexposures in our earlier studies, we have developed a reliable and simple conditioning protocol utilizing the one trial learning and one reexposure to the odor CS. The conditioned change in NK cell activity was significantly different ($P < 0.05$) from the control groups of mice. The paradigm is short and simple in that the conditioned change could be demonstrated within 3 days. We have also compared the effects of temporal association of CS and US on conditioned increase in NK cell activity. Forward conditioning (CS preceded the US) demonstrated a conditioned change, but the backward conditioning protocol did not. The paradigm provides a reliable approach to the study of mechanisms of the phenomenon of odor-NK conditioning.** [P.S.E.B.M. 1992, Vol 199]

The procedures of pavlovian conditioning provide the means for reliably demonstrating central nervous system (CNS) control over natural killer (NK) cell activity and possibly over other immune responses. Studies that deal with conditioned tolerance of drug-induced NK cell activity have been reported by Dyck *et al.* (1). They have used a protocol in which mice were given four weekly injections of polyI:C paired with a complex environmental stimulus where a reliable tolerance to polyI:C was observed. Earlier studies from our laboratory demonstrated the conditioning of a change in the NK cell response by one trial pairing of polyI:C, the unconditioned stimulus (US), with either saccharin and LiCl (2) or camphor (3) as the conditioned stimulus (CS). The model was particularly effective in that only one CS/US association was required on Day 0 and two CS reexposures were given either on Days 3 and 5 or 7 and 9. It was also found that a suboptimal dose of polyI:C injected at the time of the last CS exposure enhanced the magnitude of the response. The effects of the pairing are odor specific and do not represent sensitization to novel odors (4). While the conditioning protocol was simple and could be used to study the neuronal links between the CNS and immune system (5, 6), the requirement of two CS signals along with a suboptimal dose of polyI:C was an added complication. A shorter conditioning protocol that is simple to implement and that consistently reproduces the conditioned response would be important for studying the neuronal links between the CNS and immune system. This report describes a new, highly reproducible paradigm in which the conditioned enhancement of the NK cell response can be established with one CS/US association and in which the conditioned response can be elicited with one CS exposure, without the suboptimal dose of polyI:C. The model provides a conditioning paradigm that allows identification of the mediators and possible pathways used by the CNS in the communication between it and the immune system.

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Received April 3, 1991. [P.S.E.B.M. 1992, Vol 199]
Accepted September 16, 1991.

0037-9727/92/1992-0199$3.00/0

Materials and Methods

Mice. BALB/c female mice 6–8 weeks of age, weighing 20–24 g, were used in these studies. The mice were obtained from the National Cancer Institute (Frederick, MD). All mice were kept in standard animal

facilities with a 12:12-hr light:dark cycle and were provided food and water *ad libitum*. The mice were allowed to adapt to our facilities for at least 1 week prior to performing the experiments.

Conditioning Procedure. All conditioning procedures (association and reexposure) and the sacrificing of the animals to perform NK cell activity were started between 7:00 and 7:30 AM. The exposure to the odor stimulus and treatment with US were completed by 8:30 AM. The lights were on at 7:00 AM and off at 7:00 PM. The procedures were performed as soon as the lights were on to accommodate the diurnal cycle and low corticosteroid levels. A 1-oz block of camphor was partially dissolved in mineral oil (one block to about 150 ml of mineral oil) while stirring on low heat. Camphor exposure was carried out inside a cabinet in a different laboratory. Thirty milliliters of the camphor-mineral oil mixture in a small glass container were heated in a microwave oven for 1 min and then placed upon the cage top. Another empty cage was inverted over the cage holding the animals to contain the camphor odor. This was done inside the cabinet, away from where the other animals were housed, and care was taken to prevent the camphor odor from reaching the control animals. Mice were exposed to camphor odor in this way for 1 hr. PolyI:C was obtained in lyophilized form from Pharmacia (Uppsala, Sweden) and dissolved in sterile physiological saline (PSS) at 200 μg/ml and stored at 4°C, and 0.1 ml (20 μg) was given intraperitoneally for each mouse within 5 min following the removal from the camphor-containing cage. The mice were allowed to stay for about 3 hr in the cabinet room before they were returned to the vivarium.

Preparation of Spleen Cells. Animals of each group were sacrificed simultaneously in a box with CO_2 asphyxiation. The animals were sacrificed before 8:00 AM. It took only 5–10 min to sacrifice all four groups (CND, conditioned; NC, nonconditioned; CNDo, conditioned not reexposed to CS; and US, unconditioned). Spleens were removed immediately and placed into individual petri plates containing sterile PSS on ice. The spleen cells were expelled from the spleen sac with the help of a forceps and needle. The single-cell suspension was collected with a 23-gauge needle and a 3-ml syringe into a sterile, 15-ml tube. The tubes were filled with PSS and centrifuged at 2000 rpm for 5 min at 5°C in a Beckman centrifuge. The supernatant was discarded and the washing was repeated once more. The pellet was suspended with 1 ml of sterile PSS with a sterile pasteur pipette to remove the debris. Spleen cell counts were made in a Coulter counter following lysis of red blood cells with saponin. The whole spleen cells (with red blood cells) were used in the NK cell assay.

Assay for NK Cell Activity. YAC-1 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 100 units of penicillin, 100 μg of streptomycin, and 5×10^{-5} *M* 2-mercaptoethanol. The YAC-1 cells were cultured into fresh tissue culture flasks with fresh medium 24 hr before harvesting for the assay. With this procedure, the viability is >95% and the spontaneous release in the chromium release assay is <10%–15%. YAC-1 cells were labeled with sodium chromate (Amersham, Chicago, IL) at a ratio of 100 μCi/1 $\times$ 10^6 cells in a very small volume (total vol 0.2 ml) at 37°C in a CO_2 incubator for 30 min. The cells were washed with a large excess of medium twice and suspended at a final density of 1×10^5 cells/ml in RPMI 1640 supplemented with 5% fetal calf serum. Spleen effector cells (0.1 ml) at ratios of 200:1, 100:1, and 50:1 (effector cell to target cell [E:T] ratio) were mixed in triplicate wells with 0.1 ml of 1×10^4 ^{51}Cr-labeled YAC-1 target cells in 96-well, flat-bottomed microtiter plates (Linbro Scientific Co., Hamden, CT). Plates were incubated for 4 hr in a humidified, 37°C, CO_2 incubator. Supernatant (0.1 ml) from each well was collected after centrifugation of plates. The radioactivity of the samples was counted in a Beckman gamma counter. Maximum ^{51}Cr release from the target cells (MR) was measured after incubation in the presence of 0.2% Triton X-100 (Sigma Chemical Co., St. Louis, MO) and spontaneous release (SR) in the presence of medium. The percentage of specific ^{51}Cr release was calculated as 100 times ([test release-SR]/MR-SR).

Statistical Methods. The data were analyzed by two-factor analysis of variance, with repeated measures on one factor with an α-level of 5%. The non-repeated-measures factor was treatment group and the repeated-measures factor was the E:T ratio. To test the null hypothesis that the means of the treatment groups are not different, the F statistic was calculated. The value of F was compared with the critical value of F, with the degree of freedom associated with the treatment group as numerator and the degree of freedom associated with the residual variation among subjects within the treatment group as denominator. In addition, the Duncan test was used for the multiple comparisons of all possible pairs of means, with an overall significance level of 5% adjusted for multiple testings.

Results

The first series of experiments compared the NK cell activity of four groups (CND, CNDo, NC, and US) of animals. On Day 0, the CND and CNDo groups were conditioned by exposing the animals to the CS (odor of camphor for 1 hr), after which the animals were injected with the US (polyI:C 20 μg ip). The NC and US groups were injected with polyI:C only on this day. On Day 2, the CND and NC groups were exposed to the odor of camphor only and the animals were assayed for NK cell activity 24 hr later (Table I). The data reported in Table II compared the NK cell response to camphor odor of a CND group that had

Table I. Schedule for One Trial Learning of Odor CS and PolyI:C US Paradigm

Groups	*n*	Treatment days		
		0	2	3
CND	11	C + P[a]	C	NK
CNDo	8	C + P	—	NK
NC	10	P	C	NK
US	9	P	—	NK

[a] C, camphor odor for 1 hr in an enclosed environment; P, polyI:C 20 μg ip/mouse.

Table II. NK Cell Activity of One Trial-Learning Paradigm Mice

Groups	*n*	Percentage of ^{51}Cr-released, E:T ratio		
		200:1	100:1	50:1
CND	11	22.9 ± 1.1[a]	18.3 ± 1.0	12.7 ± 0.8
CNDo	8	18.7 ± 1.3	14.2 ± 3.0	10.3 ± 0.9
NC	10	18.5 ± 1.3	14.5 ± 1.0	10.5 ± 0.7
US	9	13.6 ± 1.0	10.7 ± 0.7	7.0 ± 0.5

[a] Values are mean ± SE. NK cell activity of the four groups was analyzed for differences and similarities with repeated-measures analysis of variance (ANOVA), with Duncan's multiple range test with a significance level of 5%. CND was significantly different from CNDo, NC, and US groups, $P < 0.05$. CNDo and NC groups were significantly different from the US group, $P < 0.05$.

previously received a single pairing of camphor and polyI:C to three control groups (CNDo, NC, and US). The CND group had the highest percentage of specific ^{51}Cr released at all E:T ratios tested. The CND group differed reliably from the CNDo, NC, and US groups (Table II). We have demonstrated in earlier studies that camphor odor itself has no stimulatory action on NK cell response (3). Since in this experiment, the NK cell activity of the US control group was statistically lower than that of the CNDo and NC control groups, a second experiment was done in which CND, NC, and US groups were compared. The schedule used was exactly the same as shown in Table I, except in this experiment, the CNDo group was omitted. There were 10 mice per group. These results again showed a conditioned change in NK cell activity of the CND group in comparison with the NC and US groups (Table III). This time there were no significant differences between the NC and US groups, while the conditioned response was consistently reproducible. Occasionally, statistically significant differences were seen among the control groups (NC and US), but these differences were not consistently present or reproducible from experiment to experiment.

The phenomenon of conditioned reflex is usually attributed to a forward temporal association between the CS and US, where the CS was preceded by the US treatment with short intervals between the CS and US (7, 8). In our paradigm, it was of further interest to determine whether the change in NK cell activity could

Table III. NK Cell Activity of CND, NC, and US Groups of a Repeat Experiment Using the Table I Schedule

Groups	*n*	Percentage of ^{51}CR-released, E:T ratio		
		200:1	100:1	50:1
CND	10	25.3 ± 0.9[a]	22.2 ± 1.1	15.1 ± 0.7
NC	10	22.3 ± 1.5	18.1 ± 1.3	12.9 ± 0.9
US	10	20.0 ± 0.7	15.4 ± 0.6	11.5 ± 0.5

[a] CND versus NC, $P < 0.05$; CND versus US, $P < 0.05$; NC versus US, not significant. NK cell activity of the CND, NC, and US groups was analyzed with repeated-measures analysis of variance with Duncan's multiple range test with a significance level of 5%.

be conditioned by varying the interval between the CS exposure and the US treatment. In this experiment, four groups were used. One group of mice (Day −1) were exposed to camphor odor 1 day prior to the treatment with the US. On Day 0, the CND group was exposed to camphor for 1 hr and injected with polyI:C immediately (2–3 min) after the CS. All of the animals in the other groups were injected with polyI:C on this day. On day +1, the backward-conditioning group mice were exposed to the odor of camphor for 1 hr, i.e., 1 day after US treatment. On Day 2, the CND, Day −1, Day +1, and NC groups were exposed to the odor of camphor. All animals were tested for their NK cell activity on Day 3 (Table IV). The results show that the CND group had considerable increase in NK cell activity in comparison with the Day +1 or NC groups (Table V). Mice in the Day −1 group also demonstrated a significant increase in NK cell activity. On the other hand, the Day +1 mice (backward conditioning) showed a limited change in NK cell activity. The Day +1 group showed a response similar to those of the NC groups that was significantly lower than those of the CND or Day −1 groups.

Discussion

The procedures of pavlovian conditioning provide the means for demonstrating CNS control over an immune response. This control was observed in terms of enhancement (3, 9, 10) and suppression of immune responses in a number of systems (1, 11, 12). Identification of the pathways used by the CNS and the immune system to maintain this communication with one another requires a conditioning paradigm that is easy to perform and that produces conditioned changes in immune function with high reliability. We have chosen the NK cell system for our conditioning studies because NK cells are a first line of defense in that they lyse tumor cells or virus-infected cells without the need for prior immunization. They play a significant role in natural immunity to tumors (13, 14), antibacterial resistance (15), and antiviral responses (16). Although relatively little is yet known about the underlying mech-

Table IV. Schedule for Testing the Effect of Different Times of Exposure to CS and Treatment with US

Groups	*n*	Treatment days				
		−1	0	1	2	3
CND	10	—	C + P[a]	—	C	NK
Day −1	10	C	P	—	C	NK
Day +1	9	—	P	C	C	NK
NC	7	—	P	—	C	NK

[a] C, camphor odor for 1 hr in an enclosed environment; P, polyI:C 20 μg ip/mouse.

Table V. Effect of Varying the Time between CS Exposure and US Treatment for CS/US Association on Conditioned Change in NK Cell Activity

Groups[a]	*n*	Percentage of ^{51}CR-released, E:T ratio		
		200:1	100:1	50:1
CND	10	19.9 ± 0.9[b]	17.3 ± 0.8	13.5 ± 0.9
Day −1	10	20.6 ± 1.2	17.0 ± 1.1	12.7 ± 0.8
Day +1	9	15.8 ± 0.9	12.7 ± 2.2	9.3 ± 0.4
NC	7	14.5 ± 1.2	13.0 ± 1.1	8.1 ± 0.9

[a] CND and unpaired Day −1 groups were significantly different from unpaired Day +1 and NC groups, $P < 0.05$, using repeated-measures analysis of variance with Duncan's multiple range test with a significance level of 5%.
[b] Values are mean ± SE.

anisms, it has been demonstrated that NK cell activity can be both suppressed and enhanced by the CNS (17, 18).

We have used the pairing of camphor odor and the injection of polyI:C to condition the changes in NK cell response in a pavlovian conditioning paradigm. PolyI:C, the unconditioned stimulus, is a double-stranded synthetic RNA that mimics infection by the double-stranded RNA virus. PolyI:C induces the expression and secretion of α- and β-interferons (19). The interferons, in turn, directly stimulate NK cell activity (20). The kinetics of this response is ideal for conditioning studies because following polyI:C injection, NK cell activity peaks within 24 hr (21) and returns to baseline within 3–5 days (22). In addition, the evidence that the CNS and immune system can signal these cells is shown by the fact that neurotransmitters and lymphokines influence NK cell activity (17, 18). For example, NK cell function is suppressed by exposure to β-adrenergic agonists or prostaglandins (23–26). Sympathetic nervous system interaction with NK cells is shown in that permanent ablation of the sympathetic nervous system with 6-hydroxydopamine also caused a significant temporal increase and decrease of the NK cell activity (27). That cells of the immune system also influence NK cell activity is shown by enhanced interaction with α- and β-interferon and interleukin 2, which are produced by macrophages and T helper cells (28), respectively.

In our standard procedure for conditioning of the NK response, the interval between odor presentation and polyI:C injection is usually 3–5 min, although the time of the effective US is unknown. In pavlovian paradigms, the strength of the association is affected by the temporal relationship between the CS and the US. The specific interstimulus intervals that yield the strongest conditioning vary with the organism and response studied (29). When the US precedes the presentation of the CS, as in backward conditioning, learning is poor. When the CS precedes the US by increasingly longer time intervals, the probability of a conditioned response also declines (29). The important features of the present paradigm were the single reexposure to odor stimuli and the absence of a suboptimal dose of polyI:C. The paradigm described here is an improvement in that it also uses one CS/US association and only one CS exposure, without the need for a suboptimal dose of polyI:C. The model was tested several times using variations of the same schedule. Interesting results were observed when the CS and US were given at different times and in a different order. Animals exposed to the CS 1 day before the US demonstrated a conditioned change in NK cell activity, as did the CND group, in which the CS/US association was made on the same day. This conditioned change was evident whether the animals were tested where the CND and Day −1 animals were kept in the same cage or where both groups were kept separated. Under either situation, both groups were conditioned to the same extent. However, the unpaired Day +1 group showed a NK cell response similar to that of animals in the NC group. Whereas forward conditioning (CS preceded by the US) consistently produced the conditioned effect, backward conditioning (US preceded by the CS) did not. This would be consistent with pavlovian training. Because of the number of groups used and the nature of this type of experiment, variation between the control groups can often occur (Table II). In any event, in each of the protocols in which the CND group was compared against its controls and, in particular, the CNDo, NC, or US groups, the CND group always evoked a conditioned response that was significantly higher than that of each of these controls (3).

There are a number of caveats and considerations

to note. These experiments were performed on different cohorts of animals obtained at different times. The variation in NK cell activity between different experiments is the result of cohort variation, which must be controlled separately for each experiment. As a result, comparisons were always made with groups within the same experiments and not between groups across experiments. For conditioning, it is essential that the strain possess a background level of NK cell activity.

It is essential to use fairly large groups of animals for statistical comparisons, usually 8–10 mice per group. Since each animal is assayed individually and the E:T ratio from each individual spleen is tested in triplicate, generally, a total of 40 mice can be managed adequately.

Finally, animals should be kept under low stress conditions at all times, in separate rooms away from activities of other investigators. During the course of an experiment, any strange sounds, strange odors, or handling of the mice by individuals not involved in the experiment can lead to uncontrolled variations in the results.

This work was supported by Grant CA37570 from the National Institutes of Health and Grant IM509 from the American Cancer Society.

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