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Indomethacin and Sodium Carbonate Effects on Conditioned Fever and NK Cell Activity

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ROGERS, C., V. GHANTA, C.-M. HSUEH, N. HIRAMOTO AND R. HIRAMOTO. *Indomethacin and sodium carbonate effects on conditioned fever and NK cell activity.* PHARMACOL BIOCHEM BEHAV 43(2) 417–422, 1992.—The augmentation of natural killer (NK) cell activity and elevation of body temperature (fever) can both be conditioned using camphor odor as the conditioned stimulus (CS) and poly I:C as the unconditioned stimulus (US). While both responses can be conditioned in parallel fashion as shown previously, our results indicate the conditioned learning of these responses may not follow along a common path. We found that injection of a 1% solution of sodium carbonate was able to consistently block the CS/US learning of the NK cell response but did not block conditioning of the fever response. In contrary fashion, mice treated with indomethacin (which inhibits prostaglandin-induced fever) dissolved in the sodium carbonate solution did not learn in consistent fashion the fever response. However, indomethacin-treated animals were able to recall the NK cell response. These results support the view that although the same mediator, IFN-β, is responsible for the conditioned learning of the NK cell and fever responses both the learning and recall of the responses are initiated along separate pathways.

NK conditioning Fever conditioning Camphor odor Indomethacin Sodium carbonate Learning block

THERE are numerous studies in the early literature on conditioning hyperthermia with low doses of morphine with repeated pairing with a conditioned stimulus (CS). Lal et al., (13) conditioned morphine-induced hyperthermia by pairing morphine injection with the odor of anise oil. After 39 pairings, the odor itself acquired a conditional property of eliciting hyperthermia. Tone and the sound of a bell have been used as well (17,22). Dyck et al. (4) have shown poly I:C (50 μg/mouse) produces a pyrexic response 4–6 h after injection. However, following repeated paired odor cue–drug (poly I:C) delivery, a compensatory hypothermic response was conditioned.

The conditioned enhancement (25) and suppression of the natural killer (NK) cell response has also been demonstrated. Dyck et al. (2,3) used a combination of odor and light cues in repeated pairings to condition tolerance of the NK cell response. Gorczynski and Kennedy (7) also showed suppression of the NK response when saccharine and LiCl was paired with poly I:C.

It is important to note that the methodology employed in the aforementioned studies differs significantly from ours in that these investigators employed multiple pairings of CS and an unconditioned stimulus (US) before they tested for the conditioned response. In some cases, the repeated pairing of CS and US resulted in a compensatory response. For example, Lal et al. (13) used 39 daily pairings of CS and US to achieve conditioned hyperthermia, whereas Dyck et al. used multiple light (or dark) and odor cues and observed a tolerance response in both NK cell activity and body temperature (hypothermia)(2–4).

In the last several years, we have attempted to describe the mechanisms of a pavlovian conditioning model to demonstrate the CNS and immune system interactions (11,25,27,29). In this model, saccharine–LiCl (10) or camphor (25,28) is used as the CS and poly I:C is used as the US. Poly I:C is a double-stranded RNA that induces the formation of interferon (IFN)-α and -β, which in turn cause a rise in NK cell activity that peaks in the spleen at 24 h (6). In our paradigm, a single-trial CS/US association appears to condition animals so that subsequent reexposure to the CS elicits an enhancement of the NK cell activity (25,28). Using this model, we investigated several questions of interest. For example, are catecholamines (11) and opioids (29) involved in conditioning? A key question was, what is the mediator from the immune system that allows the CS/US association to be made? We found that IFN-β could serve as the US in place of poly I:C (25,27). Therefore, it appears that IFN-β might be the important mediator that acts centrally to permit the CS/US learning to take place. IFN-β has been shown to induce fever (15) and we have used our paradigm to condition fever (9) as well as the NK cell

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response (28). These studies were initiated to determine if conditioning of the fever and NK cell response utilize a single common pathway or whether these responses can be dissociated.

In our past work, we treated administration of poly I:C as the US. This is a common practice but probably inaccurate. In the case of analogous paradigms such as taste aversion learning, animals learn to avoid palatable tastes on the basis of their association with drug injections that cause some discriminable internal state like nausea. In taste aversion learning, it is not the drug (cyclophosphamide) injection per se that is the US. It is more likely the nausea. Because IFN is produced in response to poly I:C injection in our fever and NK cell paradigm, it is possible that this molecule serves as the US. This is supported by the fact that IFN either centrally or peripherally will substitute for poly I:C in the conditioning paradigm (27). Because IFN is a pyrogen, it is also possible that the pyretic effect of poly I:C or IFN is a discriminable internal state that mediates the conditioning effect. A substantial amount of literature documents conditioned changes in body temperature as a result of signalled exposure to such drugs as morphine that have an effect on body temperature (13,17,22).

METHOD

Animals

Six-week-old female BALB/c mice were purchased from Charles River Breeding Laboratories (Wilmington, DE) and maintained on standard rodent chow and water ad lib with 12 L : 12 D cycle. Animals were allowed to adapt to our vivarium surroundings for at least 1 week before use in the experiments.

Conditioning Procedure

All conditioning procedures (association, reexposure) and killing animals to perform NK cell activity were started between 7:00 and 7:30 a.m. Exposure to the odor stimulus and treatment with US were completed by 8:30 a.m. Lights were on at 7:00 a.m. and off at 7:00 p.m. The procedures were performed as soon as lights were on to accommodate the diurnal cycle and low corticosteroid levels. A 1-oz block of camphor was partially dissolved in mineral oil (1 block to about 150 ml 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 was heated in a microwave oven for 1 min and then placed upon the cage top. Another empty cage was inverted over the cage holding 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 control animals. Mice were exposed to camphor odor in this way for 1 h. Poly I:C was obtained in lypholized form from Pharmacia (Piscataway, NJ) and dissolved in sterile physiological saline (PSS) at 200 μg/ml and stored at 4°C and 0.1 ml (20 μg) was given IP for each mouse within 5 min following removal from the camphor-containing cage. Mice were allowed to stay for about 3 h in the cabinet room before returning to the vivarium. Mice of each group were housed separately in individual cages of 7–10 mice/cage for 1 week prior to the performance of the experiment. The schedule used for conditioning of the NK cell and fever responses is given in Table 1.

All our comparisons in these experiments were between the conditioned group (CND) and the nonconditioned group (NC). In our previously published work, we extensively characterized this response with respect to all of the necessary controls and have repeatedly shown that the comparison between the CND and NC groups reliably reflects a conditioned response when it is present (9,25,26,28). We have found through experience that for conditioning of the NK cell activity it is essential that the mouse strain employed possess a normal background level of NK cell activity. Naive animals possessing very low or no measurable level of NK cell activity gave poor responses.

TABLE 1

PROTOCOL FOR CONDITIONING THE NK CELL RESPONSE

		Days			
Groups	*n*	0	1	2	3
CND	10	C,P		C	NK
NC	10	P		C	NK

C, exposure to the odor of camphor for 1 hr; P, injection with poly I : C 20 μg/mouse IP. On day 0, mice in the CND group are conditioned by exposure to camphor odor for 1 h in an enclosed cabinet, after which they are immediately injected with poly I : C. This constitutes the CS/US association. The NC group is injected with poly I : C only. On day 2, the CND and NC groups are exposed to camphor odor only. Animals are tested for NK cell activity on day 3.

Preparation of Spleen Cells

Animals of each group were killed simultaneously in a box with CO_2 asphyxiation. Animals were killed before 8:00 a.m. This procedure took only 5–10 min to kill both groups (CND, NC). Spleens were removed immediately and placed into individual Petri plates containing sterile 0.9% sodium chloride solution (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-ga needle and a 3-ml syringe into a sterile 15-ml tube. The tubes were filled with PSS and the mixture centrifuged at 1,800 rpm for 5 min at 5°C in a Beckman centrifuge (Beckman Instruments, Fullerton, CA). The supernatant was discarded and the washing was repeated once more. The pellet was suspended with 1 ml 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. Whole spleen cells (with red blood cells) were used in the NK cell assay.

We routinely assay 20–40 mice for each experiment (10 mice/group) and the spleens of individual mice are handled as expeditiously as possible. To obviate experimental bias, we have six people processing the spleens for teasing and cell counts. Two individuals who have no idea about the experiment or groups are responsible for making the spleen cell dilutions and dispensing them into the microtiter plates for the assay. The individuals that are processing the samples are unaware of the groups or their order. The whole processing of spleens takes about 3 h and is handled by six individuals randomly.

Assay for NK Cell Activity

YAC-1 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 100 U penicillin, 100μg

streptomycin, and 5×10^{-5} M 2-mercaptoethanol. The YAC-1 cells were cultured into fresh tissue culture flasks with fresh medium 24 h before harvesting for the assay. With this procedure, the viability is >95% and the spontaneous release in the chromium release assay is 5–15%. 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 volume is about 0.2 ml) at 37°C in a CO_2 incubator for 30 min. The cells were washed with a large excess of medium 2× and suspended at a final density of 1×10^5 cells/ml in RPMI 1640 supplemented with 5% FCS. One tenth milliliter of spleen effector cells at ratios of 200:1, 100:1, and 50:1 (E:T ratio) were mixed in triplicate wells with 0.1 ml 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 h in a humidified, 37°C, CO_2 incubator. One tenth milliliter of supernatant from each well was collected after centrifugation of plates. The radioactivity of the samples were counted in a Beckman gamma counter. Maximum [^{51}Cr] released from the target cells (MR) was measured after incubation in the presence of 0.1% HCl and spontaneous release (SR) in the presence of medium. Percent specific [^{51}Cr] release was calculated as 100 × [(test release-SR)/MR-SR].

RESULTS

During the course of studies in which the effect of indomethacin on conditioning was being investigated, we found that injection of mice with 0.2 ml of a 1% solution of sodium carbonate (pH 9.0), the vehicle used to dissolve the indomethacin, interfered with conditioning of the NK cell response. The mechanism by which sodium carbonate interferes with the CS/US association is not clear at this time, but it appears to be a potent inhibitor in that conditioning of the NK cell response was consistently and repeatedly blocked in seven consecutive trials.

Conditioning of the NK cell activity (28) and elevation of body temperature (Tc)(9) can be readily established using a short single-trial conditioning paradigm. On day 0, the CND group is exposed to camphor odor (CS) for 1 h after which animals are immediately injected with poly I:C (US). The NC control group is injected with poly I:C only at this time. On day 2, both groups are exposed to the CS and NK cell activity is measured on day 3. If conditioned enhancement of the Tc response is to be made, then rectal temperature measurement is taken on day 2 immediately after animals are removed from camphor exposure. Table 2 shows an experiment in which mice were injected with saline instead of sodium carbonate prior to the CS/US association. The CND group was conditioned in that the NK cell activity was elevated at all E:T ratios over the NC group [analysis of variance (ANOVA), $p = 0.0008$]. These experiments have been repeated many times with similar results (28).

TABLE 2

CONDITIONING OF THE NK CELL RESPONSE

		%[^{51}Cr] Released at E : T Ratios		
Group	n	200 : 1	100 : 1	50 : 1
CND	10	14.6 ± 0.8	13.6 ± 0.8	8.7 ± 0.6
NC	10	10.8 ± 0.8	9.1 ± 0.8	5.2 ± 0.6

Values are mean ± SEM. NK cell activities (% specific release of [^{51}Cr] of CND and NC groups were compared with repeated-measures ANOVA with α value of 0.05 ($p = 0.0008$).

TABLE 3

EFFECT OF ADMINISTRATION OF 1% SOLUTION OF Na_2CO_3 ON THE CONDITIONED NK CELL RESPONSE

			% [^{51}Cr] Released at E : T Ratios		
Experiment	Groups	n	200 : 1	100 : 1	50 : 1
1	CND	10	15.5 ± 1.1	13.7 ± 1.0	9.8 ± 1.0
	NC	10	14.5 ± 1.0	12.8 ± 0.9	7.8 ± 0.8
2	CND	10	15.3 ± 0.8	13.8 ± 0.8	9.3 ± 0.5
	NC	10	15.3 ± 0.7	13.9 ± 0.5	9.4 ± 0.4
3	CND	10	16.6 ± 1.1	15.8 ± 1.1	11.7 ± 0.8
	NC	10	16.1 ± 1.0	15.0 ± 1.0	10.7 ± 0.7

Values are mean ± SEM.

Initially, we tested to see if sodium carbonate or indomethacin interfered with the induction of NK cell activity by poly I:C. Injection of mice with 0.2 ml 1% solution of sodium carbonate or indomethacin IP was followed by injection of poly I:C 20 μg/mouse IP in 0.1 ml saline. Injection of sodium carbonate solution or indomethacin did not inhibit the induction of NK cell activity when measured at 24 h (data not shown). This implies that in sodium carbonate- or indomethacin-treated animals IFN-α and -β and the resulting NK cell activity are produced normally and during the CS/US association conditioned learning should proceed in normal fashion. Table 3 shows the effect of sodium carbonate on conditioning of the NK cell response. In three experiments, animals treated with sodium carbonate showed no conditioned effect in comparison with the NC group. The percent chromium released by the target cells at all E:T ratios were comparable. Table 4 shows the results of an additional four experiments in which sodium carbonate was injected into mice prior to CS/US association. In this series, animals were also tested for the conditioned fever response. Mice exposed to the CS on day 2 were measured for changes in body temperature immediately after removal from camphor exposure. While the NK cell response was again consistently not conditioned (compare CND vs. NC), changes in body temperature could become conditioned. However, in mice treated with sodium carbonate both conditioned augmentation (Experiments 2 and 4) and conditioned suppression (Experiments 1 and 3) of the body temperature was observed. The reason for this variability is not clear. The results, however, demonstrate that the fever response pathway utilized by the CNS in response to the recall stimulus (camphor odor) is not formally linked to the pathway that triggers the NK cell response. Thus, conditioning the elevation of body temperature is not a prerequisite for conditioning of the NK cell response. Interestingly, indomethacin overcomes the inhibitory effect of sodium carbonate on conditioned learning of the NK cell response. Table 5 shows the results of this experiment. Mice were injected with indomethacin solubilized in 1% sodium carbonate prior to the CS/US association. Indomethacin blocks prostaglandin-induced fever response induced by poly I:C. Therefore, while the NK cell response has recovered, no conditioned elevation of body temperature is observed. Preventing the onset of fever during the CS/US association might have aborted the conditioned fever response. These results again support the view that the NK cell

TABLE 4

EFFECT OF Na_2CO_3 ON THE CONDITIONED NK CELL RESPONSE AND BODY TEMPERATURE

Experiment	Group	n	% [^{51}Cr] Released at E : T Ratios			Body Temperature (Tc)	p*
			200 : 1	100 : 1	50 : 1		
1	CND	9	16.5 ± 0.9	15.2 ± 0.9	10.8 ± 0.9	36.11 ± 0.10	0.09
	NC	9	17.0 ± 1.1	13.6 ± 0.8	10.0 ± 0.6	36.36 ± 0.08	
2	CND	10	22.1 ± 1.5	19.8 ± 1.3	15.3 ± 1.1	34.83 ± 0.08	0.0001
	NC	10	23.2 ± 1.0	20.0 ± 0.8	14.5 ± 0.8	34.07 ± 0.08	
3	CND	10	16.3 ± 0.9	12.6 ± 0.8	9.0 ± 0.5	35.92 ± 0.13	0.0176
	NC	10	16.8 ± 1.6	12.7 ± 1.2	8.9 ± 0.7	36.29 ± 0.06	
4	CND	8	27.2 ± 1.7	24.0 ± 1.6	19.1 ± 1.5	35.97 ± 0.09	0.0001
	NC	7	26.1 ± 0.5	22.6 ± 0.6	16.3 ± 0.6	34.20 ± 0.20	

Values are mean ± SEM.
*Body temperature values are compared using two-tailed Student's t-test.

response and elevation of body temperature are not sequenced along a common pathway and might be learned and triggered through interrelated but separate pathways.

DISCUSSION

There are no good reasons to suspect that injection of 0.2 ml 1% solution of sodium carbonate (adjusted to pH 9) should interfere with the conditioning of the NK cell response. This vehicle is normally used to dissolve the drug indomethacin (12). However, indomethacin dissolved in 1% Na_2CO_3 at a final pH of 9.0 can overcome the inhibition of learning produced by the vehicle. Because the sodium carbonate solution did not interfere with the induction of NK cell response in the spleen, we feel that the disruption must be taking place within the CNS during the CS/US learning and indomethacin, which blocks the prostaglandin-induced fever, can override this disturbance.

Many cell types contain neurotransmitters, hormones, and neuromodulators that can alter the transmembrane potential as well as the levels of second messengers like cyclic adenosine monophosphate (cAMP). The effect of Na^+ ions on these functions in vitro has been investigated by a number of laboratories. Lichtshtein et al. (14) demonstrated that Na^+ (135 mM) in vitro is required for opiate, muscarinic, and adrenergic receptor-mediated reduction of cAMP levels in mouse neuroblastoma and rat glioma hybrid cells (NG108-15). The biological mechanism underlying this effect seems to involve the action of guanosine triphosphate (GTP) and Na^+ on membrane components that are separate from the receptors yet involved in transferring information from the agonist receptor complex to the catalytic subunit of adenylate cyclase. It has also been demonstrated by Simon and Groth (24) that addition of salt decreases binding of the opiate agonist etorphine. Pert and Snyder (21) found no effect of salt on the binding of the opiate agonist naloxone. Simon and Groth went on to show the effect of sodium ions is due to the conformational changes observed in the opiate receptor.

In addition to the above studies, it has been reported that rat brain synaptic vesicles treated with Na_2CO_3 (1%, pH 11.0) cause loss of adenosine triphosphate (ATP)-dependent H^+ transport and release major polypeptide components. ATPase is a major component of vesicles, consisting of about 20% of their total protein. It is also a primary pump for accumulation of neurotransmitters. Treatment with Na_2CO_3 caused almost complete inhibition of the ATP-dependent uptake of GABA and serotonin, indicating that the transport systems for glutamate, GABA, and serotonin were energetically coupled with the H^+-ATPase (19). Whether injection of 0.2 ml 1% sodium carbonate solution could bring about changes that alter neurotransmitter uptake in vivo is not known, but the fact that a specific form of learning was interrupted argues that low concentrations of this compound can act centrally.

In our studies that deal with conditioning of NK cell activity, the CS/US association is independent of opiate pathway; however, the recall of the conditioned response requires the opiate system. This requirement was demonstrated by the blocking of recall of the conditioned response with the opiate antagonist naltrexone (29). Similar changes as reported in the above-referenced systems might be taking place in the pres-

TABLE 5

EFFECT OF INDOMETHACIN ON THE CONDITIONED INCREASE IN TEMPERATURE AND NK CELL RESPONSE

Group	n	% [^{51}Cr] Released at E : T Ratios			Body Temperature (Tc)
		200 : 1	100 · 1	50 : 1	
CNDI	10	18.6 ± 1.0	15.9 ± 1.1	12.3 ± 0.8	35.64 ± 0.10
NCI	9*	12.8 ± 1.9	10.4 ± 1.5	8.1 ± 1.1	35.72 ± 0.07

CND_I vs. NC_I groups were compared with repeated-measures ANOVA with α value of 0.05 (p = 0.0081).
*One animal died from the effects of indomethacin injected at 10 mg/kg.

ence of Na^+ ions in the loss of the conditioned NK cell activity.

In mice treated with Na_2CO_3, the conditioned NK cell response was aborted but the fever response was conditioned. Alternatively, in animals treated with indomethacin dissolved in Na_2CO_3 NK cell activity was conditioned but fever was not. Both the NK cell activity and fever can be influenced in parallel fashion by many conditions. Studies have shown that stress can elevate the release of β-endorphin and corticotropin (ACTH) into the plasma (1,8,23). The release of β-endorphin by the pituitary has been shown to directly cause hyperthermia (18). β-endorphin has also been shown to elevate NK cell activity (16). Both β-endorphin and ACTH can trigger a higher NK cell response (activity) of populations showing low levels of stimulation (5). All these facts point to the possibility that fever and NK cell activity may be linked through an opioid pathway involving β-endorphin. Naltrexone has been shown to block the hyperthermia (13,20) and we have shown that naltrexone can block the NK cell conditioned response (29). In other words, when mice are conditioned and subsequently injected with naltrexone prior to exposure to the CS they no longer recall the conditioned response (CR). Therefore, naltrexone blocks an opioid pathway required for eliciting the conditioned NK cell response. Quaternary naltrexone, which does not pass the blood-brain barrier (BBB), is unable to block the CR; therefore, it appears that the central opioid pathways are involved in the recall of the CR (29).

Although we support the view that both responses are conditioned by separate pathways, there are some points of convergence. It appears that a noradrenergic pathway is present in the hypothalamus that mediates the febrile response to poly I:C or IFN. Liu et al. (15) have shown that administration of either poly I:C (0.05–50 μg) or norepinephrine (2–8 μg) into the anterior hypothalamic area produced a dose-related fever in rats. The poly I:C-induced fever was attenuated after selective depletion of norepinephrine in the hypothalamus. This selective depletion of norepinephrine from the hypothalamus did not affect the fever induced by intrahypothalamic infusion of norepinephrine. Thus, poly I:C and IFN may act to induce fever through the endogenous release of norepinephrine from the rat's hypothalamus. This implies that during the CS/US association where the US is poly I:C the pathway to fever induction is by norepinephrine and not β-endorphin. Therefore, a block of the opioid receptors with naltrexone during the CS/US association may not prevent conditioned learning of fever or the NK cell response. This also falls in line with our observation that when reserpine (11) or amantadine and DDC were used to deplete the central catecholamine levels the CS/US association was blocked (unpublished observations). These results imply that the conditioning of both the NK cell and fever response depend upon noradrenergic pathways. Whether the responses depend upon a common noradrenergic pathway is not known.

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