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Conditioned Increase in Peripheral Blood Mononuclear Cell (PBMC) Number and Corticosterone Secretion in the Rat

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BUSKE-KIRSCHBAUM, A., L. GROTA, C. KIRSCHBAUM, T. BIENEN, J. MOYNIHAN, R. ADER, M. L. BLAIR, D. H. HELLHAMMER AND D. L. FELTEN. *Conditioned increase in peripheral blood mononuclear cell (PBMC) number and corticosterone secretion in the rat.* PHARMACOL BIOCHEM BEHAV **55**(1) 27–32, 1996.—Femoral artery catheters were surgically implanted into male Lewis/N rats to allow blood sampling and drug infusion in the freely moving animal. After recovery, conditioned animals received four pairings of a peppermint odor, the conditioned stimulus (CS), and an infusion of 0.1 mg/kg nicotine bitartrate, an unconditioned stimulus (US) for an increase in the number of peripheral blood mononuclear cells (PBMC) and an increase in corticosterone concentration. When reexposed to the peppermint odor, conditioned animals showed a significant increase in PBMC number and corticosterone secretion when compared to saline and unpaired control groups and previously conditioned animals that were not reexposed to the CS. Increased PBMCs were found on the fifth unreinforced CS trial. Conditioned CORT responses were lost after the initial test trial. The data indicate that the distribution of immune cells can be influenced by learning processes and support the role of learning in the regulation of corticosterone secretion.

Classical conditioning Psychoneuroimmunology Peripheral blood mononuclear cells (PBMC)
Corticosterone Nicotine

THERE is growing evidence of functional links among the central nervous system (CNS), the endocrine system, and the immune system. Evidence for a close interaction between the CNS and immunological processes arises from experimental observations such as: 1) altered immune responses following stimulation or lesion of specific limbic or hypothalamic structures (20,34); 2) the modulation of immune processes by various stressors (4,21); 3) neurochemical alterations in the CNS following antigenic challenge or interleukin-2 administration (6,7). Additional evidence derives from studies indicating that the immune system can be manipulated by behavioral conditioning techniques. Classical conditioning of immune function is demonstrated by the ability of an initially neutral stimulus (CS) to trigger immunologic changes by virtue of a learned association with an immunomodulating biologic and/or pharmacological agent. Besides the early work of investigators in the Soviet Union (2), Ader and Cohen (3) were the first to describe classically conditioned immunosuppression in the rat. They found that after pairing saccharin (CS) with the immunosuppressive drug, cyclophosphamide (US), rats showed a marked suppression of antibody responses when reexposed to the saccharin solution. Recent experiments have shown that a range of specific and nonspecific cellular immune functions can be reduced (8,19,26) or enhanced (5,9,17,27,35) by classical conditioning processes.

However, most of the past research into this phenomenon has relied on conditioned alteration of the in vitro and in vivo (re)activity of various immune cells. In fact, it is well recognized

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that beside specific immunological responses of the single immune cell, migration of different cell types between the lymphoid and nonlymphoid tissue via the blood and lymphatic circulatory systems is an important mechanism of the host's defense against invading pathogens (11,14). In recent years, substantial evidence has emerged that the CNS might influence lymphocyte migration and distribution (29,30). The potential role of the brain in regulating immunocyte trafficking has been documented by studies showing alteration of circulating lymphocyte subset concentrations by neuroendocrine pathways such as the hypothalamus–pituitary–adrenal (HPA) axis or the sympathetic nervous system. In addition, there is accumulating evidence indicating that lymphocyte migration is responsive to behavioral processes such as stressful stimulation (10,25,28).

The present study was conducted to investigate whether lymphocyte trafficking can be manipulated by behavioral learning processes. Thus, the specific objective of the present study was to determine the acquisition and extinction of classically conditioned elevations of peripheral blood mononuclear cell (PBMC) number. In previous reports (3,22) it was suggested, however, that modulation of immune responses seen in conditioned animals might be a direct reflection of increased CORT concentration induced by a stressful conditioning protocol or due to a conditioned elevation of this steroid. Because there are several reports indicating that lymphocyte trafficking can be modulated by elevated CORT levels (31), in addition to investigating conditioned elevation of PBMC counts, the present report examines conditioned alteration of CORT secretion.

METHOD

Animals

Adult male (Lewis/N) rats, approximately 3 months old, were housed five per cage and maintained under a 12 L:12 D cycle (lights from 0800–2000 h) with free access to food and water for 2 weeks before beginning the experiment.

Surgical Procedure

Animals were anesthesized with pentobarbital (30 mg/kg IP). Polyethylene (PE) 10 tubing was surgically implanted into the right femoral artery and extended subcutaneously with PE 20 tubing, exiting at the animal's neck. A detailed description of the surgical procedure is provided elsewhere (36). After surgery, all animals were maintained in individual cages and were rested 1 week after cannula implantation to allow recovery from the surgical procedure. To ensure proper functioning of the catheters, lines were flushed daily with a 50% glucose:heparin:saline (1:1:1) solution. While flushing the catheters the animals were handled and used to injections as well as blood sampling via the catheter.The conditioning protocol began 1 week after surgery.

Behavioral Protocol

Conditioning sessions began by placing individual animals in a covered 20 × 20 × 30 cm Plexiglas experimental apparatus containing fresh bedding. To allow blood sampling and nicotine/saline infusion without handling or disturbing the animals, the previously implanted catheters were extended by PE 20 tubing and fixed outside the experimental cage. After a rest period of 1 h, a baseline blood sample was obtained (about 1000 h). Ten minutes later, conditioned animals (randomly selected) were exposed to a peppermint odor (Lorann Oils, Lansing, MI; CS) followed, 5 min later, by an infusion of 300 μl of saline containing 0.1 mg/kg nicotine bitartrate (Sigma Chemical Co., St. Louis, MO). Nicotine treatment elevates PBMC counts and CORT secretion (16,32). To avoid uncontrolled spread of the peppermint odor in the experimental room, small glass vials containing 50 μl peppermint oil spread on a paper towel (2 × 2 cm) were prepared in a fume hood in an adjacent room and capped. For CS presentation, the glass vials were placed in the experimental apparatus, and then opened. Following two additional blood samples 15 and 60 min after beginning CS presentation, the animals were returned to their home cages and the CS vials were recovered.

Placebo-treated rats ($n = 6$) received the peppermint odor, but were infused with saline rather than nicotine. These animals provided a control for the effects of the experimental procedure per se (experimental apparatus, infusion, peppermint exposure, etc.) on CORT secretion and PBMC counts. A second control group was included to determine whether presentation of the stimuli in a noncontingent manner would also result in a conditioned response. For animals in the unpaired control group ($n = 7$), the time of nicotine infusion was varied on each acquisition trial. The US was presented in randomized order across four acquisition trials, for instance, at 1400 h (day 1), at 2000 h (day 2), at 1700 h (day 3), and at 1100 h (day 4) based on the work of Rescorla (33). It should be noted that the time of CS presentation in the unpaired control group was identical to CS presentation in the conditioned group.

On the test day (day 5), the previously conditioned animals were randomly assigned to two subgroups. The critical experimental group, designated CS_+ ($n = 7$), was reexposed to the peppermint odor and infused with 300 μl of saline. The other previously conditioned animals, the CS_0 group ($n = 6$), received no further stimulation and remained in their home cages on this particular day. The placebo group and the unpaired control group were both treated as the conditioned group and were given the peppermint odor followed by saline infusion.

During the following week, animals in the CS_+ group were given four additional unreinforced presentations of the CS on days 8, 9, 10, and 11. Animals in the CS_0 group were given unreinforced CS presentations on days 9 and 11. Because the process of extinction should vary as a function of the number of unreinforced CS presentations, the magnitude of conditioned response on days 9 and 11 in the CS_+ group was expected to be significantly smaller than in the CS_0 group. The experimental protocol is summarized in Table 1.

Number of Peripheral Blood Mononuclear Cells (PBMC)

Based on the kinetics of leukocyte number after nicotine infusion (data not shown), the absolute number of PBMC was measured 10 min before and 60 min after nicotine presentation on day 3 and CS presentation on the test day (day 5) in all experimental groups, as well as on days 9 and 11 in the CS_+ and the CS_0 groups. For analysis of PBMC number, 600 μl blood was collected in heparinized tubes and diluted 1:5 with RPMI 1640 medium. Withdrawn blood volume was replaced with room temperature saline via the catheter. The cell suspension was gently layered onto 2 ml Histopaque (Sigma Chemical Co., St. Louis, MO) and centrifuged for 30 min at 2500 RPM at room temperature. PBMC were harvested from the interface, washed three times in RPMI 1640, and subsequently counted under trypan blue exclusion.

TABLE 1
EXPERIMENTAL PROTOCOL

		Conditioning				Extinction			
Group	Subgroup	Day 1	Day 2	Day 3	Day 4	Day 5 Test Day	Day 8	Day 9 Test Day	Day 10	Day 11 Test Day
Conditioned	CS_-	CS* + N†	CS + N	CS + N	CS + N	CS + Sal‡	CS + Sal	CS + Sal	CS + Sal	CS + Sal
	CS_0	CS + N	CS + N	CS + N	CS + N	H§	H	CS + Sal	H	CS + Sal
Unpaired		CS ≠ N	CS ≠ N	CS ≠ N	CS ≠ N	CS + Sal				
Placebo		CS + Sal	CS + Sal	CS + Sal	CS + Sal	CS + Sal				

CS* = conditioned stimulus (peppermint odor).
N† = nicotine.
Sal‡ = saline.
H§ = no treatment, home cage.
≠ = unpaired CS and US.

Corticosterone (CORT)

The concentration of CORT in 600 μl of peripheral blood was measured 10 min before and 15 min after nicotine presentation on acquisition day 3 and after CS exposure on the test day in all experimental groups, as well as on day 9 in the CS_+ and the CS_0 groups. Unfortunately, samples from day 11 were lost due to a technical error. Analysis of CORT levels utilized a commercially available radioimmunoassay (RIA) kit (ICN Biomedicals, Costa Mesa, CA).

Data Analysis

Analyses of variance (ANOVAs) for repeated measures were performed on PBMC and CORT responses to nicotine or saline infusion. Over the course of the experiment a few blood samples could not be obtained due to clotted catheters. Where this occurred, the number of animals per group that reflects the actual number of animals used is indicated.

RESULTS

PBMC Number

On Acquisition day 3, nicotine infusion into the CS_+ and the CS_0 groups induced significantly elevated cell numbers, with a mean increase from a baseline of 4.45 ± 0.35 to 10.62 ± 0.85 × 10^6 cells/ml, $F(1, 11) = 51.5$, $p < 0.001$, 60 min after nicotine administration.

Analysis of PBMC number on the test day (day 5, Fig. 1A) revealed a significant group × time interaction, $F(3, 22) = 7.44$, $p < 0.01$. Baseline levels did not differ among the groups. No significant increases in cell counts were found in the saline-treated placebo animals, indicating that the treatment per se (exposure to the peppermint odor, blood sampling, infusion, etc.) did not affect this parameter. Both placebo and CS_- groups were provided with peppermint odor and saline infusion on the test day but only the critical CS_+ group displayed a marked increase of PBMC counts, $F(1, 22) = 47.51$, $p < 0.001$. As in the placebo-treated group, no significant elevation was evident in the unpaired control group ($F < 1$). Animals in the CS_0 group showed a small increase in PBMC, $F(1, 22) = 4.90$, $p = 0.04$, but the cell numbers in this group (Fig. 1A) were not different from the other control groups.

Unreinforced CS trials were given to CS_+ animals on days 5, 8, 9, 10, and 11 and to CS_0 animals on days 9 and 11 (Table 2). For CS_- animals, reexposure to the CS on day 9 ($n = 5$) failed to elevate cell numbers but on day 11, reexposure to the CS increased PBMC number, $F(1, 4) = 7.39$, $p < 0.05$. Animals in the CS_0 group that were not given unreinforced CS trials on days 5, 8, or 10 had elevated PBMC on days 9 and 11 ($n = 4$) $F(1, 3) = 61.07$, $p < 0.004$, in response to CS reexposure. PBMC numbers were higher when the first unreinforced CS exposure (test trial) occurred on day 5 (CS_+) than when it occurred on day 9 (CS_0; $F(1, 9) = 7.71$, $p < 0.02$).

Corticosterone Concentration

A similar pattern of results was observed for the CORT measure. Comparable to the PBMC data, nicotine infusion on acquisition day 3 caused a significant elevation of CORT secretion from a baseline of 39.8 ± 13.8 to 176.9 ± 15.9 ng/ml, $F(1, 9) = 70.4$, $p < 0.001$, 15 min after nicotine infusion in the CS_+ ($n = 6$) and the CS_0 ($n = 5$) groups.

The CORT concentrations of all experimental groups on day 5 are summarized in Fig. 1B. Analysis of variance revealed a significant group × time interaction, $F(3, 18) = 6.22$, $p <$

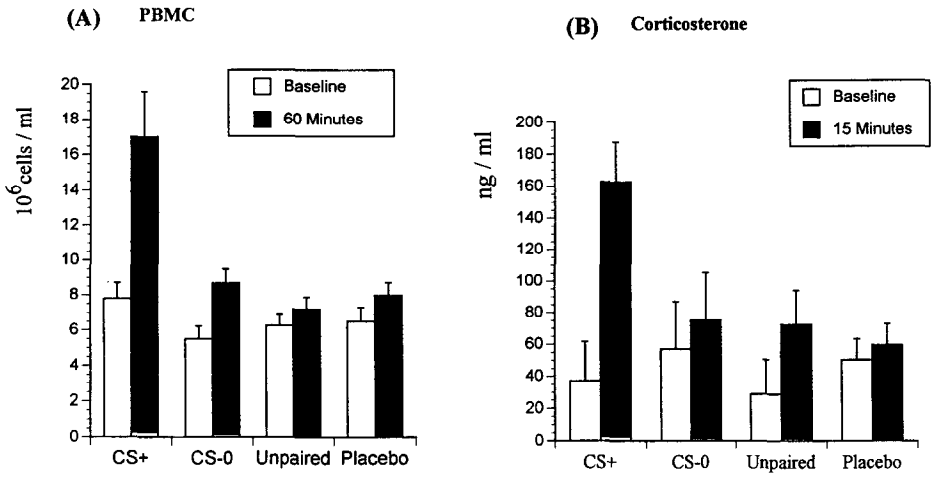

FIG. 1. Absolute number of peripheral mononuclear cells (pMNC) of all experimental groups obtained 5 min before (baseline) and 60 min after stimulus presentation on the critical test day 5 (A). Furthermore, pMNC counts of the CS_+ group and the CS_0 group on experimental day 9 (EXT A; RET A) and on experimental day 11 (EXT B; RET B). (B) Corticosterone (CORT) concentration (in ng/ml) of all experimental groups obtained 5 min before (baseline) and 15 min after stimulus presentation on the critical test day 5 (A). Furthermore, CORT concentration of the CS_+ group and the CS_0 group on the experimental day 9 (EXT A; RET A) compared to the CORT concentration on test day 5 (CS_+).

0.01. Baseline values did not differ among the groups. Saline-treated placebo animals ($n = 6$) did not show a significant increase in CORT concentration ($F < 1$), suggesting that the experimental treatment per se did not result in elevated levels of this steroid. However, the same treatment, for instance, peppermint odor and saline infusion, of the CS_+ group significantly increased CORT levels, $F(1, 18) = 40.15$, $p < 0.001$. Fifteen-minute CORT levels in the CS_0 ($n = 4$) and the unpaired control ($n = 6$) groups did not differ from the placebo values. Analysis of CORT levels on day 9 revealed no conditioned elevation of CORT secretion by either the CS_+ ($n = 6$) or the CS_0 ($n = 3$) groups.

DISCUSSION

Rats provided with nicotine infusions had significantly elevated CORT concentration 15 min and significantly increased PBMC counts 60 min later. After repeated associations of nicotine infusion with a peppermint odor, reexposure to the odor and neutral saline infusion resulted in significantly increased PBMC numbers and CORT secretion. In contrast, exposure to the odor caused no alteration of PBMC or CORT in placebo-treated or unpaired control groups, suggesting that not the experimental procedure per se but the contingent relationship between the CS and the US during acquisition is responsible for the alteration of PBMC number and CORT concentration. The CS_0 group showed a slight increase in PBMC number, whereas CORT levels in this group were unchanged. The reason for increased PBMC counts in the CS_0 group is not clear. It should be noted that the elevation of PBMC number in the CS_0 group was slight and was significantly lower than PBMC counts seen in the critical CS_+ group. The present data indicate that an association of the pepper-

TABLE 2

PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) RESPONSES TO UNREINFORCED CS REEXPOSURE ON DAYS 5, 9, AND 11

Group	Extinction Trial	n	PBMC ($\times 10^6$ cells/ml) Baseline	60 min	p
CS_+					
Day 5	1*	7	7.78 ± 0.93	17.01 ± 2.61	< 0.001
Day 9	3	5	5.50 ± 0.86	6.96 ± 0.54	NS
Day 11	5	6	5.12 ± 0.63	9.33 ± 0.84	< 0.05
CS_0					
Day 9	1*	4	3.92 ± 0.63	8.92 ± 0.78	< 0.005
Day 11	2	4	5.06 ± 0.77	9.52 ± 1.01	< 0.005

*The first extinction trial is also the test trial for determining the response to CS alone.

mint odor and nicotine infusion was established during the acquisition trials, resulting in a conditioned increase in PBMC number and CORT secretion upon reexposure to the CS.

These data suggest that peripheral leukocyte number can, in fact, be modulated by classical conditioning techniques and support previous observations of conditioned manipulation of leukocyte counts. Thus, after conditioning rats with a saccharin/vanilla solution and cyclophosphamide (US), conditioned animals showed significantly reduced leukocyte number when reexposed to the CS (22). Furthermore, Husband and colleagues demonstrated that after association of saccharin (CS) with the immunomodulating drug levamisole (US), subsequent presentation of saccharin produced conditioned modulation of the helper:suppressor T cell subset ratio (19).

Classical conditioning of endocrine responses has been evaluated by others. There are some reports showing that reexposure of a taste (CS) previously associated with illness-inducing food or drinking fluid (US) resulted in increased CORT secretion in rats (1,18). Furthermore, more recent experiments suggest that reintroduction to various stimuli previously paired with a stressor, for instance, shock exposure, induced marked elevation of CORT levels (13,23). These results are extended by other experiments demonstrating conditioned alteration of CORT concentration using corticotropin-releasing-factor (CRF) or interleukin-1 (IL-1) as the unconditioned stimulus (15,24). Most interestingly, Caggiula and co-workers demonstrated conditioned tolerance of nicotine-induced CORT secretion. Thus, after repeated pairing of nicotine injection (US) in a specific environment (CS) rats showed a decrease of the CORT stimulating effect of nicotine injection as indexed by plasma CORT levels (12). These data are in contrast to our findings showing a compensatory CR, for instance, a decrease of CORT secretion, instead of a mimicking CR, which resembles the UR. It should be noted, however, that in both studies different numbers of acquisition trials as well as different dosis of nicotine bitartrate have been used.

Taken together, the data discussed above support the present findings suggesting that distribution of PBMC and CORT secretion are, indeed, responsive to behavioral learning processes. The fact that changes in both responses were found in a single conditioning paradigm does not imply that altered CORT levels induced altered PBMC distribution or vice versa. It is possible that these two phenomena are independently derived and involve separate CNS pathways or mechanisms. In our study, the imposition of unreinforced CS trials did not eliminate conditioned PBMC responses that were observed on the fifth extinction trial. In contrast, conditioned CORT responses were not observed after the initial test trial; conditioned CORT responses were lost with the passage of time. This observation would also be consonant with previous reports documenting differential extinction rates for different conditioned responses (8,26). Additional studies that include more extinction trials with a larger (n) will be necessary to establish extinction of conditioned PBMC responses in our learning protocol.

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