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Effect of Reserpine on Retention of the Conditioned NK Cell Response

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HIRAMOTO, R., B. SOLVASON, V. GHANTA, J. LORDEN AND N. HIRAMOTO. *Effect of reserpine on retention of the conditioned NK cell response.* PHARMACOL BIOCHEM BEHAV **36**(1) 51–56, 1990.—The effect of reserpine and 6-hydroxydopamine on the learned conditioned natural killer (NK) cell response was investigated in mice. Reserpine given at 2.5 mg/kg, 24 hr prior to reexposure to camphor-conditioned stimulus on days 6 and 8 blocked the recall of conditioned NK cell response to a significant extent. In other words, the NK cell activity of conditioned mice, treated with reserpine and reexposed to the conditioned stimulus, was similar to the nonconditioned (NC) group. A conditioned increase in NK cell response was still evident in mice treated with 6-OHDA.

Conditioned response Camphor odor Reserpine 6-Hydroxydopamine

WE have used the pairing of camphor odor and injections of Poly I:C to condition the NK cell response in a Pavlovian conditioning paradigm (13,38). The mechanism by which the NK cell response is learned and subsequently triggered by the camphor odor (CS) is unknown. However, learning and memory must take place by changes occurring in the brain that lead to the encoding and storage of information in a form that can subsequently be retrieved (27). Learning processes produce many effects within the organism and are greatly influenced by various external treatments including electrical stimulation, lesions, drugs and hormones (18, 30, 24, 26, 39). Once the memory of even a relatively simple event has been stored, environmental stimuli (perhaps the effect of a drug or neurohormone) associated with the event might reinstate a complex series of physiological events that may be modified by the motivational or arousal state of the organism. Events such as these might produce "recall" and activate systems in the brain and pituitary resulting in release of a variety of neuroactive compounds by these organs.

Despite the complexity of the processes involved in learning and memory, recent neurobiological work in both vertebrate and invertebrate models has affirmed the idea that critical pathways can be identified that carry essential information about the elements involved in the associative learning paradigms such as Pavlovian conditioning (5, 9, 41). In attempting to uncover the mechanisms underlying the conditioning of the natural killer (NK) cell response, we adopted a strategy that has proven successful in studying other learned responses (9). That is, we have attempted to identify neurochemically defined pathways that may mediate the expression of the CR. Brain biogenic amines (e.g., norepinephrine, dopamine) have been implicated in a variety of processes that can affect learning and memory. Evidence even suggests that catecholamines such as norepinephrine play an important role in memory formation. It has been demonstrated that amnesia resulting from the administration of puromycin can be reversed by a variety of drugs that compete for adrenergic receptor sites (34).

We chose to use the NK system for conditioning studies because of the critical roles played by the NK cells in vivo. Natural killer cells have been demonstrated to play a significant role in natural immunity against lymphoid derived and other tumors (16,43) and to be involved in antibacterial (19) and antiviral host responses (4). Poly I:C the unconditioned stimulus in these studies, is a nonantigenic, double-stranded synthetic RNA that mimics infection by double-stranded RNA virus. Poly I:C induces the expression and secretion of interferons (IFN) alpha and beta by interaction with cell membranes and inclusion into the cytoplasm, (33); IFN in turn directly stimulates NK activity (10). Following poly I:C injection, NK cell activity peaks within 24 hr (14) and returns to baseline within 3 to 5 days (12).

Sieden and Peterson (36) have shown that reserpine and α-methyl-p-tyrosine, both of which decrease the concentration of amines in the brain, cause a temporary failure to perform a well-learned conditioned avoidance response. Randt, Quartermain, Goldstein and Anagnoste (32) demonstrated that inhibition of norepinephrine synthesis in the brain with diethyldithiocarbamate (DDC) at the dopamine β-hydroxylase (which decreases the brain concentration of NE) stage is associated with early enhance-

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ment and later impairment of memory for one trial passive avoidance response. It has also been shown that the peripheral effects of reserpine on catecholamines might be responsible for the amnesic effects of reserpine (3). We investigated the effect of reserpine on the retrieval of a conditioned NK cell response. The peripheral effect of 6-hydroxydopamine (6-OHDA) on the Pavlovian conditioned NK cell response was also reported.

METHOD

Animals

BALB/c female mice, 6–8 weeks of age, weighing 20–24 grams, 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 12-hr light/dark cycle, and food and water ad lib.

Conditioning of Mice

Animals were divided into conditioned (CND) and nonconditioned (NC) groups. On day 0 the CND group was placed into a cabinet, and one small jar of camphor dissolved in mineral oil, preheated for 1 min in a microwave oven, was placed on the cage top. A separate cage of the same size was inverted over the first cage to keep the camphor odor confined. The cabinet doors were closed and the animals were allowed to remain in the presence of the camphor odor for 1 hr, after which time the mice were immediately injected with poly I:C (20 μg/mouse IP). Animals were placed into clean cages (tops, food, water) and returned to the home facility. The NC group was treated on day 0 with poly I:C only. Subsequently, mice in the CND and NC groups were reexposed to the CS (camphor) on days 7 and 9. All animals were sacrificed by ether anesthesia and tested for NK cell activity on day 10. The use of two CS exposures on days 7 and 9 has often been misunderstood and has been interpreted as an extinction procedure. We have found that these two exposures to CS one day apart provided a more consistent response to the CS than a single exposure to CS because of the slow return of the NK cell activity to baseline after stimulation.

Assay for NK Cell Activity

Spleen effector cells at ratios of 200:1, 100:1, and 50:1 (E:T ratio) were mixed in triplicate wells with 1×10^4 ^{51}Cr-labeled Yac-1 target cells in 96-well, flat-bottomed microtiter plates (Linbro Scientific Co., Hamden, CT) in a total volume of 0.2 ml. Plates were incubated for 4 hr in a humidified, 37°C, CO_2 incubator. One-tenth ml of supernatant 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. Percent specific ^{51}Cr-release was calculated as 100 times (test release-SR)/MR-SR.

Determination of Norepinephrine Levels in Spleen and Hypothalamus

Tissue samples were assayed using high performance liquid chromatography with electrochemical detection (HPLC-EC) following alumina extraction (29). The citric acid-phosphate buffer mobile phase described by Kontur *et al.* (20) was optimized for experimental conditions. The chromatography system consisted of a Beckman 112 pump, a 5 μm C_{18} reverse-phase chromatography column, and Bioanalytical Systems amperometric detector with a glass carbon electrode. The working electrode was maintained at an oxidation potential of +0.72V against a Ag/AgCl reference electrode. The flow rate was 1.0 ml/min and the injection volume, 20 μl. Levels of NE were calculated using 3,4-dihydroxybenzylamine as an internal standard. Recovery of NE with this method was 55–60%.

TABLE 1

SCHEDULE OF TREATMENT FOR CONDITIONING NK CELL ACTIVITY AND TREATMENT WITH RESERPINE

	Treatment Days					
Groups	0	6	7	8	9	10
CND	C+P	V	C	V	C	NK assay
NC	P	V	C	V	C	NK assay
CNDr	C+P	R	C	R	C	NK assay
NCr	P	R	C	R	C	NK assay

C = camphor; P = poly I:C; V = vehicle used to dissolve reserpine (200 mg ascorbic acid and 100 μl Tween 80 per 20 ml sterile water); R = 2.5 mg/kg reserpine.

RESULTS

Effect of Reserpine in Conditioned Mice

These studies were initiated to determine if central catecholamines are involved in the conditioned NK cells response. It has been shown that reserpine (2.5 mg/kg) depletes 90–95% of the brain catecholamines 24 hr following its administration (3). The purpose of the first experiment was to determine if depletion of catecholamines in the brain produces amnesia causing a block of the conditioned response.

In this study, 4 groups were compared, the conditioned (CND) group was compared with a control nonconditioned (NC) group to establish that conditioning had taken place. In parallel with the CND and NC groups a conditioned group treated with reserpine (CNDr) was compared to control (NCr) group to determine the effect of reserpine on the conditioned response. The protocol for the conditioning of NK cell activity and treatment with reserpine is given in Table 1.

On day 0, the CND and CNDr groups were conditioned using camphor as the CS and poly I:C (P) injection as the US. The control groups (NC and NCr) received P injection only. Subsequently, the CND and NC groups were injected with vehicle (V) only on days 6 and 8 and received CS exposure on days 7 and 9. The CNDr and NCr groups were injected with reserpine (R) on days 6 and 8 to deplete catecholamine levels in the brain. They were exposed to CS on days 7 and 9, i.e., 24 hr after reserpine injection. All animals were sacrificed and their spleen cells were tested on day 10. The results of the spleen NK cell activity were presented in Table 2.

The results show that the CND group upon reexposure to camphor produced the conditioned response. A statistically significant, $F(3,30) = 5.66$, $p<0.0085$, increase in NK cell activity was measured at all E:T ratios tested when compared with the NC control. On the other hand, treatment with reserpine 24 hr prior to reexposure to the CS led to a block in the conditioned response (compare CND vs. CNDr and CNDr vs. NCr). These results support the hypothesis that depletion of catecholamines produces a

TABLE 2

EFFECT OF DEPLETION OF CATECHOLAMINES WITH RESERPINE ON THE CONDITIONED NK CELL ACTIVITY

Groups	n	Percent ^{51}Cr-Released, E:T Ratio 200:1	100:1	50:1
CND	5	31.3 ± 5.4*	22.8 ± 5.1	11.5 ± 3.4
NC	4	14.6 ± 2.1	10.6 ± 2.2	6.0 ± 1.4
CNDr	5	11.0 ± 2.7	8.3 ± 2.2	4.7 ± 1.8
NCr	5	13.6 ± 2.6	11.5 ± 2.0	7.0 ± 1.4

*Values are mean ± SE.

Statistical analysis was performed by ANOVA where CND vs. other groups was, $F(3,30) = 5.66$, $p = 0.0085$, statistically significant. CNDr vs. NCr was not significant.

block of the CND efferent pathway of the conditioned response.

Lack of Effect of Chemical Sympathectomy With 6-OHDA on the Conditioned Response

Reserpine depletes both central and peripheral catecholamine levels. The use of 6-hydroxydopamine (6-OHDA) to block the effects of the peripheral sympathetic nervous system was therefore initiated. The protocol for the treatment of groups of mice was given in Table 3.

In this study a complete chemical sympathectomy was maintained through the CS/US pairing and during reexposure to the CS. 6-OHDA was given on days 0 and 4 at 150 mg/kg IP. Animals were conditioned (CND) on day 1 and reexposed to the CS (CND and NC) on days 4 and 6. All mice were assayed for NK cell activity on day 7.

Animals in this study were chemically sympathectomized throughout the experiment. Dr. Felton (University of Rochester, personal communication) has shown that this sympathectomy lasts 3–5 days postinjection of 150 mg/kg 6-OHDA. Our results show chemically sympathectomized mice were not blocked in their ability to respond to the CS. It appears that a block of the peripheral sympathetic nervous system does not prevent the conditioned response (Table 4).

In a second study animals were conditioned on day 0 and reexposed to the CS (CND and NC) on days 3 and 5. 6-OHDA was given on days 2 and 4 at 150 mg/kg IP. US group treated with 6-OHDA was also included. All mice were assayed for NK cell

TABLE 3

PROTOCOL FOR TREATMENT WITH 6-OHDA PRIOR TO ASSOCIATION AND ALONG WITH ONE CS REEXPOSURE

Groups	Treatment Days 0	1	4	6	7
CND	6-OHDA	C+P	6-OHDA +C	C	NK assay
NC	6-OHDA	P	6-OHDA +C	C	NK assay

C = camphor; P = poly I:C. These animals were treated with suboptimal dose of 1 μg P on day 6 to augment the NK response.

TABLE 4

EFFECT OF CHEMICAL SYMPATHECTOMY WITH 6-OHDA ON CONDITIONED ENHANCEMENT OF NK ACTIVITY PRIOR TO ASSOCIATION AND ALONG WITH ONE CS REEXPOSURE

Groups	n	Percent ^{51}Cr-Released, E:T Ratio 200:1	100:1	50:1
CND	5	52.8 ± 1.5*	44.0 ± 2.0	29.9 ± 1.5
NC	5	40.9 ± 3.5	33.1 ± 4.2	21.9 ± 2.9

*Values are mean ± SE.

Statistical analysis was performed by ANOVA where the CND vs. NC group was, $F(1,22) = 13.22$, $p = 0.0039$.

activity on day 6 (Table 5). Table 6 shows that animals chemically sympathectomized through the CS reexposure did not prevent the conditioned response.

These observations (6-OHDA and reserpine studies) taken together indicate that the peripheral sympathetic nervous system might not be involved in the CS signal transmission. This tentative conclusion was based on the fact that a conditioned increase in NK cell response was still evident in mice treated with 6-OHDA. Reserpsine, on the other hand, blocked the "recall" of the augmentation of NK cell activity in conditioned animals. These results indicate that conditioning of the NK cell response might be mediated through central processes which lead to stimulation and release of endorphins or other mediators that stimulate NK cell activity in vivo.

The Effect of Reserpine on NK Response

There is a possibility that reserpine might directly suppress NK

TABLE 5

CHEMICAL SYMPATHECTOMY TREATMENT SCHEDULE

Groups	n	Treatment Days 0	2	3	4	5	6
CND	9	C+P	6-OHDA	C	6-OHDA	C	NK
NC	9	P	6-OHDA	C	6-OHDA	C	NK
US	4	P	6-OHDA		6-OHDA		NK

These animals were treated with suboptimal dose of 1 μg P on day 5 to augment the NK response. 6-OHDA was given at a dose of 150 mg/kg, IP on days 2 and 4.

TABLE 6

RESULTS OF 6-OHDA CHEMICAL SYMPATHECTOMY AFTER CS AND US ASSOCIATION AND PRIOR TO ONE CS REEXPOSURE

Groups	Percent ^{51}Cr-Released, E:T Ratio 200:1	100:1	50:1
CND	10.83 ± 2.87*	8.66 ± 1.99	5.52 ± 1.40
NC	5.17 ± 0.67	4.62 ± 0.49	3.14 ± 0.51
US 6-OHDA	4.07 ± 1.02	4.18 ± 0.41	2.36 ± 1.00

*Values are mean ± SE.

Statistical analysis was performed by ANOVA where the CND vs. NC group was, $F(2,36) = 4.15$, $p = 0.032$.

TABLE 7

EFFECT OF RESERPINE AND VEHICLE ON POLY I:C STIMULATED AND BACKGROUND NK CELL ACTIVITY

Groups	Percent ^{51}Cr-Released, E:T Ratio 200:1	100:1	50:1
Reserpine ×2 Poly I:C 20 μg	42.16 ± 2.49*	32.92 ± 1.77	23.92 ± 1.68
Vehicle ×2 Poly I:C 20 μg	40.56 ± 2.18	32.23 ± 1.98	22.50 ± 1.68
BG	13.90 ± 1.12	10.9 ± 1.05	7.74 ± 0.77

*Values are mean ± SE. Statistical analysis of effect of reserpine and vehicle on poly I:C-induced NK cell activity was performed by ANOVA and found no statistically significant difference between the two treatments, $F(2,16)=0.205$, $p=0.6627$.

activity. The effect of reserpine on NK cells was tested therefore under two separate conditions. In one experiment, 5 mice were given 2.5 mg/kg reserpine on day 0, rested one day and reinjected once again with reserpine on day 2. The animals were also injected IP with 20 μg/mouse poly I:C. A control group was treated in the same fashion except the controls received vehicle. The results are shown in Table 7. Animals receiving R had NK activity of 42.16, 32.92 and 23.92 at E:T ratios 200:1, 100:1 and 50:1, respectively. Those receiving vehicle only and poly I:C on day 2 showed NK activity of 40.56, 32.23 and 22.50. Animals not treated at all had background NK activity of 13.90, 10.9 and 7.74. Poly I:C treatments elevated NK activity over the untreated animals. Reserpine had no effect on this response. In the second experiment, the effect of R was tested in three groups of mice: CNDr, NCr and US (Table 8). The CNDr (n = 10) received C+P on day 0 and were exposed to the CS on days 3 and 5. Twenty-four hr before each CS (day 2 and 4) the mice were treated with 2.5 mg/kg R.

The NCr (n = 10) mice received P only on day 0 and received CS on days 3 and 5, and twenty-four hr before the CS they were injected with vehicle only. The US (n = 5) groups received P only on day 0. All animals were sacrificed on day 6 and tested for NK activity (Table 8). The NK activity in the reserpine-treated CNDr and NCr were essentially identical. However, the NK activity was slightly higher than in the US group. These results show resperine by itself had no suppressive influence on the NK activity.

To demonstrate that 6-OHDA depletes norepinephrine (NE) and to show that conditioning and reexposure to camphor did not alter the peripheral or central NE levels, two parallel studies were

TABLE 8

EFFECT OF RESERPINE ON CONDITIONED INCREASE IN NK CELL ACTIVITY

Groups	Percent ^{51}Cr-Released, E:T Ratio 200:1	100:1	50:1
CNDr	26.86 ± 1.17*	19.71 ± 0.97	12.64 ± 0.79
NC	26.18 ± 1.40	19.26 ± 1.07	11.97 ± 0.79
US	22.47 ± 1.52	16.61 ± 1.20	9.59 ± 0.60

*Values are mean ± SE. Statistical analysis was performed by ANOVA of CNDr and NC groups, $F(2,36)=0.126$, $p=0.7268$.

TABLE 9

NE LEVELS IN SPLEEN AND HYPOTHALAMUS

Group	μg/g of Tissue Spleen	Hypothalamus
Saline	0.533 ± 0.084*	2.33 ± 0.76
6-OHDA	0.142 ± 0.142	1.84 ± 0.17
CND	0.569 ± 0.081	1.67 ± 0.57
NC	0.585 ± 0.086	1.76 ± 0.38

*Values are mean ± S.D. The data was analyzed by the Student *t*-test for variance in catecholamine (norepinephrine, NE) levels of 6-OHDA-treated and untreated mice. Spleen NE levels of 6-OHDA-treated vs. untreated animals ($p<0.001$). NE levels of hypothalamus of 6-OHDA-treated vs. untreated mice ($p>0.10$).

done. In one experiment, 5 animals (controls) were treated with saline on days 0 and 1 and exposed to camphor odor on days 3 and 4 and immediately tested for NE levels in the hypothalamus and spleen. A second group of 5 animals were treated with 100 mg/kg 6-OHDA on days 0 and 1 and exposed to camphor on days 3 and 4 and tested for NE.

In a separate but parallel experiment we tested whether NE concentration would be affected in conditioned vs. nonconditioned Balb/c (n = 4 per group). The conditioned group was exposed to camphor and poly I:C on day 1. The NC group was injected with P only on day 1. On day 4, both groups were exposed to the CS (camphor odor). Both CND and NC groups were sacrificed immediately after the reexposure to the CS to see if NE levels will be altered.

The results show 6-OHDA caused a significant drop (75%) in NE levels in the spleen but not in the hypothalamus. The CND and NC mice exposed to camphor odor and poly I:C and reexposed to the CS showed no appreciable decline of NE in the spleen or hypothalamus (Table 9).

DISCUSSION

Once an association between a conditioned stimulus (CS, camphor) and unconditioned stimulus (US, poly I:C) has been made, the presentations of the CS alone is sufficient to evoke the conditioned response (CR, NK cell activity). The CS presentation might trigger the pathways that eventually carry a message to the spleen. Two pathways from the brain are potentially involved in the expression of the CR, the pituitary and the autonomic nervous system. Compounds associated with these efferent pathways, such as ACTH, epinephrine, norepinephrine, vasopressin, endorphin, and enkephalins have all been implicated in learning and memory. The identification of pharmacologic agents that can enhance or retard the expression of the CR might help to elucidate the mechanisms and pathways involved in triggering the learned response.

A common strategy for describing neurobiological mechanisms of memory formation is to investigate the behavioral consequences of amnesia-inducing drugs and relate them to the drugs' known mechanism of action. The rationale inherent in this approach is that a reliable correlation between the drugs' pharmacodynamics and its effects on behavior will provide significant information about the biological processes involved in learning and memory. This experimental strategy, along with other lines of indirect support (1, 11, 42), have led to the hypothesis that the catecholamines, norepinephrine (NE) and dopamine (DA) are critically involved in memory formation (15, 23, 31). If such is the case it

is likely that these neurotransmitters might likewise be critical to retrieval of the memory.

Reserpine injections (2.5 mg/kg) result in 90–95% depletion of brain catecholamines by 24 hr following its administration (3). This provides a means to test whether in CND animals depletion of the central catecholamine levels would affect retrieval of memory. By comparing the behavioral response of CND animals treated with reserpine vs. 6-OHDA we support the conclusion that the central catecholamines are specifically involved in evoking the conditioned enhancement of NK cell response. 6-OHDA is a catecholamine-specific neurotoxin. It is preferentially taken up by adrenergic neurons where its cytotoxic action destroys the presynaptic terminals (22,40). Following systemic administration, the drug produces long lasting depletion of peripheral NE without altering the levels or functional integrity of central catecholamine processes. If peripheral adrenergic mechanisms are necessary for triggering the memory recall of the conditioned NK response, then peripherally administered 6-OHDA should produce an impairment of this response. Since reserpine pretreatment impairs the CND response and 6-OHDA did not, it appears that the central catecholamines are important. However, because 6-OHDA does not modify peripherally the adrenal medulla, the results are not conclusive.

Reserpine interferes with the intraneuronal binding of catecholamines (17,37) and allows the amines to diffuse freely in the cytoplasm where they result in inactivation by deamination by mitochondrial monoamineoxidase and depletion in the tissue (8,21). Reserpine-induced depletion of catecholamines is associated with decreased brain levels of norepinephrine, dopamine and serotonin (2,6). Reserpine-induced depletion of central catecholamines has been shown to suppress the conditioned avoidance response (34–36).

Other studies have shown amino acid precursors of catecholamines and serotonin to animals previously given reserpine, can raise the concentration of the respective monoamines in the brain (7). The administration of dihydroxyphenylalanine, a catecholamine precursor, reverses the effect of reserpine and restores the conditioned avoidance response. The serotonin precursor 5-hydroxytryptophan does not restore normal functioning (7,28). Our studies show depletion of central catecholamine suppresses the conditioned natural killer cell response and is in line with the observations of previous investigators.

In the experiments cited on memory retention, treatments are administered shortly after training for the following reasons: 1) this is to obviate problems associated with heightened or lowered performance of the animal if treatment is given before training, and 2) the influence of postraining treatment effects would be greatest when memory was being acquired and thus when the hormone systems are acting during this process. Treatment at this stage is believed to influence the consolidation processes that follow the learning experience. In our experiments we are not so much interested in memory acquisition as we are in blocking or interfering with the pathway after the memory has been acquired. Therefore, our studies are aimed at administering the drugs just prior to reexposure of the conditioned animals to the CS, i.e., several days after the memory has been acquired and stored. Whether the acquired response elicited is inhibited or enhanced might provide clues to the pathways involved in the CR. This approach is consistent with the views proposed by McGaugh (25) that treatments given after the learning trial affect brain processes that are related to memory and has an affect on the processes which consolidate and help to retain the learned experience. However, treatments given shortly before retention testing (i.e., testing for recall of the learned experience) may influence retrieval processes. Our results show that disruption of the central catecholamine levels after memory has been acquired interrupts the pathways through which camphor odor signals activate the conditioned NK cell response.

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