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Hsueh, Chi-Mei Ko, Ph.D.

University of Alabama at Birmingham, 1992

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EFFERENT MECHANISMS RESPONSIBLE FOR ELICITING THE CONDITIONED ENHANCEMENT OF NATURAL KILLER CELL ACTIVITY

by

CHI-MEI HSUEH

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biology in the Graduate School, The University of Alabama at Birmingham

BIRMINGHAM, ALABAMA

1992

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree Ph.D. Major Subject Biology

Name of Candidate Chi-Mei K. Hsueh

Title Efferent mechanisms responsible for eliciting the conditioned enhancement of natural killer cell activity

The mechanisms responsible for recalling the conditioned natural killer (NK) cell response were investigated. Conditioning requires animals to learn the associative relationship between the conditioned stimulus (CS) and the unconditioned stimulus (US). Neither the CS/US learning nor recall of the conditioned response (CR) was dependent on the consciousness of the animals. Both learning and recall could be elicited under anesthesia. It was also confirmed that a naltrexone (NTX) sensitive opioid pathway was involved centrally in recall of the conditioned NK cell response. Injection of methionine-enkephalin (Met-Enk) directly into the cisterna magna of the brain, resulted in a significant enhancement of NK cell activity, and this activity was specific to the N-terminal tyrosine residue of Met-Enk. The enhancement was blocked by opiate antagonists, NTX and quaternary naltrexone. These results indicate that the activation of a centrally located opioid pathway is capable of activating pathways that stimulate the NK cell response in the periphery. The hypothalamus is involved in regulating and maintaining the homeostasis of the organ systems. It is likely that in the recall of the CR the hypothalamus-pituitary-adrenocortical (HPA) axis is involved. It has been known that opioids have the potential to modulate the activity of the HPA axis. Blocking of the CR with dexamethasone at the conditioned recall but not at the association stage supports the view that the HPA axis is utilized in recall of the CR. Adrenocorticotropin (ACTH), β-endorphin (β-End) and

interferon (IFN) have all been reported to stimulate NK cells directly and/or indirectly. Since the neuroendocrines permit the central nervous system (CNS) to effectively communicate with the NK cell system the plasma levels of ACTH and β-End were measured by radioimmunoassay. These neurohormones which are released from the HPA axis might be directly involved in the regulation of the NK cell activity in conditioned animals. In addition, northern hybridization analysis was applied to detect the IFN message expression in the spleens of conditioned animals. The direct role of IFN in mediation of the NK cell activity was therefore assessed by monitoring IFN gene expression. An increase in plasma levels of ACTH and IFN-α message in spleen cells were found in conditioned mice. Therefore, it is possible that the effector pathway utilized by conditioned animals in recall of the CR is mediated by an opioid-driven signal which activates the HPA axis, and results in the release of ACTH in the plasma and an upregulation of IFN-α in the spleen.

Abstract Approved by: Committee Chairman Vithal K. Ghanta

Program Director [signature]

Date 12/8/92 Dean of Graduate School [signature]

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GENERAL INTRODUCTION

Classical conditioning was first described by Russian physiologist, I. P. Pavlov in 1927. It is one of the oldest and most systematically studied phenomena in psychology. It represents the simplest type of "associative learning" (Aszodi & Friedrich, 1987). The essential parameter in conditioning is the pairing of two stimuli. One, initially neutral in that it elicits no response, is the conditioned stimulus (CS); the other, which consistently elicits a response, is called the unconditioned stimulus (US). The response elicited by the US is the unconditioned response (UR). As a result of the pairing of the CS and the US, the previously neutral CS comes to elicit the response, the conditioned response (CR) (Morgan & King, 1966). In general, the criteria (Aszodi & Friedrich, 1987; Abrams & Kandel, 1988; Morris *et al.*, 1988) for any mechanistic model that is to account for classical conditioning include: (1) without input signal (US or CS) it must not induce responses; (2) the US alone should induce a response; (3) the CS must not give the response in the "naive" state, i.e., before pairing CS and US; (4) after the association of CS and US, some lasting modification ("memory") should occur such that the CS alone can elicit the response during the recall stage; (5) the CS always precedes by a certain critical period prior to the presentation of US; and (6) there is a predictive relationship between the CS and US, because they are positively correlated. Although classical conditioning has been observed for many years, the underlying mechanisms are still unclear and are currently under investigation.

Many responses have been subjected to conditioning including the immune responses. By pairing saccharin flavored solution (CS) with the immunosuppressive drug cyclophosphamide (US), a conditioned suppression of antibody production can be elicited

by the CS alone (Ader & Cohen, 1982; Kusnecov *et al.*, 1989). Changes in cell-mediated immune response were also observed, in that mitogen-induced spleen cell proliferation and NK cell cytotoxicity were suppressed by reexposing conditioned animals to the saccharin solution (CS) (Kusnecov *et al.*, 1989, 1990; O'Reilly & Exon, 1986). With the same CS/US pairing, conditioned suppression of the plaque-forming-cell response (McCoy *et al.*, 1986) and conditioned enhancement of delayed-type hypersensitivity were also described (Bovbjerg *et al.*, 1987).

A conditioned immune response requires two directions of communication: (1) US signals from the immune system (IS) communicate with the central nervous system (CNS) during the formation of the conditioned association with the CS (afferent communication), and (2) signals from the CNS communicate with the IS during the conditioned recall and modulate immune function (efferent communication).

Previously, in our laboratory a series of studies dealing with conditioning the natural killer (NK) cell activity were conducted (Hiramoto *et al.*, 1987; Ghanta *et al.*, 1987; Solvason *et al.*, 1992). During the course of these studies, a simple and highly reproducible conditioning paradigm was developed, in which the conditioned enhancement or suppression of the NK cell response can be established with one association of the CS with US and in which the CR can be measured anywhere between 3 to 6 days (Solvason *et al.*, 1992). Camphor odor and poly I:C were used as the CS and US, respectively. Poly I:C is a double-stranded synthetic RNA that mimics infection by viral RNA. Poly I:C induces the expression and secretion of interferon (IFN)-α and -β (Riordan & Pitha-Rowe, 1985), which stimulates NK cell activity (Djeu *et al.*, 1979). The kinetics of the response are ideal for conditioning studies because following poly I:C injection, NK cell activity peaks within 24 hr (Gidlund *et al.*, 1978) and returns to baseline within 3-5 days (Ghanta *et al.*, 1987). Camphor odor or saccharin-LiCl serving as the CS can be paired with the poly I:C (US) to condition the enhancement of the NK cell activity (Solvason *et al.*, 1988). The observed conditioned enhancement of the NK cell activity in conditioned animals is not

due to any nociceptive properties of the CS, as the smell of camphor had no effect on the NK cell activity (Hiramoto *et al.*, 1992). This conditioning model is used to study molecular mechanisms that participate in the conditioned NK cell response.

It has been known that IFN-β (but not -α) could replace poly I:C as the US (Solvason *et al.*, 1988, 1991b). In addition, the conditioned augmentation of NK cell response demonstrated odor specificity and was dependent on the distinct odor used as CS, such that the CR was only elicited by the odor used in the formation of the conditioned association (Solvason *et al.*, 1991a).

When animals were treated with reserpine, to deplete 95% of catecholamines centrally and peripherally, both the CS/US associative learning and the recall of the CR were prevented. On the other hand, when 6-hydroxydopamine was used to block the peripheral β-adrenergic sympathetic neurons, it did not prevent CS/US learning or recall (Hiramoto *et al.*, 1990). These results suggested that central catecholamines were induced in the conditioned learning and recall of the NK cell response. The peripheral sympathetic nervous system appears not to be responsible for mediating the signals to and from the CNS during CS/US learning or CS recall.

In other studies the conditioned augmentation of NK cell response was blocked by the naltrexone (NTX) at the CS recall but not at the CS/US association step. The recall of the CR, however, was not blocked by the quaternary naltrexone (QNTX) (Solvason *et al.*, 1989). Naltrexone blocks the opioid receptors both centrally and peripherally, whereas QNTX blocks opioid receptors in the periphery only. These results indicated a centrally located opioid pathway might play an important role in triggering the CR during the conditioned recall stage.

From the summary of these observations mentioned above, the mechanism(s) of conditioned NK cell response is far more complex. In this dissertation study, the nature of this model was further characterized by focusing on identifying the events/signals involved in the effector pathway (CNS to IS) of conditioned recall.

In the standard procedure for conditioning of the NK response, the interval between the odor presentation (CS) and the poly I:C injection (US) is usually 2 to 3 min. In classical Pavlovian paradigm, the strength of the association is affected by the temporal relationship between the CS and the US and the strength of the stimuli. Although the specific CS/US inter-stimulus interval (ISI) that yields the strongest condition varies with the organism and response studied, for every response there is some optimal ISI. In general, 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 CR also declines.

An earlier study from our laboratory (Hiramoto *et al.*, 1992) demonstrated that conditioning of the NK cell response could be effected even when the CS/US inter-stimulus interval was separated by 1 to 2 days. This unusually long interval between the CS and US indicated that this learning might be different from the conscious learning where the organism seeks information using logical perceptual relations among events. The goal oriented emotions and motivations of the animals appeared not to be heavily or directly involved in this delayed associative learning. Therefore, in this dissertation study it was proposed that the CS/US learning must be taking place unconsciously and the animals should be conditioned even when they are under anesthesia. The effect of anesthesia on the conditioned enhancement of NK cell response was investigated by anesthetizing animals either at the CS/US association or at the CS recall stage. Conditioning under anesthesia can provide more information and opportunities to manipulate the conditioned animals without stressing them with handling.

While IFN-β was demonstrated to be the afferent signal that allows CS/US association to occur, the pathways which are involved in the CS recall of the CR remain to be identified. Since the conditioned recall but not the association can be blocked by treatment with NTX, a centrally mediated opioid pathway was suggested as being involved in regulating the CR (Solvason *et al.*, 1989). To further document the involvement of

opioid peptides in the pathway resulting in the CR, an opioid peptide, methionine-enkephalin (Met-Enk), was injected directly into the brain via the cisterna magna (CM) to see its effect on NK cell activity. Many reports (Gacel *et al.*, 1979; Nikiforovich *et al.*, 1990) indicated that the amino terminal (N-terminal) end of the opioid peptides was responsible for the opioid receptor binding activity, therefore, a N-terminal tyrosine-deprived Met-Enk (Des-Tyr-Met-Enk or DT-Met-Enk) was used to verify that the effect of Met-Enk is an opioid-specific event. Methionine-enkephalin was selected because it is a brain-derived endogenous opioid peptide, and it has the potential to stimulate the NK cell activity both in humans (Krout & Greenberg, 1986; Oleson & Johnson, 1988) and in mice (Ghanta *et al.*, 1991; Faith *et al.*, 1987).

It has been known that opiates can affect the functional activity of the hypothalamus-pituitary-adrenocortical (HPA) axis (Grossman *et al.*, 1982; Cohen *et al.*, 1985; Smriti *et al.*, 1987; Odio & Brodish, 1990; Siegel *et al.*, 1982), although the exact role (stimulatory or inhibitory) played by the opioid system on resting and stress-induced activity of the HPA axis remains uncertain due to conflicting results. Earlier evidence (Solvason *et al.*, 1989) supports the contention that an endogenous opioid peptide may be released during the conditioned recall, which might play an important role in triggering the HPA axis to release neurohormones that can stimulate the NK cell activity directly or indirectly. To demonstrate that the conditioned NK cell response is mediated through the neuroendocrine system (HPA axis), particularly at the recall stage, the effect of dexamethasone (Dex) on NK cell activity at the CS/US association and the CS recall levels was investigated. Dexamethasone was chosen because it has been shown to inhibit the HPA axis (Calogero *et al.*, 1990; Imaki *et al.*, 1991; Sapolsky *et al.*, 1990) and is a potent synthetic glucocorticoid which can suppress plasma corticosteroid level by means of negative feedback inhibition on the HPA axis (Kusnecov *et al.*, 1990). Several reports indicated that the release of adrenocorticotropin (ACTH) and β-endorphin (β-End) from the pituitary gland were also inhibited by Dex treatment (Britton *et al.*, 1985; Kant *et al.*, 1989;

Rossie *et al.*, 1979). Therefore, if the HPA axis is involved in the CR, pretreatment with Dex at the recall step should block the conditioned enhancement of NK cell activity.

Some peptides released from the HPA axis (eg. ACTH and β-End) have been demonstrated to possess neuroimmunomodulatory activity (Blalock, 1989; McCann *et al.*, 1990), and stimulate the NK cell activity in human (Kay *et al.*, 1987; Mandler *et al.*, 1986) and murine systems (Krout & Greenberg, 1986). Adrenocorticotropin and β-End are concomitantly released from the pituitary (Guillemin *et al.*, 1977) and have been reported to stimulate the NK cell activity *in vivo* and/or *in vitro* (Gatti *et al.*, 1990; McGlone *et al.*, 1991; Mandler *et al.*, 1986; Mathews *et al.*, 1983). Plasma β-End and ACTH levels in conditioned and control mice were measured to prove whether β-End or ACTH is the efferent signal released from the HPA axis that is involved in the conditioned enhancement of NK cell activity.

Natural killer cell activity can be enhanced by many biological agents (Shau & Golub, 1985), such as IFN, IFN inducer (poly I:C), and interleukin-2 (IL-2). β-Endorphin can enhance both NK cell activity (Mathews *et al.*, 1983) and the production of IFN by NK cells (Mandler *et al.*, 1986; Brown & Van Epps, 1986) and IL-2 by lymphocytes (Gilmore & Weiner, 1988). In light of these observations it would be pertinent to establish whether the conditioned enhancement of NK cell activity correlates with elevation of β-End and/or ACTH and with a concomitant rise in IFN-α or IFN-β.

If β-End or ACTH is released during the recall of the CR, the changes in β-End or ACTH levels should be detected in the plasma of the conditioned mice. In addition, by measuring the IFN gene expression (both α- or β- type) in spleen cells at different times after the CS recall it will be possible to establish the relationships among β-End, ACTH, IFN and NK cell activity in conditioned mice. Radioimmunoassays (RIA) were used to determine the β-End and ACTH concentrations and northern hybridization analysis was used to detect the IFN gene expression.

The overall goal of this dissertation research is to explore the basic molecular mechanisms involved in the conditioned increase in NK cell activity. In order to establish the roles of efferent signals, the following objectives will be investigated: (1) to test whether the signals induced during the conditioned association and recall in the anesthetized animals can cause an enhancement of conditioned NK cell response; (2) to specify the role of Met-Enk which might be released from the CNS during the recall that might be responsible for the enhancement of the NK cell activity; (3) to demonstrate the role of the HPA axis in the conditioned NK cell response; and (4) to target the specific factors which are responsible for triggering the conditioned NK cell response in the periphery.

Understanding the mechanisms of conditioned enhancement of NK cell response is important in many aspects. Since CR requires animals to learn and to remember the associative relationship between the CS and the US, mechanisms that deal with conditioning can help us understand details about learning and memory of a physiological function. Natural killer cells have been demonstrated to play a significant role in natural immunity against lymphoid derived and other tumors (Herberman & Ortaldo, 1981; Wiltrout *et al.*, 1985) and to be involved in antibacterial (Kearns & Leu, 1983) and antiviral host response (Bukowski *et al.*, 1985). Because NK cells are the first line of defense for the host, conditioned learning of NK response might permit the host through signals perceived by the CNS to make immediate responses to its environment and to infections. This response can take place within hours even before the specific immune system is prepared to make a suitable response. Such conditioned defensive reflex responses might play an important role in maintaining host resistance and survival.

ACQUISITION OF ENHANCED NATURAL KILLER CELL ACTIVITY UNDER ANESTHESIA

CHI-MEI HSUEH, JOAN F. LORDEN, RAYMOND N. HIRAMOTO, AND VITHAL K. GHANTA

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An increase in natural killer (NK) cell activity can be conditioned with a one trial learning paradigm to demonstrate the interaction between the central nervous system (CNS) and the immune system. In order to demonstrate learning possibilities during "non-conscious" state, mice were anesthetized with a ketamine/rompun mixture and underwent one trial learning with odor cue as the conditioned stimulus (CS) preceding the unconditioned stimulus (US). The results indicate that mice that are exposed to camphor odor cue under the influence of anesthesia can associate the signal with the poly I:C unconditioned stimulus and are able to recall the conditioned response upon reexposure to the CS. Secondly, the conditioned association made in a conscious state can be recalled by exposure to the same olfactory odor cue in a "non-conscious" state. The increase in the conditioned change in NK cell activity of both situations was significantly higher than the control group. The results demonstrate that learning can take place and the learned response can be recalled under the reduced awareness caused by anesthesia. The findings reported here are unusual and novel in that they demonstrate that the CNS can learn new associations under conditions where the host is apparently unaware of the signals being linked. Anesthesia combined with the long interstimulus interval indicate that certain neuronal pathways in the CNS are receptive to second signals (elicited by the US) even when the second signal is separated by one day. This means the conditioned learning of a physiological response can take place unconsciously at a separate level and under situations where the host is totally unaware of the events which the brain is processing and linking as incoming information.

INTRODUCTION

Much evidence exists that the CNS communicates with the immune system. The pathways by which the CNS and the immune system maintain this communication with one another can be identified using conditioning paradigms which are easy to perform and which produce conditioned animals with high reliability. The NK cell system has been utilized for 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[20,32], antibacterial resistance[23], and antiviral response[5].

By pairing of camphor odor (CS) and the injection of poly I:C (US), an increase in NK cell activity was conditioned based on a Pavlovian conditioning paradigm. Camphor alone has no effect on NK cell activity[30]. Poly I:C, the unconditioned stimulus, is a double stranded synthetic RNA that mimics infection by double stranded RNA virus. Poly I:C induces the expression and secretion of interferons (IFN) -α and -β[27]. The IFN in turn directly stimulates NK cell activity[7]. The kinetics of this response are ideal for conditioning studies because following poly I:C injection, NK cell activity peaks within 24 hr[13] and returns to baseline within 3 to 5 days[12].

The evidence that the CNS and immune system can signal the NK cells is shown by the fact that neurotransmitters and lymphokines influence NK cell activity. For example, *in vitro* NK cell function is suppressed by exposure to β-adrenergic agonists or prostaglandins[2,3,17,19]. Depletion of catecholamines with 6-hydroxydopamine caused significant temporal increase and decrease of the NK cell activity[26]. Interferon-α and -β and IL-2, which are produced by macrophages and T-helper cells, can also influence NK cell activity[28].

Earlier studies demonstrated that the conditioning of enhanced NK cell activity could be effected even when the CS/US interstimulus interval was separated by 1 to 2 days[21]. This unusually long interval between the CS and US indicated that the learning differed from conscious learning where the organism seeks information using logical perceptual relations among events. The delayed association learning goes beyond a simple contiguous temporal relation and that the goal oriented emotions and motivations of the animals appear not to be heavily or directly involved. In light of these observations, it is reasoned that the CS/US learning must be taking place unconsciously and that if this is true CS/US linkage should be possible even when the host is under anesthesia. The present

studies were conducted to explore the effects of anesthesia on learned enhancement of NK cell activity by anesthetizing animals during either training (CS/US association) or test presentations of the stimulus odor (CS recall).

MATERIALS AND METHODS

Mice

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 libitum*. A 12 hr light/dark cycle was in effect with lights on at 7:00 am. The animals were adapted to the vivarium for at least one week before being used in the experiments.

Conditioning procedure

All conditioning procedures and sacrifice of the animals for measurement of NK cell activity were carried out between 7:00 and 8:30 am. This time was chosen because circulating levels of corticosteroids are lowest at this point in the diurnal cycle. On the training and test days, animals to be exposed to camphor were transported to a room outside the vivarium. Camphor was prepared by partially dissolving a 1 oz block in 150 ml of mineral oil while stirring over low heat. Thirty ml of the mixture was heated in a microwave oven for 1 min and then placed on the top of the mouse cage. A second cage was inverted over this to contain the camphor odor. Mice were exposed to camphor in this way for 30 min. Poly I:C was obtained in lyophilized form from Pharmacia and dissolved in physiological saline at 200 µg/ml and stored at 4°C. Immediately following exposure to the camphor odor, mice were injected with 20 µg (0.1 ml) poly I:C. Mice were placed in clean cages kept in the same room for 3 hr before being returned to the vivarium to allow any odor of camphor remaining on their fur to dissipate. In addition, previous results have shown that it is not possible to condition other animals to camphor using residual camphor on the fur of camphor-exposed mice as a stimulus[21]. Conditioned (CND) groups received exposure to camphor (day -1) followed one day later by injections of poly I:C (day 0). Control animals (NC) received only injections of poly I:C (day 0). Half the animals in each

group were anesthetized with ketamine (85 mg/kg) and rompun (13 mg/kg) ip on day -1. These groups were designated $CND_{K/R}$ and $NC_{K/R}$ (Table I). On day 2 all animals were exposed to camphor and on day 3, sacrificed for measurement of NK cell activity. On day -1, camphor exposure was initiated as soon as the mice were inert from anesthesia. When the mice became somnolent, they were tested for the presence of a leg flexion reflex in response to pinching the foot. Absence of the reflex was considered evidence of deep anesthesia. Camphor exposure lasted for 30 min. Animals in the NC anesthesia group were monitored during this time for recovery. At the doses of ketamine and rompun used adequate levels of anesthesia were maintained for the duration of camphor exposure. In separate groups of animals, the effects of anesthesia during the test presentation of camphor were examined (Table II). Groups and conditions were identical to those described above, except that animals in the anesthesia groups were anesthetized only during the day 2 camphor exposure.

Preparation of spleen cells

Animals of each group were killed simultaneously in a box with CO_2 asphyxiation. The animals were sacrificed before 8:00 am. This procedure took only 5 to 10 min to sacrifice all four groups (CND, NC, $CND_{K/R}$ and $NC_{K/R}$). Spleens were removed immediately and placed into individual petri-plates containing sterile 0.9% sodium chloride solution 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 saline, centrifuged at 1800 rpm (566 g) 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 saline 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.

TABLE I

Schedule for one trial association of odor CS and Poly I:C US with/without anesthesia.

a. BALB/c mice were anesthetized with ketamine/rompun (K/R) mixture of 85 mg/kg K and 13 mg/kg R, 0.1 ml intraperitoneally; as soon as the mice were inert they were exposed to the odor of camphor (CND) for 30 min. b. Poly I:C, 0.1 ml containing 20 μg was given intraperitoneally. c. Four groups of mice were exposed to the odor of camphor for 1 hr. C. represents the camphor smell. P. represents the poly I:C injection.

	Days			
Groups	*-1*	*0*	*2*	*3*
$CND_{K/R}$	K/R + C[a]	P[b]	C[c]	NK
$NC_{K/R}$	K/R	P	C	NK
CND	C	P	C	NK
NC	-	P	C	NK

TABLE II

Schedule for a learned NK cell activity and recall in a non-conscious state[a]

a. BALB/c mice of all four groups were handled similar to standard CND and NC groups on day -1 and day 0. On day 2, $CND_{K/R}$ and $NC_{K/R}$ were anesthetized with ketamine/rompun mixture and made sure that the animals were inert prior to exposure to camphor odor. All of the other treatments were similar to Table I. Each group had 10 mice.

	Days			
Groups	*-1*	*0*	*2*	*3*
$CND_{K/R}$	C	P	K/R + C	NK
$NC_{K/R}$	-	P	K/R + C	NK
CND	C	P	C	NK
NC	-	P	C	NK

Assay for NK cell activity

YAC-1 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 100 U penicillin, 100 μg streptomycin, and 5 x 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 was >95%. YAC-1 cells were labelled with sodium chromate (Amersham, Chicago, IL) at a ratio of 100 μCi/1 x 10^6 cells in a very small volume (total volume was 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 twice and suspended at a final density of 1 x 10^5 cells/ml in RPMI 1640 supplemented with 5% FCS. One-tenth ml 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 of 1 x 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. One-tenth ml of supernatant from each well was collected after centrifugation of plates. The radioactivities of the samples were counted in a Beckman gamma counter. Maximum ^{51}Cr-released from the target cells was measured after incubation in the presence of 0.1N HCl and spontaneous release in the presence of medium. Percent (%) specific release = 100 x [(test release-spontaneous release)/(maximum release-spontaneous release)].

Statistical analysis

A repeated-measures analysis was used to analyze this factorially designed experiment. Mice were assigned to the four different groups (CND, NC, $CND_{K/R}$ and $NC_{K/R}$) and repeats for each mouse were at the three E:T ratios. A split-plot design was used with mice as the main plot and mice at each E:T ratio as a subplot unit. This particular design takes into account the fact that different experimental subjects can have very different mean responses because of between-subject variability and not necessarily because of treatment effects. The various treatments were compared individually using

Duncan's Multiple Range Test to control for the multi-comparisons with the appropriate error variance utilized for making the comparisons[10,14].

RESULTS

Single trial learning of NK cell activity and effect of anesthesia on the associative process

BALB/c mice can be trained to associate an olfactory cue (camphor odor) with poly I:C which is an interferon producer and NK cell activator. This simple and reproducible conditioning paradigm was used to establish the abilities of mice to make an association between the odor cue and poly I:C in a non-conscious state. Four groups of mice with 9 mice/group were included in this experiment (Table I).

Overall, there were significant differences between the treatment groups ($F = 9.37$, $P < 0.001$). Duncan's Test (controlling for the Type I comparison-wise error rate) indicated no difference between CND and $CND_{K/R}$ groups or between NC and $NC_{K/R}$; however, the NK cell activity of the $CND_{K/R}$ group was different from the $NC_{K/R}$ group and the CND group differed from the NC group (Table III).

Effect of anesthesia on the recall of learned association between camphor odor and poly I:C

To examine the effects of anesthesia or the non-conscious state on the recall of conditioned association response, mice in the CND and $CND_{K/R}$ groups were subjected to one trial conditioned association as described in Materials and Methods and Table II. On day 2 $CND_{K/R}$ and $NC_{K/R}$ groups were anesthetized prior to exposure to camphor odor.

CND and NC groups served as controls and also to establish the reproducibility of the conditioned response. The results showed that there were large differences between the treatment groups ($F = 18.83$, $P < 0.001$). Duncan's Test (controlling for the Type I comparison-wise error rate) indicated statistically significant differences between the NK cell activity for $CND_{K/R}$ vs. $NC_{K/R}$, $CND_{K/R}$ vs. CND, and CND vs. NC (Table IV).

TABLE III

NK cell activity of one trial learning paradigm of mice with/without anesthesia

a. Values were mean for each group ± SEM.

	Percent ^{51}Cr-released, E:T ratio		
Group	*200:1*	*100:1*	*50:1*
$CND_{K/R}$	17.9 ± 1.4[a]	14.6 ± 1.0	10.4 ± 0.8
$NC_{K/R}$	12.9 ± 0.8	10.5 ± 0.7	7.3 ± 0.7
CND	15.6 ± 0.9	13.1 ± 0.8	8.7 ± 0.7
NC	11.3 ± 0.7	8.9 ± 0.7	6.1 ± 0.5

TABLE IV

NK cell activity of mice where learning was performed in conscious state and recall in non-conscious state.

a. Values were mean for each group ± SEM.

	Percent ^{51}Cr-released, E:T ratio[a]		
Groups	*200:1*	*100:1*	*50:1*
$CND_{K/R}$	16.8 ± 0.9[a]	15.4 ± 0.8	11.4 ± 0.7
$NC_{K/R}$	7.0 ± 0.8	6.4 ± 0.6	5.3 ± 0.5
CND	13.8 ± 1.0	12.5 ± 0.9	9.2 ± 0.7
NC	9.6 ± 1.3	8.9 ± 1.2	6.5 ± 0.8

DISCUSSION

The results clearly demonstrate that an association between the CS and the US can take place even while the mouse is in deep sleep induced by anesthetics that interfere with normal perception. Secondly, a conditioned increase in the NK cell activity was observed when the association between the CS and US was made in a conscious state and reexposed to the CS in a state of unconsciousness. This paradigm is useful to demonstrate the state of unconsciousness both on association and recall of physiological response is independent of the state of the animal. A number of studies have been reported demonstrating conditioned responses under anesthesia in support of the observations reported here. Edeline and Neuenschwander-El Massioui[9] demonstrated retention of a tone (CS) with a footshock (US) association learned under ketamine anesthesia. The learning under these conditions is strong enough to trigger an adaptive response even after a change in the state of consciousness and a context modification between conditioning and test. Bermudez-Rattoni *et al.*[1] demonstrated acquisition of conditioned flavor aversions under anesthesia using "Innovar-vet" and rompun. Weinberger *et al.*[31] reported an acquisition of Pavlovian fear conditioning with epinephrine under sodium pentobarbital with chloral hydrate anesthesia. They have also demonstrated a number of aspects of this conditioned response: animals that received epinephrine at the time of training under anesthesia demonstrated very good conditioned suppression when tested 2 or 7 days later. Their results provide support for the view that learning can occur under anesthesia with appropriate hormonal conditions.

Behaviorally modified NK cell activity demonstrated that the odor of camphor was detected and processed by the CNS in anesthetized mice so that when the second signal (poly I:C) was injected one day later it could still be linked to the CS. Although the analgesic properties of ketamine can be explained by its interaction with serotoninergic and opiate receptors[24,29], ketamine may reduce neuronal excitations mediated by the N-methyl-D-aspartate (NMDA) receptor[9]. The hippocampal formation and the olfactory area are regions particularly enriched in binding sites for phencyclidine and ketamine[22]. The results

presented in this paper indicate that blocking NMDA receptors with ketamine does not prevent acquisition of delayed association learning. Subjects usually become aware of the CS/US pairing during acquisition and modify their behavior accordingly. As demonstrated by the present observations and other reported data[11] the subjects can be "unconscious" of the associative processes. The results also demonstrate that the conditioning of an immune function (NK cell activity) is independent of the conscious state of the animal both at the association and the recall stages.

These studies are unique and significant in that one trial association learning is applied and secondly there is no change in the context between the learning and test. The findings have also demonstrated that: 1) the animals can learn under anesthesia and recall the learned physiological effect in a conscious state, 2) learning took place under normal circumstances and recall of the learned response was tested under unconscious conditions, and 3) it is believed that the learned response can be recalled if both manipulations are done under anesthesia with the results observed under the above two different circumstances. It might be possible in this experimental system that epinephrine is released during exposure to camphor odor cue which facilitates learning and recall of the response. This possibility has been suggested by Gold *et al.*[16] that some surgical episodes result in the release of epinephrine after anesthesia has been induced and that in such cases patients can learn and remember events taking place in the operating room.

It is of interest to demonstrate learning of a physiological function can take place during non-conscious states because sensory systems can process stimuli during states of deep anesthesia. Most knowledge of sensory neurophysiology is based on electrical recordings obtained from anesthetized animals. The results of such sensory processing apparently are not remembered or at least not retained in a form that is later expressed in behavior. Only anecdotal reports or ambiguous data of learning in unconscious patients[4,18] or under general anesthesia[6,8] are available. In animal studies, some positive results have been reported in such situations that did not support unequivocally in favor of learning in a

non-awake subject. There are a number of recent reports that attempt to demonstrate learning under anesthesia. A variety of anesthesia, such as a combination of pentobarbital supplemented with chloral hydrate[16,31] and ketamine[9] were used for this purpose. Learning and memory can be facilitated by pharmacological agents and hormones[25]. It has been shown that epinephrine can facilitate memory storage in several situations in which memory storage is deficient[15]. Weinberger *et al.*[31] and Gold *et al.*[16] combined these principles to demonstrate the possibility of associative learning under barbituric anesthesia supplemented with epinephrine. The results reported here support these observations and suggest that physiological processes are taking place normally and conditioned association and recall of a physiological process is expressed similar to the conscious state.

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THE CENTRAL EFFECT OF METHIONINE-ENKEPHALIN ON NK CELL ACTIVITY

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The central effect of opioid peptide on natural killer (NK) cell activity in BALB/c mice was investigated. Injection of methionine-enkephalin (Met-Enk), 0.02 μg/mouse or 1 μg/kg, directly into the cisterna magna (CM) of the brain, resulted in a significant enhancement of NK cell activity. This enhancement was blocked by opiate antagonists, naltrexone and quaternary naltrexone. The same dose of Met-Enk had no effect on NK cell activity when given to the mouse intraperitoneally or intraveneously. Moreover, des-tyrosine-methionine-enkephalin injected into the CM at 1 μg/kg, had no effect on NK cell activity. The results indicate that activation of an opioid mediated pathway in the central nervous system is capable of activating the pathways that stimulate the NK cell response in the periphery.

INTRODUCTION

The enkephalins were originally described as the endogenous ligands for the morphine receptors in the brain by Hughes *et al.*[15]. In addition to their analgesic, antidepressant, antianxiety, and anticonvulsant activity[30], an immunoregulatory action of the enkephalins was first observed by Wybran *et al.*[39] in 1979. In Wybran's report, the receptors for morphine and methionine-enkephalin (Met-Enk) on normal human blood T-lymphocytes were suggested. Since this initial report there has been an increase in interest in the effect of Met-Enk on immune functions. Jankovic and Maric have summarized their groups' extensive studies that dealt with the potentiating and suppressive activities of Met-Enk in a variety of immunological functions[19].

Recent work in this laboratory[35] has demonstrated that a naltrexone (NTX) sensitive opioid mediated pathway in the central nervous system (CNS) was involved in the conditioned enhancement of NK cell response at the recall step. This observation suggested endogenous opioid peptides may be responsible to trigger the conditioned response. In order to confirm this observation, an opioid peptide, Met-Enk, was injected directly into the brain via the cisterna magna (CM) to see its effect on NK cell activity.

Whether injection of Met-Enk into the CM can mimic the effect produced by the conditioned stimulus (CS) that can augment the NK cell activity was also examined.

Methionine-enkephalin was selected for several reasons: 1) it is a brain-derived opioid peptide, 2) it has the potential to regulate the hormone secretion from the hypothalamus-pituitary-adrenal (HPA) axis[13], which is a major pathway linking the CNS and the immune system (IS), and 3) it can stimulate NK cell activity in human[4,22,29] and murine systems[6].

MATERIALS AND METHODS

Animals

BALB/c female mice used in this study were 6 weeks old and were obtained from Charles River Breeding Company (Wilmington, DE). All mice were kept in standard animal facilities, on 12 hr light/dark cycle with *ad libitum* food and water. Mice were separated into groups of 10 per cage at least one week before the experiments were started.

Drugs

Methionine-enkephalin, des-tyrosine-methionine-enkephalin (DT-Met-Enk) and naltrexone (NTX) were purchased from Sigma Chemical Co., St. Louis, MO. Quaternary naltrexone (QNTX) was a kind gift of Dr. Merz (Boehringer-Ingelheim, West Germany).

Treatment schedule

In most of the studies a three day protocol was employed. Mice were injected ip on day 0 with 20 μg poly I:C to induce an NK cell response. Drugs were injected ip, iv or via the CM on day 2 at a time when NK cell response was declining[9]. NK cell assays were performed on day 3.

Cisterna Magna injection

On day 2 poly I:C treated mice were anesthetized with 0.1 ml of ketamine/rompun mixture (85 mg/kg and 13 mg/kg), respectively. Ten minutes later when the animals were inert injections were made into the CM with a 250 μl capacity Hamilton syringe with 5 μl steps using a 27 gauge needle. Prior to delivery, Met-Enk or DT-Met-Enk, was diluted in

sterile saline to 4 μg/ml so that a 5 μl injection would contain 0.02 μg of the drugs. Mice in control group were injected with saline at 5 μl/mouse.

Preparation of Spleen Cells

One day after the CM, iv or Met-Enk ip injection, mice were killed with CO_2 asphyxiation. The animals were sacrificed before 8:00 am. This procedure took only 5 to 10 min to sacrifice all the animals. Spleens were removed immediately and placed into individual petri-plates containing sterile physiological saline (0.9% NaCl) 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 1800 rpm (566 g) 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 saline 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.

NK Cell Assay

YAC-1 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 100U penicillin, 100 μg 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 5 to 15%. YAC-1 cells were labelled with sodium chromate (Amersham, Chicago, IL) at a ratio of 100 μCi/1×10^6 cells in a very small volume (total volume is about 0.2 ml) at 37°C in 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% FCS. One-tenth ml 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 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. One-tenth ml of supernatant from each well was collected after centrifugation of plates at 1400 rpm (345g) for 5 min at 25°C in a Beckman centrifuge. The radioactivities of the samples were counted in a Beckman gamma counter. Maximum ^{51}Cr-released (MR) from the target cells was measured after incubation in the presence of 0.1N HCl and spontaneous release (SR) in the presence of medium. Percent specific ^{51}Cr-release was calculated as 100 x [(test release-SR)/MR-SR].

Statistical analysis

Statistical analysis of the percent specific ^{51}Cr-released was performed using a repeated measures of Analysis of Variance (ANOVA) test with Duncan's multiple range comparisons with an α value of 0.05.

RESULTS

Previous results have demonstrated that NK cell activity can be enhanced *in vivo* by Met-Enk if the mice have been injected with a suboptimal dose of poly I:C[10]. These results have also shown that NTX but not QNTX can block the recall of the conditioned response (CR) indicating that a central opioid mediated pathway is activated which in turn plays a significant role in the activation of NK cells in the spleen. In an effort to test for such an opioid mediated pathway in the CNS, Met-Enk was injected into the CM at low doses. Initial studies were done to test the effect of different doses of Met-Enk via the CM route on NK cell activity. Three of the groups were injected into the CM with Met-Enk at 0.002 µg, 0.02 µg, and 0.2 µg per mouse. The fourth (control) group of mice were injected into the CM with saline, the diluent for Met-Enk. All groups were injected with a suboptimal dose of 1µg poly I:C ip immediately after the injection into the CM. NK cell activity was assessed after 24 hr (Table I). Animals injected into the CM with Met-Enk at a dose of 0.002 µg and 0.02 µg showed an enhanced NK cell activity. At the higher dose of Met-Enk (0.2 µg) this enhancement was lost as the NK cell activity approached that of the saline control. There was an inverse relationship between dose and NK cell activity.

TABLE I

Effect of Met-Enk CM injection on Poly I:C boosted NK cell activity

a. Values are Mean for the group ± SEM. All groups were injected with a suboptimal dose of 1 μg poly I:C (ip) immediately after the Methionine-Enkephalin or saline injection into the cisterna magna. NK cell assay was conducted on the next day. Statistical analysis was performed by repeated measures of ANOVA with α value of 0.05, where Met-Enk 0.002 vs Saline group was, $F(1,8) = 13.891$, $P < 0.01$. Met-Enk 0.02 vs saline group was, $F(1,8) = 2.265$, $P > 0.05$, not significant. Met-Enk 0.2 vs saline group was, $F(1,7) = 0.108$, $P > 0.05$, not significant. *Represents the significant difference that existed between the experimental and control groups.

		Percent ^{51}Cr-released, E:T Ratio		
Dose (μg/mouse)	*n*	*200:1*	*100:1*	*50:1*
0.002	5	30.5 ± 3.2[a],*	21.1 ± 1.4*	14.4 ± 1.7*
0.02	5	20.9 ± 2.2	15.7 ± 1.8	11.0 ± 1.1
0.2	4	16.9 ± 2.5	12.9 ± 2.5	8.3 ± 1.8
Saline	5	15.8 ± 3.0	11.7 ± 2.3	7.3 ± 1.6

To show that stimulation of NK cells by Met-Enk required poly I:C activation, two groups were injected into the CM with 0.002 μg and 0.02 μg Met-Enk. The third group served as control. These results showed no elevation of NK cell activity and indicated that activation of opioid pathway was not sufficient to activate resting NK cells (background, Table II). Preliminary tests have demonstrated that injection of Met-Enk into the CM had no effect on basal level of NK cell activity. However, injection of Met-Enk into the CM stimulated the NK cell activity in a dose dependent fashion, when mice had been treated with a suboptimal dose of 1μg/mouse of poly I:C injected ip. A dose of 0.02 μg/mouse of Met-Enk was selected for injection into the CM based on preliminary trials.

Effect of Met-Enk on mice whose NK cells had been activated with poly I:C

Two different ways of activating the NK cells were tested. In one protocol the scheduling mimicked the 3 day experimental paradigm which had been developed for conditioning studies[34]. In this paradigm mice were injected with 20 μg poly I:C on day 0 and on day 2, Met-Enk was injected directly into the CM of the animals. In the second protocol, mice were injected into the CM with Met-Enk followed immediately by injection of a suboptimal dose of 1 μg poly I:C to activate NK cells. The two treatment protocols were shown in Table III. Table IV showed that: 1) with the 3 day schedule animals in both control and experimental groups had lower NK cell activity than that of animals in the 1 day schedule, 2) the injection of Met-Enk into the CM stimulated the NK cell activity of animals in both paradigms, however, the effect was more significant in animals on the 3 day schedule. Due to the effectiveness of the 3 day schedule, the subsequent studies were carried out using the 3 day paradigm.

The injection of Met-Enk into the CM, but not ip or iv injections, significantly enhanced the NK cell activity of poly I:C boosted animals

A 3 day schedule was used where 20 μg of poly I:C was injected on day 0 and Met-Enk (0.02 μg/mouse) on day 2. Table IV showed that Met-Enk injected into the CM significantly enhanced the NK cell activity ($P < 0.003$), when compared to the effect of

TABLE II

Effect of Met-Enk CM injection on the basal level of NK cell activity

a. Values are Mean for the group ± SEM. All groups were given Methionine-Enkephalin or saline directly into the cisterna magna. Twenty-four hr later NK cell assay was done. Statistical analysis was performed by repeated measures of ANOVA with α value of 0.05, where Met -Enk 0.002 vs Saline group was, $F(1,8) = 6.987$, $P < 0.05$. Met-Enk 0.02 vs saline group was, $F(1,9) = 3.183$, $P > 0.05$, not significant. *Represents significant difference that existed between the experimental and control groups.

		Percent ^{51}Cr-released, E:T Ratio		
Dose (μg/mouse)	*n*	*200:1*	*100:1*	*50:1*
0.002	5	2.2 ± 0.5[a],*	1.6 ± 0.7*	1.2 ± 0.4*
0.02	6	2.4 ± 0.5	2.3 ± 0.7	2.3 ± 0.4
Saline	5	3.8 ± 0.6	3.8 ± 0.6	3.1 ± 0.5

TABLE III

Schedule of Met-Enk CM injection to determine its effect on NK cell activity in two different paradigms

a. Mice in group P_{20}-Met and P_{20}-Saline were treated with 20 µg poly I:C intraperitoneally on day 0. On day 2, Methionine-Enkephalin (1 µg/kg) and saline were given intracisternally to group P_{20}-Met and group P_{20}-Saline, respectively. b. Mice in group P_1-Met and P_1-Saline were treated with lower dose of poly I:C (1 µg, ip) following the intracisternal injection of Met-Enk (1 µg/kg) and saline on the same day. NK cell assay was conducted on the next day.

	Treatment days		
Groups	*0*	*2*	*3*
P_{20} - Met[a]	Poly I:C	Met-Enk	NK
P_{20} - Saline[a]	Poly I:C	Saline	NK
P_1 -Met[b]	-	Met-Enk Poly I:C	NK
P_1 - Saline[b]	-	Saline Poly I:C	NK

TABLE IV

Effect of Met-Enk CM injection on poly I:C boosted NK cell activity in two different paradigms

a. Values are Mean for the group ± SEM. Mice in group P_{20}-Met and P_{20}-Saline were treated with 20 μg poly I:C ip on day 0. On day 2, P_{20}-Met group received 0.02 μg of Methionine-Enkephalin and P_{20}-Saline group received 5 μl saline intracisternally. All of the mice were sacrificed on day 3 for the analysis of NK cell activity. Mice in group P_1-Met and P_1-Saline were treated with 1 μg poly I:C ip following the intracisternal injection of 0.02 μg Met-Enk (P_1-Met group) and saline (P_1-Saline group). All the mice were sacrificed on the next day for the analysis of NK cell activity. Statistical analysis was performed by repeated measures of ANOVA with α value of 0.05 where P_{20} - Met vs P_{20} - Saline group was, $F(1,18) = 11.364$, $P < 0.003$, statistically significant. P_1 - Met vs P_1 - Saline group was, $F(1,18) = 3.557$, $P > 0.05$, not significant. *Represents significant difference that existed between the experimental and control groups.

		Percent ^{51}Cr-released, E:T Ratio		
Groups	*n*	*200:1*	*100:1*	*50:1*
P_{20} - Met	10	16.8 ± 2.0[a],*	14.5 ± 1.8*	10.8 ± 1.3*
P_{20} - Saline	10	9.8 ± 0.7	8.4 ± 0.7	6.2 ± 0.5
P_1 - Met	10	21.5 ± 1.8	16.8 ± 1.4	12.4 ± 0.9
P_1 - Saline	10	16.4 ± 2.0	12.8 ± 1.5	9.7 ± 1.3

saline injected into the CM. However, injection of the same dose of Met-Enk by the ip and iv routes produced no significant elevation of NK cell activity over saline control (data not shown). The findings indicate that Met-Enk can stimulate the NK cell activity through a centrally-mediated pathway and the leakage of Met-Enk into the periphery can not be responsible for the enhanced NK cell activity.

CM injection of DT-Met-Enk had no effect on NK cell activity

To show that the effect produced by Met-Enk was not non-specific, DT-Met-Enk was injected into the CM. The effect of this tyrosine deleted peptide on NK cell activity compared to that of the saline treatment was not significant (Table V, $P > 0.05$). The observation suggested that the tyrosine residue of Met-Enk played an important role in mediating the Met-Enk stimulatory effect on NK cells. Without tyrosine at its amino terminal (N-terminal), Met-Enk lost its potential to modulate the NK cell activity. This finding also indicates that an opioid receptor binding event may be involved, and the N-terminal tyrosine residue is needed for the opioid receptor binding activity[8,28].

The NK cell activity of animals that received both NTX (ip) and DT-Met-Enk (CM) treatments were the same as that of control animals (Table V). There was no synergistic or antagonistic interaction between NTX and DT-Met-Enk. This finding further confirmed that the blocking effect of NTX is specific to the binding of tyrosine at the opioid receptor.

NTX and QNTX blocked the stimulatory effect of Met-Enk on NK cell activity

The effect of Met-Enk was tested in mice pretreated with NTX. Naltrexone blocks both central and peripheral opioid receptors. The Sal-Met and NTX-Met groups were injected into the CM with 0.02 μg Met-Enk and the NTX-Sal group was injected into the CM with saline. NK cell activity was assessed on day 3. The data showed that NTX blocked the enhancing effect of Met-Enk (Table VI, $P < 0.001$). Quaternary naltrexone also blocked the enhancement of NK cell activity by Met-Enk (Tables VII, $P < 0.001$). These results indicate that both central and peripheral opioid receptors may be involved in mediating the Met-Enk effect on NK cells. The data from the QNTX experiments suggest

TABLE V

Effect of DT-Met-Enk on poly I:C boosted NK cell activity, and the effect of naltrexone on the DT-Met-Enk treatment[a]

a. All three groups of mice were treated with 20 μg poly I:C intraperitoneally on day 0. On day 2, Sal-DT-Met group received 0.1 ml of saline ip and 0.02 μg of DT-Met-Enk by the CM route. Sal-Sal group received an ip and a CM injection of saline. NTX-DT-Met group received an ip injection of 10 mg/kg naltrexone, 15 min prior to anesthesia, followed by a 0.02 μg of des-tyrosine-methionine-enkephalin into the cisterna magna. Mice were sacrificed on day 3 for the analysis of NK cell activity. b. Values are Mean for the group ± SEM. Statistical analysis was performed by repeated measures of ANOVA with α value of 0.05, where Sal-DT-Met vs Sal-Sal group was, $F(1,17) = 1.084$, $P > 0.05$, not significant. Sal-DT-Met vs NTX-DT-Met group was, $F(1,17) = 1.650$, $P > 0.05$, not significant. Sal-Sal vs NTX-DT-Met group was, $F(1,18) = 0.098$, $P > 0.05$, not significant. Sal = Saline; NTX = Naltrexone; DT-Met = DT-Met-Enk.

		Percent ^{51}Cr-released, E:T Ratio		
Groups	*n*	*200:1*	*100:1*	*50:1*
Sal-DT-Met	9	7.9 ± 0.7[b]	7.8 ± 0.5	5.4 ± 0.5
Sal-Sal	10	8.3 ± 0.8	9.2 ± 1.0	6.5 ± 0.6
NTX-DT-Met	10	9.3 ± 1.0	9.5 ± 1.0	6.4 ± 0.7

TABLE VI

The stimulatory effect of Met-Enk CM injection on the boosted NK cell activity can be blocked by naltrexone[a]

a. Mice were treated with 20 µg of poly I:C on day 0. On day 2, Sal-Met group was treated with saline (ip) and Met-Enk (1 µg/kg, CM); NTX-Met group was treated with naltrexone (10 mg/kg, ip) 15 min before anesthesia and with Met-Enk (1 µg/kg, CM); NTX-Sal group was treated with naltrexone (10 mg/kg, ip) 15 min before anesthesia and with saline (CM). Mice were sacrificed on day 3 for the analysis of NK cell activity. b. Values are Mean for the group ± SEM. Statistical analysis was performed by repeated measurements of ANOVA with α value of 0.05, where Sal-Met vs NTX-Met group was, $F(1,18) = 54.247$, $P < 0.001$. Sal-Met vs NTX-Sal group was, $F(1,18) = 54.247$, $P < 0.001$. NTX-Met vs NTX-Sal group was, $F(1,18) = 0.994$, $P > 0.05$, not significant. Sal = Saline; NTX = Naltrexone; Met = Met-Enk. *Represents significant difference that existed between the experimental and control groups.

		Percent ^{51}Cr-released, E:T Ratio		
Groups	*n*	*200:1*	*100:1*	*50:1*
Sal-Met	10	12.1 ± 0.5[b,*]	10.2 ± 0.6*	7.3 ± 0.4*
NTX-Met	10	6.7 ± 0.6	5.3 ± 0.4	3.5 ± 0.3
NTX-Sal	10	7.3 ± 0.4	5.9 ± 0.6	4.1 ± 0.4

TABLE VII

The stimulatory effect of Met-Enk CM injection on the boosted NK cell activity can be blocked by quaternary naltrexone peripherally[a]

a. Mice were treated with 20 μg of poly I:C (ip) on day 0. On day 2, Sal-Met group was treated with saline (ip) and Met-Enk (1 μg/kg, CM); Sal-Sal group was treated with saline (ip) and saline (5 μl, CM); QNTX-Met group was treated with quaternary naltrexone (10 mg/kg, ip) 15 min before anesthesia and with Met-Enk (1 μg/kg, CM); QNTX-Sal group was treated with quaternary naltrexone (10 mg/kg, ip) 15 min before anesthesia and with saline (CM). Mice were sacrificed on day 3 for the analysis of NK cell activity. b. Values were Mean for the group ± SEM. Statistical analysis was performed by repeated measures of ANOVA with α value of 0.05, where Sal-Met vs Sal-Sal group was, $F(1,18) = 5.745$, $P < 0.02$. Sal-Met vs QNTX-Met group was, $F(1,17) = 14.425$, $P < 0.001$. QNTX-Met vs QNTX-Sal group was, $F(1,17) = 0.074$, $P > 0.05$, not significant. QNTX-Sal vs Sal-Sal group was, $F(1,18)=1.898$, $P > 0.05$, not significant. Sal = Saline; NTX = Naltrexone; Met = Met-Enk. *Represents significant difference that existed between the experimental and control groups.

		Percent ^{51}Cr-released, E:T Ratio		
Groups	*n*	*200:1*	*100:1*	*50:1*
Sal-Met	10	14.4 ± 1.5[b,*]	12.2 ± 1.4*	8.2 ± 1.0*
Sal-Sal	10	9.6 ± 1.2	8.7 ± 1.2	5.0 ± 0.6
QNTX-Met	9	7.6 ± 0.9	6.4 ± 0.8	3.8 ± 0.5
QNTX-Sal	10	7.8 ± 0.8	6.4 ± 0.6	4.3 ± 0.5

that in the periphery an endogenous opioid peptide might be responsible for increasing the activity of the preactivated NK cells.

In other studies (data not shown) NTX was given to the poly I:C treated animals before the CM injection of saline was performed, and the data showed no effect of NTX on NK cell activity. Another preliminary data (data not shown) also indicated that CM injection of saline alone had no effect on NK cell activity. Therefore, the basal level of NK cell activity of mice in the NTX-Sal group in Table VI was representative of the NK cell activities of mice in Sal-Sal group.

DISCUSSION

Earlier work demonstrated that a central opioid mediated pathway is involved in regulating the conditioned NK cell activity at the recall step[35]. To further establish the involvement of opioid peptide(s) in this conditioned response, the effect of Met-Enk on NK cell activity following poly I:C pretreatment was examined. In order to determine whether the effect of Met-Enk can mimic the involvement of opioid pathway at the recall stage of conditioning, the experimental design was based on the 3 day paradigm used in conditioning experiments[34]. The results have demonstrated that infusion of a small dose of Met-Enk (1 μg/kg) into the cisterna magna significantly enhanced the activity of suboptimally activated NK cells. In the 3 day schedule mice were injected with 20 μg poly I:C on day 0, followed by Met-Enk on day 2, at a time when the NK cell activity was declining[9]. The immunopotentiating activity of this dose of Met-Enk is in agreement with the immunopotentiating activity observed by Jankovic *et al.*[20].

Opiates and enkephalins possess bidirectional immunomodulatory effects on various immune functions[17,19-21,36,37]. Many factors can affect the ability of Met-Enk to modulate the immune functions. Concentration of Met-Enk is an important variable responsible for its bidirectional immunomodulation[20]. Normally, fmol/ml of β-End and Met-Enk can be found in the human plasma or cerebrospinal fluid, and it is also known that the concentrations of these opioid peptides vary among different species. In rats, for

example, higher levels of these opiates are observed. Foris *et al.*[7], showed that the antibody-dependent cell mediated cytotoxicity (ADCC) of human polymorphonuclear leukocytes was stimulated by lower concentration of Met-Enk and suppressed by the higher concentration of Met-Enk. Both humoral immune response and cell mediated immunity can be modulated by Met-Enk *in vivo*[17,24]. Jankovic *et al.* demonstrated that the route of the injection also plays an important role on the regulatory effect of Met-Enk[19]. In general, higher doses of Met-Enk are required peripherally than centrally to conduct its effect on many immune responses. Raymond *et al.*[31], reported that when the immune response of murine spleen cells was strong (as represented by a strong plaque forming cell response), Met-Enk suppressed the immune response. Conversely, when the plaque forming cell response was suppressed by large concentration of antigen, Met-Enk overcame the suppression. Therefore, level of the immune activity plays another important role in directing the immunoregulatory effect of Met-Enk on the cells. By measuring the NK cell activities of human T cells and large granular lymphocytes, Oleson and Johnson[29] also demonstrated *in vitro* that Met-Enk stimulated the cytolytic capacity of cells with lower NK activity level, but suppressed the cytolytic capacity of cells with higher level of NK activity. It is interesting that although the studies presented in this paper were done in the murine system, the results were similar to the human NK system reported by Olsen and Johnson.

Delivery of Met-Enk directly into the brain via the CM can stimulate the elevation of NK cell activity in mice whose NK cells have been preactivated with poly I:C. Since systemic administration (ip and iv) of Met-Enk, at the same dose, did not stimulate the NK cell activity, the possibility that Met-Enk itself might leak out of the brain to activate NK cells peripherally was not supported. These findings indicated that activation of the opioid mediated pathway in the CNS can activate NK cells in the spleen. Many reports[12,13,16] indicate that Met-Enk and its analogues can modulate the functional activity of the hypothalamic-pituitary-adrenocortical (HPA) axis. Both β-End and Met-Enk have the potential to stimulate NK cell activity *in vitro* [5,26,29] and *in vivo* [6,22]. It is possible that

Met-Enk through its action in the brain signals the HPA axis to release β-End from the pituitary, or Met-Enk from adrenal medullary chromaffin cells[38] and through these peripherally released factors, NK cell activity can be up-regulated.

Methionine-enkephalin has been reported to bind to the μ- and δ-opioid receptors[8]. Naltrexone is a peripheral and central opiate receptor antagonist[11,23]. Quaternary naltrexone (naltrexone methobromide) is a peripheral opiate receptor antagonist due to the fact that the higher polarity will not allow it to readily cross the blood-brain barrier[1-3,27]. Therefore, NTX and QNTX are commonly used in determining sites of action of opiates in the CNS and the periphery. Because the stimulatory effect of Met-Enk was blocked by NTX and QNTX it suggests that both central and peripheral opioid receptors may be involved. The activation of a peripheral opioid pathway by the central administration of Met-Enk further supports the hypothesis that a peripherally released opioid peptide may mediate the enhancement of NK cell activity in the spleen.

The inability of DT-Met-Enk to raise NK cell activity demonstrates that the mechanism is specific and requires opioid receptors and shows that the N-terminal tyrosine residue of Met-Enk is responsible for the opioid receptor binding activity. Without this amino acid, the immunomodulatory capability of Met-Enk was lost. This observation was consistent with the reports of others[8,28]. Gacel *et al.*[8], believed that the tyrosine moiety could be a key topological anchor for the proper orientation of the entire Met-Enk molecule at the receptor. Results of the DT-Met-Enk treatment indicated that an opioid receptor binding event was centrally involved in mediating the effect of Met-Enk. Since all the opioid peptides share the same amino acid sequence with Met-Enk at their N-terminal, it is worth investigating whether other opioid peptides can replace Met-Enk in up-regulating the NK cell activity.

In the 3 day conditioning paradigm the CS, camphor odor, is paired with poly I:C injection (US) on day 0. The CS is then readministered on day 2 to initiate the conditioned response (CR) and NK cell activity is measured on day 3[34]. The aquisition (CS/US

association) is not blocked by NTX but the expression of the CR is blocked by NTX. However, QNTX does not block the expression of CR[35]. This implies that in conditioning the camphor odor activates the conditioned recall of the response through a centrally mediated opioid pathway. Since QNTX is unable to interfere with the recall of the CR, it is possible that in conditioning the NK cell activity in the periphery (spleen) is activated by opiates and non-opiates. Therefore the studies indicate that Met-Enk mediated activation of NK cells is blocked by both NTX and QNTX whereas only NTX blocks conditioned increase of NK cell activity. The studies where opiates are injected into the CNS confirm that an opioid mediated pathway can be triggered to drive the NK cell activity in the spleen. It appears that camphor (CS) in conditioned animals or Met-Enk injected into the CM can signal an opioid pathway in the CNS that can trigger the release of neuroendocrine mediators which can elevate NK cell activity in the spleen.

In many *in vitro* studies[4,5,7,29] the modulatory effect of Met-Enk on NK cell activity was a direct effect. Faith *et al.*[6], demonstrated significant enhancement of NK cell activity in mice with subcutaneous injection of Met-Enk. These observations suggested that an opioid receptor existed peripherally, and by binding at this receptor, Met-Enk exerted its effect on NK cells. However, when mice were injected intraperitoneally and intravenously with Met-Enk (1 μg/kg), no stimulatory effect was seen. Since the concentration of Met-Enk used was much lower than that (3-10 mg/kg) used by Faith et al.[6] dose effect may be the reason for this difference. At higher dose, Met-Enk is able to enhance the NK cell activity of preactivated NK cells peripherally. Detailed studies by Jankovic and his colleagues demonstrated dose- and route-dependent modulation of a number of immune responses and development and protection against the autoimmune disease experimental allergic encephalomyelitis[17,18,24,25].

The significance of this study is to identify a CNS mediated pathway involved in stimulating the NK cell activity. Despite the fact that, in the brain, Met-Enk could be rapidly degraded by multiple enkephalin degrading enzymes[21], the results show that the

NK cell activity can be elevated significantly at a dose of 1 μg/kg. The conditioning model has been used to delineate pathways that allow communication between CNS and the immune system[14,32,33,35]. This study provides direct evidence that the opioid mediated pathway alluded to by the conditioning studies can be triggered by introducing opiates directly into the CNS. It also opens the possibility that direct CNS stimulation with drugs of certain neuronal pathways might be used to heighten resistance to cancer and other diseases, or lower autoimmune responses which are detrimental to the host.

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EFFECT OF DEXAMETHASONE ON CONDITIONED ENHANCEMENT OF NATURAL KILLER CELL ACTIVITY

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Will submit to Brain Behav. Immun.

The involvement of hypothalamic-pituitary-adrenocortical (HPA) axis in conditioned enhancement of natural killer (NK) cell response was examined. The effect of dexamethasone (Dex) on conditioned stimulus/unconditioned stimulus (CS/US) association and on test reexposure to the CS was investigated. Conditioned enhancement of NK cell response was blocked by Dex given at recall but not at the association stage. The results indicate that activation of the HPA activity is required to elicit the conditioned NK cell response at recall but not at the association level. However, the central stimulatory effect of methionine-enkephalin (Met-Enk) on NK cell activity was not blocked by prior injection of Dex. Methionine-enkephalin might act through the HPA axis by overriding the inhibitory effect of Dex on the HPA axis or acts at a site which is beyond the Dex block, in which case the Met-Enk pathway is uninterrupted.

INTRODUCTION

A simple and reliable conditioning model has been developed in this laboratory to study the interaction between central nervous system (CNS) and immune system (IS). There is strong evidence that these two systems can communicate with each other, through the hypothalamus-pituitary-adrenocortical (HPA) axis. A number of peptides that are released from the HPA axis have been demonstrated to posses neuroimmunomodulatory activity[2,7,19]. The activity of the HPA axis is positively modulated by many factors such as corticotropin-releasing factor (CRF), arginine vasopressin, oxytocin, epinephrine, interleukin-1, opiates and negatively by glucocorticoids.

Previous work from this laboratory[12,32] demonstrated that an opioid pathway is involved in regulating the conditioned enhancement of NK cell activity during recall of the conditioned response (CR). This indicates that an endogenous opioid peptide is released during this phase of the response. Since opioid peptides have the abilities to modulate the HPA axis[8,16,22,30,31], it was hypothesized that the HPA axis may play a vital role in mediating the conditioned increase in NK cell activity. In order to demonstrate the

involvement of the HPA axis, the effect of dexamethasone (Dex) on NK cell activity at the CS/US association and the CS recall levels was investigated.

Dexamethasone was chosen because it has been shown to inhibit the HPA axis[5,13,26] and is a potent synthetic glucocorticoid which can suppress plasma corticosteroid level by means of negative feedback inhibition on the HPA axis[18,33]. Many reports have indicated that the release of adrenocorticotropin (ACTH) and β-endorphin (β-End) from the pituitary gland are also inhibited by Dex treatment[3,15,25]. Dexamethasone is believed to bind to type II corticosteroid receptor which is located in many regions of the brain and on the pituitary gland and other peripheral tissues[29]. Several reports have demonstrated that Dex can inhibit both the gene expression and release of CRF by the hypothalamus[4,9,13,17] and it can interfere with the binding of CRF to its target cells in the pituitary[14,28]. Therefore, Dex by suppressing CRF can exert its blocking effect on HPA activity, and reduce the release of ACTH and β-End from the pituitary and corticosteroid from the adrenal gland. A dose of 100 μg/kg, was used in all of the experiments based on the available information that this dose suppressed effectively corticosterone release from the HPA axis[18].

Opioid peptides which modulate the HPA activity[8,16,22,30,31] appear to alter NK cell activity. Earlier results have shown that NK cell activity can be stimulated by Met-Enk when given into the cisterna magna (CM)[12]. It was of interest to see whether the opioid induced activation of NK cell activity could be interrupted in animals pretreated with Dex.

MATERIALS AND METHODS

Animals

BALB/c female mice used in this study were 6 weeks old and were obtained from Charles River Breeding Company (Wilmington, DE). All mice were kept in standard animal facilities, on 12 hr light/dark cycle with *ad libitum* food and water. Mice were separated into groups of either 8 or 9 per cage at least one week before the experiments were started.

Drugs

Dexamethasone phosphate disodium salt (Dex) and methionine-enkephalin (Met-Enk) were purchased from Sigma Chemical Co (St. Louis, MO) and dissolved in sterile saline (0.9% NaCl) prior to delivery. Poly I:C was obtained from Pharmacia, and dissolved in sterile saline at 200 μg/ml and stored at 4 °C.

Conditioning procedure

A three day conditioning paradigm was employed in the present studies. Camphor odor was used as the CS and 20 or 36 μg of poly I:C as the US. The dose of poly I:C used is based on the prior establishment of basal levels of NK cell activity. For exposure to the camphor odor, the mice were placed inside a dark cabinet, a fuming camphor bottle was placed on the cage top, and a second cage was inverted over the cage holding the mice. The animals were left undisturbed for 1 hr. On day 0, mice in conditioned group (CND) were exposed to the camphor odor and then followed immediately by an injection with poly I:C. Mice in the nonconditioned group (NC) received an ip injection of poly I:C only. Both groups were exposed to the odor CS on day 2 for 1 hr and injected with a suboptimal dose of 1 μg poly I:C to slightly elevate NK cell activity from its low background level in order to increase the sensitivity of the CR.

Cisterna magna injection of methionine-enkephalin

A three day protocol was used in these studies in order to relate the information to the conditioning data. Mice were injected ip on day 0 with 36 μg poly I:C to induce an enhanced NK cell response. NK cell assay was performed on day 3. On day 2, poly I:C treated mice were anesthetized with 0.1 ml of ketamine/rompun mixture (K/R, 85 mg/kg and 13 mg/kg, respectively). Five minutes later when the animals were unresponsive to toe pressing, injections were made into the CM with a 250 μl capacity Hamilton syringe using a 27 gauge needle. Methionine-enkephalin was diluted in sterile saline to 4 μg/ml so that a 5 μl injection would contain 0.02 μg of the drug. Control mice were injected with saline at

5 μl/mouse. Following the CM injection, a suboptimal dose of 1 μg poly I:C ip injection was given to all the animals.

Dexamethasone treatment of poly I:C preactivated mice

To determine whether Dex had any direct effect on NK cell activity, mice were treated with Dex according to the 3 day protocol. Two groups of mice received 36 μg poly I:C ip on day 0. On day 2, one group was given Dex, 100 μg/kg, ip and the other group received saline ip injection. Mice in both groups were then injected with 1 μg poly I:C ip. NK cell assay was performed on the next day.

Dexamethasone treatment of conditioned mice

To determine whether Dex can block the conditioned increase in NK cell activity, mice were treated according to the schedules given in Table I or II. Conditioned mice were injected intraperitoneally with sterile saline or Dex (100 μg/kg). Dexamethasone was given immediately before the camphor (CS) exposure on either the association day (day 0) or the recall day (day 2). Nonconditioned groups were also treated with saline or Dex in the same manner as the conditioned animals. On day 3, NK cell assay was performed.

Dexamethasone treatment of Met-Enk injected mice

To determine the effect of Dex on the central stimulatory effect of Met-Enk on NK cell activity, mice were treated with Dex or saline according to the schedule given in Table III. Mice were divided into 4 groups of 8 mice each and were injected ip with 36 μg poly I:C on day 0. On day 2, group 1 mice were injected with Met-Enk CM and served as a positive control, group 2 received an injection of saline CM and served as a negative control group. Both groups were given saline ip before the CM injection. Group 3 received Dex ip followed by an injection of Met-Enk CM. Group 4 received Dex ip and injection of saline CM and served as a Dex control group. Injection of Dex or saline ip was given before the anesthesia and injection of Met-Enk or saline CM was given when mice were anesthetized with K/R. NK cell assay was performed on day 3.

TABLE I

Schedule of treatments to determine the effect of dexamethasone ip injection on conditioned NK cell activity at the recall step.

a. Camphor odor exposure was for 1 hr, in a dark cabinet, followed by poly I:C ip injection, at 20 μg on day 0 and 1 μg on day 2. b. Splenic NK cell activity was assayed in a 4 hr ^{51}Cr release assay. c. Dexamethasone was given, at 2 μg/0.1 ml/mouse (or 100 μg/kg), 15 min before the camphor exposure on day 2. All drugs and saline were administered via ip.

		Treatment days		
Groups	*n*	*0*	*2*	*3*
CND	7	Camphor[a] Poly I:C	Saline Camphor Poly I:C	NK[b]
NC	7	- Poly I:C	Saline Camphor Poly I:C	NK
CND_{dex}	8	Camphor Poly I:C	Dex[c] Camphor Poly I:C	NK
NC_{dex}	8	- Poly I:C	Dex Camphor Poly I:C	NK

TABLE II

Schedule of treatments to determine the effect of dexamethasone ip injection on conditioned NK cell activity at the association step

a. Camphor odor exposure was for 1 hr, in a dark cabinet, followed by poly I:C ip injection, at 36 µg on day 0, or 1 µg on day 2. b. Splenic NK cell activity was assayed in a 4 hr ^{51}Cr release assay against labeled YAC-1 tumor targets. c. Dexamethasone was given, at 2 µg/0.1 ml/mouse (100 µg/kg), 15 min before the camphor exposure on day 0. All drugs and saline were administered ip.

		Treatment days		
Groups	*n*	*0*	*2*	*3*
CND	9	Saline Camphor[a] Poly I:C	Camphor Poly I:C	NK[b]
NC	9	Saline Poly I:C	Camphor Poly I:C	NK
CND_{dex}	8	Dex[c] Camphor Poly I:C	Camphor Poly I:C	NK
NC_{dex}	8	Dex Poly I:C	Camphor Poly I:C	NK

TABLE III

Schedule of treatments to determine whether dexamethasone ip injection can block the enhancing effect of methionine-enkephalin CM injection on NK cell activity

All mice were given poly I:C (36 µg) ip injection on day 0. On day 2, 15 min before ketamine/rompun ip injection animals received either saline, 0.1 ml, or dexamethasone (100 µg/kg) ip injection. Mice were anesthetized with K/R and the CM injections were performed as follows: animals in Met-Enk and Met-Enk$_{dex}$ groups received methionine-enkephalin, 0.02 µg/5 µl/mouse (1 µg/kg); animals in Saline and Saline$_{dex}$ groups received saline, at 5 µl. Immediately after CM injection, animals were given 1 µg poly I:C ip. NK assay was performed on day 3.

			Treatment days	
Groups	*n*	*0*	*2*	*3*
Met-Enk	8	Poly I:C	Saline K/R Met-Enk Poly I:C	NK
Saline	8	Poly I:C	Saline K/R Saline Poly I:C	NK
Met-Enk$_{dex}$	8	Poly I:C	Dex K/R Met-Enk Poly I:C	NK
Saline$_{dex}$	8	Poly I:C	Dex K/R Saline Poly I:C	NK

Preparation of spleen cells

On day 3, mice were killed with CO_2 asphyxiation. Spleens were removed immediately and placed into individual petri-dishes containing sterile saline on ice. The spleen 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 saline and centrifuged at 1800 rpm (566 g) for 5 min at 5°C in a Beckman centrifuge. The supernatant was discarded and the washing was repeated. The pellet was suspended in 1 ml of sterile saline with a sterile Pasteur pipette. 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.

NK cell assay

YAC-1 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 100 U penicillin, 100 µg 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%. YAC-1 cells were labeled with sodium chromate (Amersham, Chicago, IL) at a ratio of 100 µCi/1 x 10^6 cells in a total volume of 0.2 ml at 37°C in CO_2 incubator for 30 min. The cells were washed twice with a large excess of medium and suspended at a final density of 1 x 10^5 cells/ml in RPMI 1640 supplemented with 5% FCS. One-tenth ml of spleen effector cells were mixed with target cells at ratios of 200:1, 100:1, and 50:1 (E:T ratio) in triplicate wells containing 0.1 ml of 1 x 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. One-tenth ml of supernatant from each well was collected after centrifugation of plates at 1400 rpm (345g) for 5 min at 25°C in a Beckman centrifuge. The radioactivity of the samples was counted in a Beckman gamma counter. Maximum ^{51}Cr-released (MR) from the target cells was measured after incubation in the presence of 0.1N HCl and

spontaneous release (SR) in the presence of medium. Percent specific ^{51}Cr-release = [(test release-SR)/(MR-SR)] x 100.

Statistical analysis

Statistical analysis of the percent specific ^{51}Cr-release was calculated by using a repeated measures of Analysis of Variance (ANOVA) test and Duncan's multiple range test with an α value of 0.05.

RESULTS

Dexamethasone has no effect on poly I:C preactivated NK cell activity via iv or ip injection

Using a 3 day protocol, NK cells were preactivated with an ip injection of 36 μg poly I:C. Two days later the effect of Dex on the preactivated NK cell activity was tested. Table IV showed that iv injection of Dex did not have any effect on the NK cell activity when compared to the saline treated control group. Intravenous injection of Dex was performed in ketamine/rompun anesthetized mice to avoid the stress effect caused by iv injection. Statistical analysis showed no significant difference between the Dex and saline-treated mice ($P > 0.9$). The effect of Dex via the ip route has also been tested. Table V showed that Dex had no effect on the NK cell activity by this route also. Statistical analysis, with $P > 0.4$ again showed that the difference between Dex and saline treated groups was not significant.

Although the routes of administration often play an important role in drug treatments, the results from Tables IV and V indicated that neither iv nor ip injection of Dex showed any effect on poly I:C preactivated NK cell response. Since ip injection is a less stressful way to deliver drug, the ip route was selected to administer Dex for further experiments.

Dexamethasone blocked the conditioned enhancement of NK cell activity at the recall step

By following the 3 day conditioning schedule as shown in Table I, Dex was given prior to exposure to the odor (CS) on the recall day. Table VI showed that the NK cell activity was elevated in the CND group when compared to the control NC group.

TABLE IV

Effect of dexamethasone on NK cell activity via intravenous (iv) injection[a]

a. All mice were given poly I:C (36 µg) ip injection on day 0. On day 2, mice were anesthetized with ketamine/rompun mixture injected ip. After the animals were unresponsive to toe pressing, iv injections were performed: animals in Dex group received dexamethasone at 2 µg (or 100 µg/kg), and animals in saline control group received 0.1 ml saline. Immediately after iv injection, all mice were given poly I:C ip injection at 1 µg. NK cell assay was performed on the next day. b. Values were mean for each group ± SEM. Statistical analysis was performed by repeated measures of ANOVA with α value of 0.05, where Dex vs Saline group was not statistically significant ($P > 0.9$).

		% specific ^{51}Cr released at E:T ratios		
Groups	*n*	*200:1*	*100:1*	*50:1*
Dex	8	7.0 ± 0.6[b]	6.0 ± 0.6	4.1 ± 0.3
Saline	8	7.1 ± 0.6	6.3 ± 0.5	3.7 ± 0.4

TABLE V

Effect of dexamethasone on NK cell activity via intraperitoneal (ip) injection[a].

a. All mice were given poly I:C (36 μg) ip injection on day 0. On day 2, animals in Dex group received dexamethasone ip, at 2 μg (or 100 μg/kg), and animals in saline control group received 0.1 ml saline ip injection. Immediately after ip injection, all mice were given poly I:C ip injection at 1 μg. NK cell assay was performed on the next day.
b. Values were mean for each group ± SEM. Statistical analysis was performed by repeated measures of ANOVA with α value of 0.05. Dexamethasone vs Saline group was not statistically significant ($P > 0.4$).

		% specific ^{51}Cr released at E:T ratios		
Groups	*n*	*200:1*	*100:1*	*50:1*
Dex	8	11.8 ± 0.6[b]	10.3 ± 0.6	6.9 ± 0.5
Saline	8	10.7 ± 0.7	9.6 ± 0.8	6.8 ± 0.5

TABLE VI

Dexamethasone blocked the conditioned enhancement of NK cell activity at the recall step.

a. Values were mean for each group ± SEM. Statistical analysis was performed by repeated measures of ANOVA and Duncan's multiple range tests. There is a significant difference between groups overall ($F = 10.78$, $P < 0.001$). From the test significant differences were found between the CND and NC ($P < 0.05$), CND vs CND_{dex}, CND vs NC_{dex}, NC vs CND_{dex} and NC vs NC_{dex} groups (at the $\alpha = 0.05$ significance level). However, there was no significant difference between the CND_{dex} and NC_{dex} groups ($P > 0.3$). *Represents the significant difference from the other 3 groups.

		% specific ^{51}Cr released at E:T ratios		
Groups	*n*	*200:1*	*100:1*	*50:1*
CND	7	14.7 ± 1.0*[a]	15.3 ± 0.8*	10.7 ± 0.7*
NC	7	13.0 ± 0.5	12.3 ± 0.4	8.1 ± 0.5
CND_{dex}	7	10.5 ± 0.7	10.2 ± 0.8	6.7 ± 0.6
NC_{dex}	7	9.0 ± 1.1	8.6 ± 1.1	5.8 ± 0.7

Duncan's multiple range test indicated a significant difference between groups overall ($P < 0.001$). From the test, significant differences were found between the CND vs NC groups ($P < 0.05$), CND vs CND_{dex} groups, and NC vs NC_{dex} groups (at the $\alpha = 0.05$ significant level). However, in the Dex-treated groups, CND_{dex} and NC_{dex}, the conditioned enhancement of NK cell activity was blocked by giving Dex prior to the odor exposure on the test day (CS recall step). The NK cell activity between CND_{dex} and NC_{dex} groups showed no significant difference ($P > 0.3$). Interestingly, the overall NK cell activities in Dex-treated groups were significantly decreased when compared to the saline-treated groups. However, when the experiment was repeated, this suppression was not observed consistently while the blocking effect of Dex still remained.

Dexamethasone did not prevent the conditioned association

The protocol for the conditioned association and treatment with Dex was given in Table II. Duncan's multiple range test indicated a significant difference between groups overall ($P < 0.01$), and the differences were found between the CND vs NC, CND_{dex} vs NC_{dex}, and NC vs CND_{dex} groups (where $\alpha = 0.05$). In contrast to the results in Table VI, Table VII showed that when Dex was injected into mice prior to the CS/US association the conditioned enhancement of NK cell response was not blocked. The Dex-treated conditioned group (CND_{dex}) when compared to Dex-treated nonconditioned group (NC_{dex}) showed a conditioned response ($P < 0.05$). Conditioning was also observed in the CND over the NC group ($P < 0.05$). The overall NK cell activity in Dex-treated groups was higher than that in saline-treated groups (CND_{dex} vs CND and NC_{dex} vs NC), although Duncan's multiple range test indicated that the differences were not statistically significant.

Dexamethasone did not block the enhancement of NK cell response induced by injection of Met-Enk into the CM

To test whether Dex can block the enhancement of NK cell activity induced by Met-Enk, Dex was given prior to Met-Enk or saline CM injection (Table III). The stimulatory

TABLE VII

Dexamethasone did not block the conditioned NK cell activity at the association step

a. Values were mean for each group ± SEM. Statistical analysis was performed by repeated measures of ANOVA and Duncan's multiple range tests. There is a significant difference between groups overall ($F = 4.9$, $P < 0.01$). From the test, significant differences were found between the CND vs NC groups ($P < 0.05$), CND_{dex} vs NC_{dex} groups ($P < 0.05$) and NC vs CND_{dex} (at the $\alpha = 0.05$ significance level). *Represents the significant difference that existed between the experimental and control groups.

		% specific ^{51}Cr released at E:T ratios		
Groups	*n*	*200:1*	*100:1*	*50:1*
CND	9	10.8 ± 1.6*[a]	10.2 ± 1.5*	7.8 ± 0.9*
NC	9	6.7 ± 0.5	6.6 ± 0.3	5.2 ± 0.3
CND_{dex}	8	11.7 ± 1.3*	12.6 ± 1.5*	9.1 ± 0.9*
NC_{dex}	8	8.8 ± 0.6	8.7 ± 0.6	6.4 ± 0.4

effect of Met-Enk on NK cell activity was not altered in the Dex-treated Met-Enk group ($Met\text{-}Enk_{dex}$) when compared to Dex-treated saline control group (Sal_{dex}) (Table VIII). Statistical analysis of the data showed significant difference between the groups overall ($P < 0.001$). The differences were found between the Met-Enk vs saline groups ($P < 0.05$), $Met\text{-}Enk_{dex}$ vs Sal_{dex} groups ($P < 0.01$), Met-Enk vs $Saline_{dex}$ and Saline vs $Met\text{-}Enk_{dex}$ (at the $\alpha = 0.05$ significant level).

DISCUSSION

Earlier work demonstrated that a central opioid mediated pathway was involved in regulating the conditioned increase in NK cell activity at the recall step[32]. Further studies have identified that Met-Enk plays a stimulatory role on NK cell response and could be one of the opioids released in the recall of the conditioned response[12]. These observations suggest the possibility that the neuroendocrine system and in particular the HPA axis, might play an important role in mediating the conditioned recall of the NK cell response. This is based on the evidence that opioid peptides are important modulators of the HPA axis which directs the release of ACTH and β-End from the pituitary. Both ACTH and β-End are capable of stimulating NK cell activity in the spleen. To determine whether the HPA axis is involved, the effect of Dex on the conditioned NK cell response was examined.

Since Dex has been known to block the HPA activity at both central (hypothalamus and other brain regions) and peripheral (pituitary etc.) levels, the direct effect of Dex on NK cells was tested in order to eliminate the possibility that Dex might block the CR by directly acting at the splenic NK cells rather than through the HPA. Other groups[6,10,11,23,24] have indicated that Dex can suppress the NK cell activity *in vitro* and *in vivo*. However, results from the present studies indicated that, at 100 μg/kg, Dex had no suppressive effect on NK cell activity when it was given via ip or iv routes to mice which were pretreated with 36 μg poly I:C two days before the drug treatment. Preliminary data (not shown) also demonstrated that Dex had no direct suppressive effect on splenic NK cell

TABLE VIII

Dexamethasone did not block the enhancement of NK cell activity caused by methionine-enkephalin CM injection

a. Values were mean for each group ± SEM. Statistical analysis was performed by repeated measures of ANOVA and Duncan's multiple range tests. From the test there is a significant difference between the groups overall ($F = 6.74$, $P < 0.001$). Significant differences were also observed between the Met-Enk vs Saline ($P < 0.05$), Met-Enk$_{dex}$ vs Saline$_{dex}$ ($P < 0.01$), Met-Enk vs Saline$_{dex}$ and Saline vs Met-Enk$_{dex}$ groups (at $\alpha = 0.05$).
*Represents the groups that differed significantly from control groups.

		% specific ^{51}Cr released at E:T ratios		
Groups	*n*	*200:1*	*100:1*	*50:1*
Met-Enk	8	10.0 ± 1.0*[a]	7.8 ± 0.6*	5.8 ± 0.3*
Saline	8	7.0 ± 0.5	6.5 ± 0.5	4.8 ± 0.3
Met-Enk$_{dex}$	8	9.1 ± 0.7*	7.6 ± 0.6*	6.2 ± 0.6*
Saline$_{dex}$	8	5.4 ± 0.4	5.2 ± 0.4	4.6 ± 0.3

activity *in vitro* even after 5 hr of coincubation. Holbrook and others[11] clearly demonstrated that *in vitro* treatment of human peripheral blood leukocytes for 18 to 24 hr with physiological concentration of glucocorticoids resulted in a marked decrease in NK cell activity. They further demonstrated that the effect of Dex on NK cell activity was both dose and time dependent. This might explain the discrepancy found in this laboratory and theirs. In the present *in vivo* assay, Dex at 100 µg/kg, is much lower than that used by Parrillo and Fauci[23] in the human system (12 mg), and that used by Hochman and Cudkowicz[10] in mice (125 mg/kg). Although this small amount of Dex did not suppress the NK cell activity *in vitro*, it was sufficient to reduce the plasma corticosteroid level[18] and indicated that the HPA axis was down regulated (or blocked) by this dose. Since Dex, at 100 µg/kg, can block the HPA axis but can not suppress the NK cell activity directly, it is the appropriate dose to detect the involvement of HPA axis in the conditioned NK cell response.

Dexamethasone blocked the conditioned enhancement of NK cell activity at recall stage but it did not block the enhancement when given at the association step. These results indicate that activation of the HPA axis is required in recall of the CR but not during the CS/US association step. The glucocorticoid analogue, Dex, is capable of modulating the CRF-induced ACTH/β-End secretion from the pituitary[1,13,15,26], it also negatively regulates the proopiomelanocortin (POMC) gene expression in the pituitary[20,25]. Whether the mechanism by which Dex exerts its inhibitory activity on conditioned NK cell response during recall is by blocking CRF-induced production of ACTH/β-End from the pituitary or by blocking the expression of POMC remains to be determined. Although Dex is known to bind to multiple sites within the brain and the pituitary[4,17,27,29], it is not known which site(s) is important in mediating the observed blocking of the CR.

Previous findings[32] demonstrated that an opioid pathway is involved only in the recall step and not the association step. Therefore it is highly possible that at the recall step, the odor (CS) signal caused the release of endogenous opioids which in turn act at the HPA

axis and led to the enhancement of NK cell response. Many endogenous opioid peptides could be released centrally and peripherally during the recall of a conditioned response; some of them may be responsible for the activation of HPA activity whereas others may not. Methionine-enkephalin has been reported earlier to have the ability to enhance the NK cell activity when injected directly into the brain (CM)[12]. This response appears to mimick the conditioned recall of the NK cell response.

Although Met-Enk may be one of the factors involved in the regulation of the conditioned NK cell activity[12], its central stimulatory effect was not blocked by Dex. There are two ways that Met-Enk can bypass the blocking effect of Dex on the HPA axis: (a) It is possible that the dose of Met-Enk (1 μg/kg) given into the CM can override the blocking effect of Dex, therefore, Met-Enk can counteract the suppression caused by Dex and reactivate the HPA axis in order to enhance the NK response. (b) The action of Met-Enk could be located at a site which is independent from the Dex block, in which case the Met-Enk pathway is uninterrupted. It is possible that activation of central opioid receptors (μ and δ) by Met-Enk can stimulate the release of hormones from the HPA axis, which in turn can activate the NK cells. However, these actions probably are not mediated through the CRF-dependent pathway as indicated by Nikolarakis *et al.*[21]. Therefore, Dex can not block the Met-Enk effect on NK cell activity.

It has been demonstrated that CS/US association can be made with doses of 20 or 36 μg of poly I:C but not with suboptimal doses of 1, 5, or 10 μg poly I:C. Since basal levels of NK cell activity of different groups of animals vary due to individual differences, 36 μg poly I:C (Table II) served as the US in the conditioning experiment when animals had lower NK levels and 20 μg poly I:C (Table I) when the basal levels were higher. Basal levels of NK cell activity were measured for different batches of the animals before conditioning experiments were started.

The significance of this study is to ascertain whether the HPA axis is involved in regulating the conditioned enhancement of NK cell response. Like naltrexone (an opioid

antagonist), Dex also blocked the CR at the recall but not at the association step, despite the fact that Dex did not block the central stimulatory effect of Met-Enk on NK cells. Since the CS/US association step is where conditioned learning takes place, it is believed that learning of the conditioned NK cell response in BALB/c mice requires no direct involvement of the HPA axis. These results do not eliminate the possibility that endogenous opioid(s) might positively regulate the NK cell activity by activating the HPA axis. Further study of the interrelationships among opioids, the HPA axis, and NK cell activity will help to clarify the molecular basis of this conditioned response and might provide a better way to regulate the immune system via the neuroendocrine system.

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THE EFFERENT SIGNAL(S) THAT ARE RESPONSIBLE FOR THE CONDITIONED AUGMENTATION OF NATURAL KILLER CELL ACTIVITY

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Will submit to J. Immunol.

The possible efferent signals involved in the conditioned increase of natural killer (NK) cell activity were investigated. The signaling molecules that were considered were adrenocorticotropin (ACTH), β-endorphin (β-End) and interferon (IFN). The plasma levels of ACTH and β-End were quantitated by the radioimmunoassay. Changes in IFN message in the spleen cells was determined by the northern hybridization analysis. Results showed that both ACTH level and IFN-α gene expression were higher in the conditioned animals than controls. These studies support the view that ACTH released from the pituitary gland might be involved in the upregulation of IFN-α which in turn stimulates the NK cells in the spleen.

INTRODUCTION

Pavlovian conditioning is a form of "associative learning" in which the temporal pairing of conditioned stimulus (CS) with the unconditioned stimulus (US) results in a behavioral change such that the unconditioned response previously produced only by the US will now be elicited by the CS[1,4,47,55]. Although classical conditioning has been observed for nearly a hundred years, the underlying cellular and molecular mechanisms are still unclear. Many responses including the immune responses have been subjected to conditioning[10,23,38,43,49]. A simple and reproducible conditioning paradigm was used, in which the conditioned enhancement of the natural killer (NK) cell response can be established by one CS/US association and the conditioned response (CR) can be measured within 3-6 days[20,27,61]. With this paradigm the pathways of communication between the immune system and the central nervous system (CNS) have been investigated. It has been demonstrated that interferon-β (IFN-β), an afferent signal generated by poly I:C is the true US which interacts with the CNS and allows the CS/US association to take place[59,60]. It has also been shown that a naltrexone sensitive opioid mediated pathway is involved centrally in recall of the CR[30,62]. Opioid peptides have the abilities to modulate the hypothalamic-pituitary-adrenocortical (HPA) axis[24,36,48,57,58]. This pathway is believed

to be responsible for recall of the enhancement of conditioned NK cell response and can be blocked with dexamethasone (Dex)[31].

A great deal of evidence suggests that the CNS and immune system can communicate via common signals and receptors[7,8,68]. Interferon has been shown to be a common mediator shared by these two systems[6,9]. Besides having anti-viral and anti-tumor activities, IFN has the potential to be a neurotransmitter and/or a hormone to modulate the neuroendocrine systems centrally and peripherally[6,9,54]. Interferon can also stimulate NK cell activity directly in the periphery[16,22,39]. In addition, β-endorphin (β-End) has been shown to stimulate NK cell activity in humans[33,41,42] and mice[37] and can induce production of IFN by NK cells[12,41]. Adrenocorticotropin (ACTH) has also been reported to stimulate NK cell activity *in vivo* [44]. However, in the murine model, opioids (methionine-enkephalin) or ACTH appear not to elevate NK cell activity when these cells are cultured with these mediators[19,21]. These results imply that while the conditioned recall response might depend on release of endorphins and ACTH from the pituitary gland, the products are incapable of directly activating NK cells and consequently must induce other cells to produce IFN to activate NK cells. The present report explores the possible roles of β-End, ACTH, and IFN in eliciting the conditioned NK cell activiy.

MATERIALS AND METHODS

Mice

Six weeks old, female BALB/c mice obtained from Charles River Breeding Company (Wilmington, DE) were used in this study. Mice were kept in standard animal facilities, on 12 hr light/dark cycle with food and water *ad libitum*. At least one week prior to the start of the experiments animals were separated into groups of 8 or 9 per cage. The animal facility at the University of Alabama at Birmingham guarantees strict compliance with regulations established by the ILAC animal welfare act.

Kinetics of poly I:C induced IFN expression

BALB/c mice were injected with 20 μg poly I:C ip and at 2, 4, 6, 8, 10, 12, 24, 48, and 72 hr post injection, two animals were sacrificed at each time point and their spleens were collected, rinsed with sterile phosphate buffered saline (PBS) and stored immediately in liquid nitrogen for later RNA extraction. Mice in the control group received saline ip and their spleens were collected only at 72 hr.

Dose response of poly I:C induced IFN expression

Expression of IFN gene in response to different doses of poly I:C was tested under conditions similar to the 3 day conditioning paradigm. On day 0, all of the mice received 36 μg poly I:C. On day 2, varying doses of poly I:C (1, 5, 10, and 20 μg) or saline were given to different groups of mice (6 mice/group). Two animals from each group were sacrificed at 2, 4, and 6 hr after the second injection and the spleens were collected and frozen in liquid nitrogen until they were used for RNA extraction.

General conditioning procedure

A three day conditioning paradigm was employed for this study. Camphor odor was used as the CS and poly I:C (20 or 36 μg) as the US. For exposure to the camphor odor, mice in their home cages were placed inside a dark cabinet, a fuming camphor bottle was placed on the cage top, and a second cage was inverted over the cage holding the mice. The animals were left undisturbed for 1 hr. On day 0, mice in the conditioned group (CND) and camphor group were exposed to the camphor odor (for 1 hr). Following exposure to camphor the CND but not the camphor group was injected with the US. Mice in the nonconditioned (NC) and US groups received only the US injection ip. On day 2, all the groups except the US group were exposed to the odor CS for 1 hr. Immediately following the CS exposure on day 2, all of the mice were injected with a suboptimal dose of 1 μg poly I:C to slightly activate NK cell activity from its low background level. It has been demonstrated that this suboptimal activation makes the cells more receptive to signals

derived from the CNS[52] and increases the sensitivity of the CR. NK cell assay was performed on day 3.

Spleen collection for RNA extraction

Mice were conditioned according to the protocol described in Table I. On day 2, two animals from each group (CND and NC) were sacrificed at 0, 2, 4, and 6 hr and their spleens were collected, rinsed with sterile PBS, and stored immediately in liquid nitrogen for later RNA extraction. Spleens from two animals were pooled to extract the total RNA. On day 3, eight of the remaining animals from the CND and NC groups were analyzed for NK cell activity.

Preparation of probes

cDNA probes for IFN-α and IFN-β were kindly provided by Dr. Sidney Pestka of Department of Molecular Genetics and Microbiology, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ. A single 2.6 Kb EcoRI-PstI fragment which contains the entire sequence of the murine IFN-α gene was subcloned into the recombinant plasmid pBR322, for amplification in the E. coli culture[50]. Another recombinant plasmid, pMβ-3 which carries cDNA inserts that correspond to murine IFN-β was also amplified in the E. coli culture as described by Higashi *et al.*[26]. After digestion with restriction enzymes EcoRI and PstI for IFN-α cDNA and PstI for IFN-β cDNA, the inserts were isolated by electrophoresis and purified using the geneclean kit (BIO 101). The size of the inserts which contain IFN-α cDNA and IFN-β cDNA are 2.6 Kb and 0.68 Kb, respectively. The purified cDNA inserts were labeled with ^{32}P-dCTP using the oligolabelling kit (Pharmacia).

Isolation and analysis of RNA

Total RNA was isolated from frozen spleens according to the methods of Chomczynski *et al.*[13] and Sambrook *et al.*[56]. Briefly, for the northern blot analysis, total RNA (30 or 40 μg) was denatured by formaldehyde treatment, electrophoresed through 1% agarose gel with 3% formaldehyde, transferred to nylon membranes (Dupont,

TABLE I

Schedule of conditioning to determine its effect on the IFN gene expression and the NK cell response

a. On day 0, BALB/c mice in CND group were exposed to the camphor odor for 1 hr, followed by poly I:C (20 μg) ip injection. The NC mice were given poly I:C only. b. On day 2, immediately following 1 hr camphor exposure and 1 μg poly I:C ip, 2 mice from each group were sacrificed at time 0, 2, 4 and 6 hr; spleens were pooled and extracted for RNA analysis. c. After the camphor and poly I:C treatment on day 2, eight mice from each group were analysed for NK cell activity.

	Days			
Groups	*0* [a]	*1*	*2* [b]	*3* [c]
CND	Camphor Poly I:C		Camphor Poly I:C	NK
NC	Poly I:C		Camphor Poly I:C	NK

Biotechnology systems), and hybridized with ^{32}P-labeled cDNA probes. The labeled fragments were visualized by autoradiography.

Plasma collection for radioimmunoassay

To avoid the stress effect caused by the bleeding procedure, animals were exposed to the CS (odor of camphor) under anesthesia on day 2 (Table II). It has been demonstrated that the NK cell activity can be conditioned under the influence of anesthesia at either the association or the recall stage[32]. Additionally, a delay of one day between the exposure to CS (day -1) and US (day 0) can be used to condition NK cell activity[28]. BALB/c mice in CND and camphor groups were exposed to the camphor odor for 1 hr on day (-1). The next day, mice in all of the groups except the camphor group received 36 μg poly I:C ip. On day 2, all of the mice were anesthetized with ketamine/rompun (K/R) mixture (85 mg/kg K and 13 mg/kg R), followed by Heparin (1000 U/kg) ip. As soon as the mice were unresponsive to toe pressing they were exposed to the camphor odor for 30 min. After camphor exposure, six mice from each group were decapitated and the trunk blood was collected. Blood from two animals was pooled, centrifuged at 1000 g for 10 min, and the plasma was separated and stored at -70° C until used for radioimmunoassay (RIA). For these animals the suboptimal dose of 1 μg poly I:C ip was omitted since detection of ACTH and β-End in the plasma did not require NK cell activation. On day 2, eight of the remaining mice from each group were exposed to camphor for 30 min followed by a suboptimal injection of 1 μg poly I:C and their spleen cells were assayed for NK activity on day 3.

Radioimmunoassay

Plasma concentrations of β-End and ACTH were determined using RIA kits from Peninsula Laboratories, Inc., Belmont, California. The protocol provided by the company was followed for the analysis. Cross-reactivity of β-lipotropin with the antiserum directed against β-End was not determined. Adrenocorticotropin and β-End exhibited 0% and 100% cross-reactivity with the anti-β-End antibody, respectively. Cross-reactivity of

TABLE II

Schedule of conditioning to determine its effect on the NK cell activity and plasma levels of ACTH and β-endorphin

a. BALB/c mice in CND and camphor groups were exposed to the camphor odor for 1 hr on day -1. The next day, mice in all of the groups except the camphor group received 36 μg poly I:C ip. b. On day 2, all of the mice were anesthetized with ketamine/rompun (K/R) mixture (85 mg/kg K and 13 mg/kg R) and received heparin (1000 U/kg) ip. After camphor exposure (30 min) plasma was analyzed by the radioimmunoassay for ACTH and β-End levels. c. Also on day 2, eight mice from each group received1 μg poly I:C ip after the 30 min camphor exposure, and remained undisturbed for NK assay on day 3.

	Days				
Groups	*-1*[a]	*0*	*1*	*2*[b,c]	*3*
CND	Camphor	Poly I:C		K/R Heparin Camphor	NK
NC	-	Poly I:C		K/R Heparin Camphor	NK
Camphor	Camphor	-		K/R Heparin Camphor	NK

α-melanocyte stimulatory hormone and β-End with the antiserum directed against ACTH were 0% and ACTH had a 100% cross-reactivity with this anti-ACTH.

Preparation of spleen cells for NK assay

On day 3, mice were killed with CO_2 asphyxiation. Spleens were removed immediately and placed into individual petri-dishes containing sterile saline on ice. Cell suspension of spleen was made with the help of a forceps and syringe. The spleen 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 saline and centrifuged at 566 g for 5 min at 5°C in a Beckman centrifuge. The supernatant was discarded and the washing was repeated. The pellet was suspended in 1 ml of sterile saline with a sterile Pasteur pipette. 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.

NK cell assay

YAC-1 cells were cultured in RPMI 1640 (Mediatech, Washington, DC) supplemented with 10% fetal calf serum (FCS), 100 U penicillin, 100 μg 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%. YAC-1 cells were labelled with sodium chromate (Amersham, Chicago, IL) at a ratio of 100 μCi/1×10^6 cells in a total volume of 0.2 ml at 37°C in CO_2 incubator for 30 min. The cells were washed twice with a large excess of medium and suspended at a final density of 1×10^5 cells/ml in RPMI 1640 supplemented with 5% FCS. One-tenth ml of spleen effector cells were mixed with target cells at ratios of 200:1, 100:1, and 50:1 (E:T ratio) in triplicate wells containing 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. One-tenth ml of supernatant from each well was collected after centrifugation of plates at 345g for 5 min at 25°C in a

Beckman centrifuge. The radioactivity of the samples were counted in a Beckman gamma counter. Maximum ^{51}Cr-released (MR) from the target cells was measured after incubation in the presence of 0.1N HCl and spontaneous release (SR) in the presence of medium. Percent specific ^{51}Cr-release was calculated as [(test release-SR)/MR-SR] x 100.

Statistical analysis

Statistical analysis of the percent specific ^{51}Cr-release and the levels of plasma ACTH and β-End were calculated by using Analysis of Variance (ANOVA) and Duncan's multiple range comparison tests with an α value of 0.05.

RESULTS

IFN gene expression was stimulated by poly I:C in a time dependent fashion

Type I IFN (α- and β-type) has the potential to stimulate the NK cell activity directly and it can be induced by many factors including poly I:C. Twenty and 36 μg of poly I:C have been used in the conditioning experiments as the US. Initially, the kinetics of IFN mRNA expression were assessed to determine a suitable time for mRNA analysis during the conditioned recall. Results clearly demonstrated that 20 μg poly I:C stimulated the RNA synthesis of IFN in a time dependent manner (Fig. 1). Interferon-α gene expression was first detected at 2 hr after poly I:C injection and was not detectable at 48 hr. The response peaked at 4 hr and started to decline gradually (Fig. 1A). Interferon-β gene expression was also detected at 2 hr after injection with poly I:C and declined progressively. The hybridization band was not present at 48 hr (Fig. 1B).

Detection of IFN gene expression is determined by the dose of the inducer(poly I:C) when tested under conditions similar to the 3 day conditioning paradigm

In animal or cultured cells it has been shown that IFN gene expression can be detected only if it has been induced. The dose of the inducer (such as poly I:C) determines the level of IFN gene expression. Results from Figs. 2 and 3 indicated that under conditions mimicking the time frame of the 3 day paradigm the minimal amount of poly I:C required to induce IFN RNA expression (both α- and β-type) at a detectable level was 20

Figure 1. Analysis of IFN gene expression in poly I:C stimulated spleen cells. Cytoplasmic RNA was isolated from the spleens at various times after the poly I:C (20 μg) stimulation. RNA isolated from the saline group served as the negative control. RNA (30 μg)was electrophoresed through 1% agarose gel with 3% formaldehyde, and analyzed. Nylon membranes were hybridized with either the IFN-α cDNA (A) or IFN-β cDNA (B).

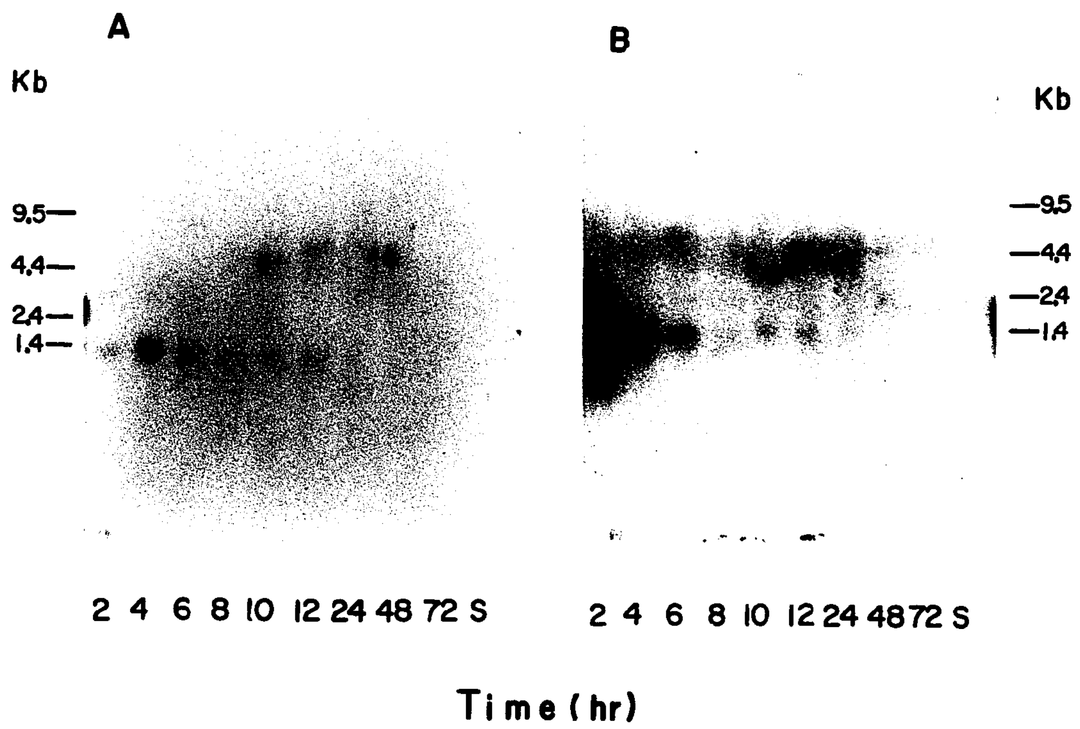

A
B
Kb
9.5
4.4
2.4
1.4
Kb
9.5
4.4
2.4
1.4
2 4 6 8 10 12 24 48 72 S
2 4 6 8 10 12 24 48 72 S
Time (hr)

Figure 2. Effect of poly I:C dose on the IFN-α gene expression. On day 0, mice in all groups received 36 μg poly I:C ip injection. Two days later, different doses of poly I:C (1, 5, 10 and 20 μg) were given to each group. Control group received saline ip at this time. RNA was isolated at various times after the second poly I:C injection or saline injection. RNA (40 μg)was separated through electrophoresis and transferred to the nylon membrane. Nylon filters then were hybridized with IFN-α cDNA probe. Arrow indicates the observed IFN-α transcript (1.2 Kb). P. represents the positive control for IFN-α RNA.

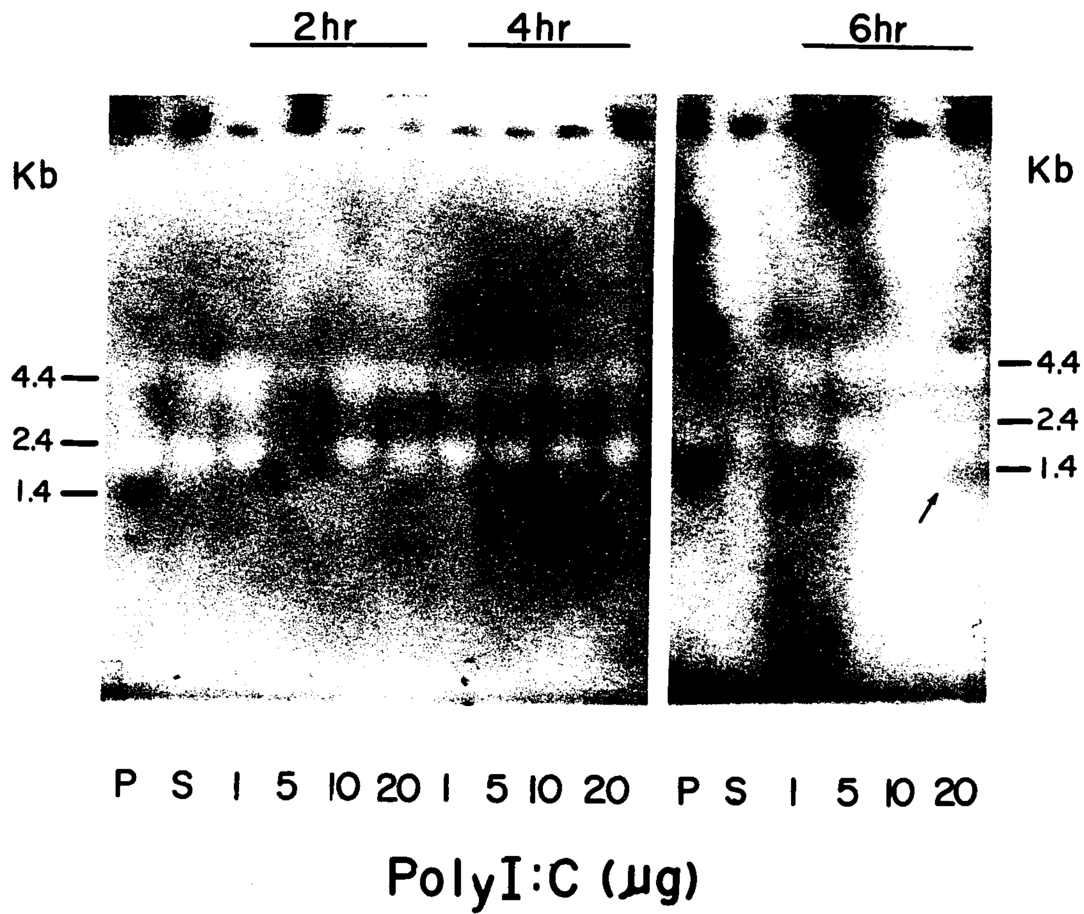
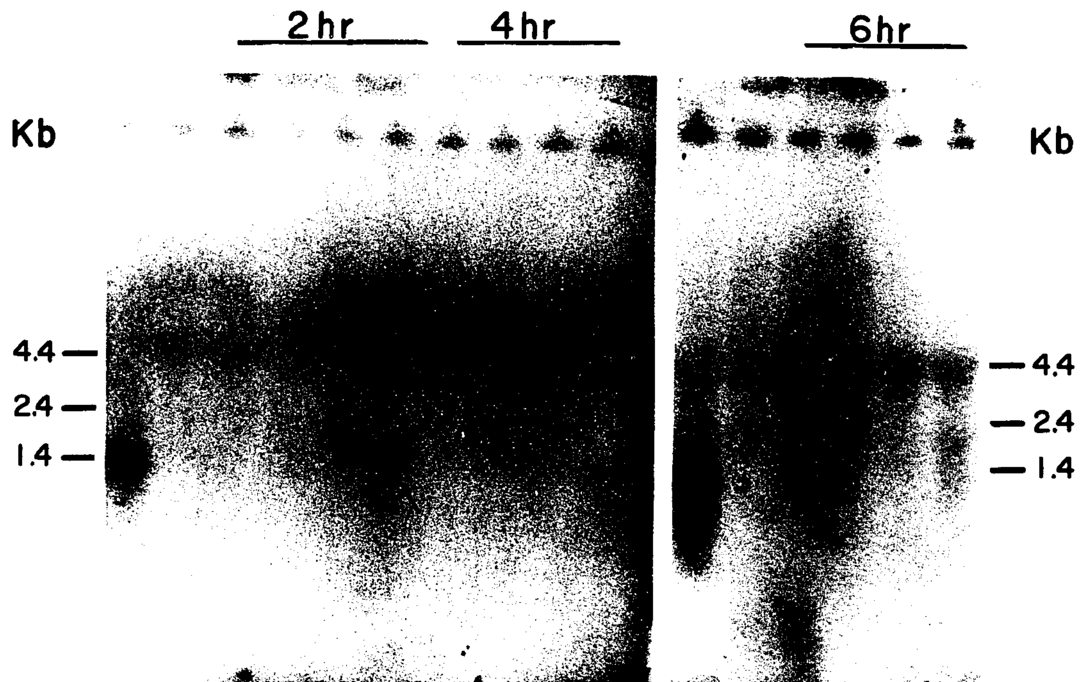

2hr
4hr
6hr
Kb
4.4
2.4
1.4
Kb
4.4
2.4
1.4
P S 1 5 10 20 1 5 10 20 P S 1 5 10 20
PolyI:C (µg)

Figure 3. Effect of poly I:C dose on the IFN-β gene expression. Five groups of BALB/c mice received poly I:C (36 μg) ip injection on day 0. Different doses of poly I:C was then given to each group on day 2, except the control group only received the saline injection. Cytoplasmic RNA was isolated from the spleen cells at various times after the second poly I:C or saline injection. RNA (40 μg)was separated through electrophoresis and then transferred to the nylon filter. IFN-β cDNA was used as the probe to hybridize with the membranes. P. represents the control for IFN-β RNA.



P S 1 5 10 20 1 5 10 20 P S 1 5 10 20

Poly I:C (µg)

μg. Suboptimal doses of 1, 5, or 10 μg poly I:C did not stimulate the IFN gene expression at a measurable level. Under the time course of this paradigm IFN-α was first detected at 6 hr after the 20 μg poly I:C injection (Fig. 2). Interferon-β was detected 2 hr after the injection of poly I:C and was undetectable at 4 and 6 hr (Fig. 3). In control animals which received 36 μg of poly I:C ip on day 0 and saline ip on day 2, no IFN RNA could be detected 48 hr after the first poly I:C injection. Therefore, the IFN gene expression observed on day 2 in the experimental group was induced by the second injection of 20μg poly I:C, but not due to the initial 36 μg poly I:C injection.

Detection of IFN gene expression in the spleens of conditioned and nonconditioned animals

To determine whether IFN gene expression could be detected in the spleens of conditioned or nonconditioned mice, total RNA was extracted from the spleen. The RNA was fractionated by electrophoresis in denaturing agarose gels, transferred to nylon filters and hybridized to either IFN-α or IFN-β cDNA probes. A single distinct band of about 2.8 Kb that hybridized to the IFN-α cDNA was detected in conditioned mice but not in nonconditioned mice (Fig. 4). It was found that IFN-α gene expression can first be detected at 6 hr (Fig. 4) after the CS exposure on day 2. Assessment at longer intervals was not performed. Surprisingly, the expression of IFN-β gene was detected in both CND and NC groups (Fig. 5). Besides IFN-β RNA (about 1.2 Kb), the 28S RNA and the 18S RNA also appeared to be hybridized with the IFN-β cDNA probe nonspecifically. The expression of IFN-β gene was detected at 0, 2, 4 and 6 hr after the CS exposure on day 2 in both groups. Whether IFN gene expression can be detected beyond 6 hr remains to be determined. The NK cell activity was significantly higher in the CND vs NC group ($P < 0.05$) (data not shown).

Adrenocorticotropin (ACTH) but not β-endorphin levels were elevated in the conditioned animals

Animals in the conditioned group had the highest ACTH levels in the plasma when compared to two other control groups (Fig. 6). Statistical analysis using Analysis of

Figure 4. IFN-α mRNA expression in the spleen cells of the conditioned mice. Two groups of BALB/c mice (CND and NC) were treated based on the 3 day paradigm (Table I). On day 2, cytoplasmic RNA was isolated from spleens of CND and NC mice at various times after exposure to camphor and poly I:C boosting. RNA (30 μg)was separated by electrophoresis and transferred to the nylon membrane. Nylon filter was hybridized with IFN-α cDNA probe. P. represents IFN-α RNA induced in the poly I:C treated spleen cells. N. represents the negative control. Arrow indicated the observed large fragment (approximately 2.8 Kb) of the IFN-α transcripts.

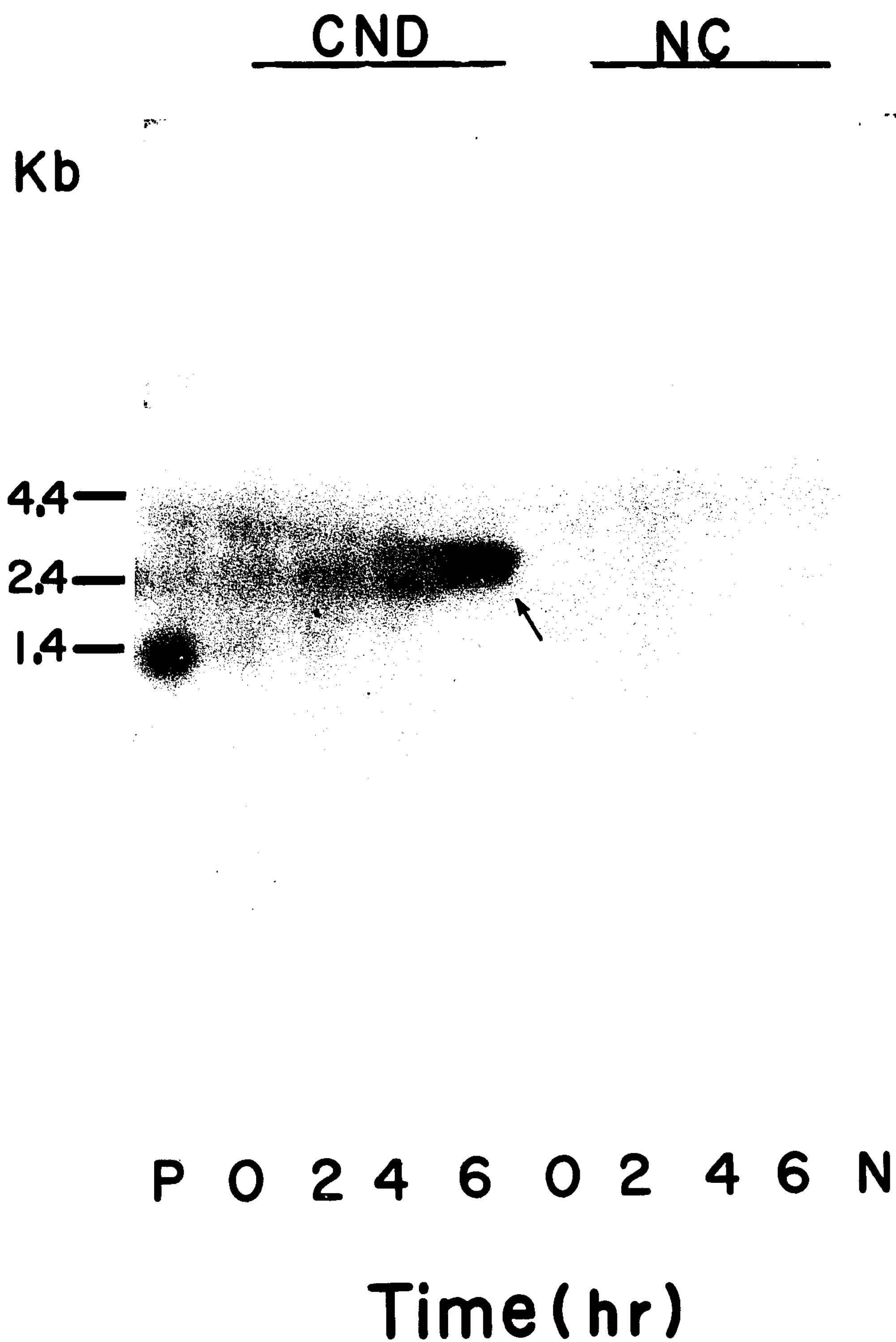
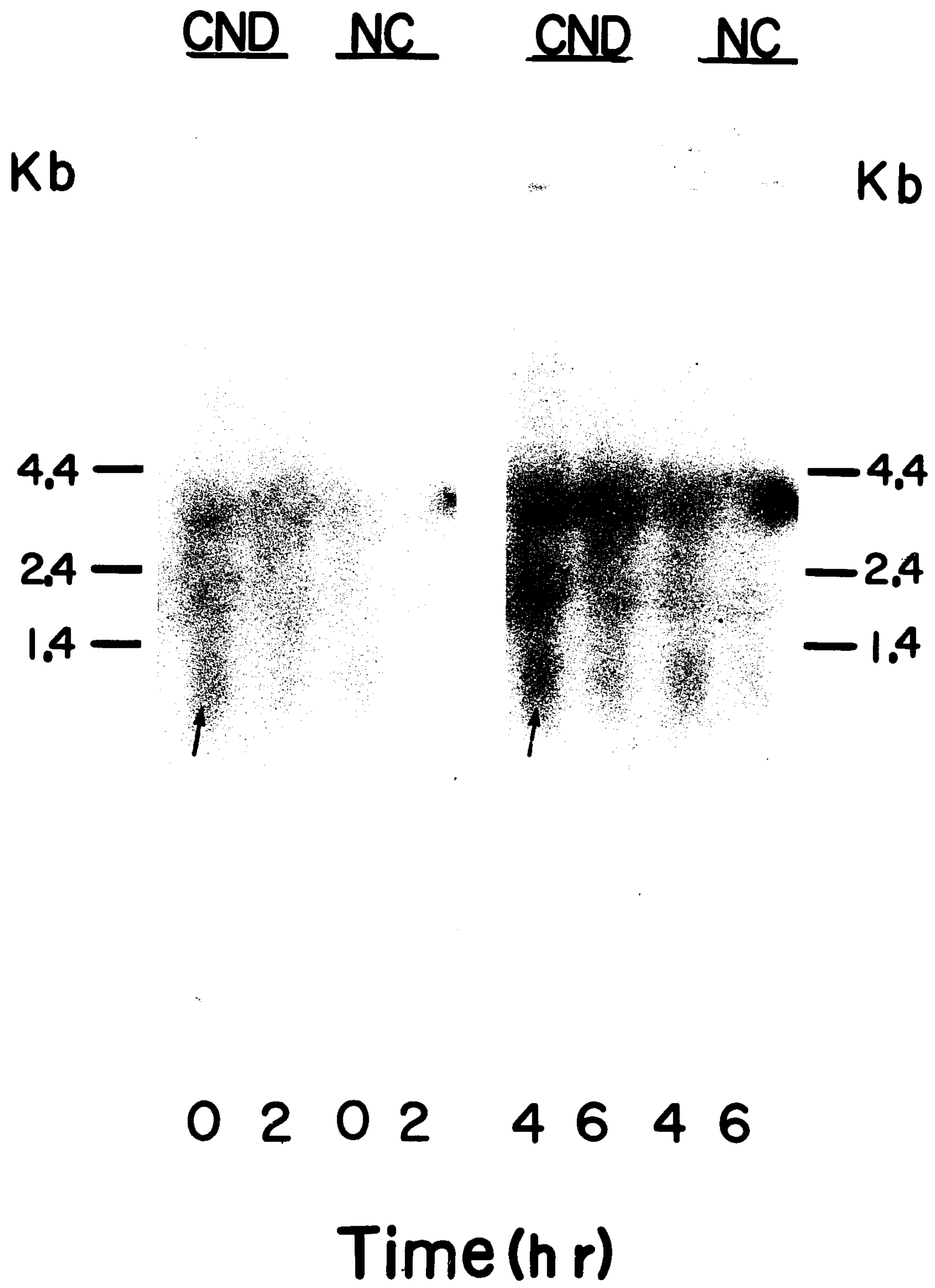
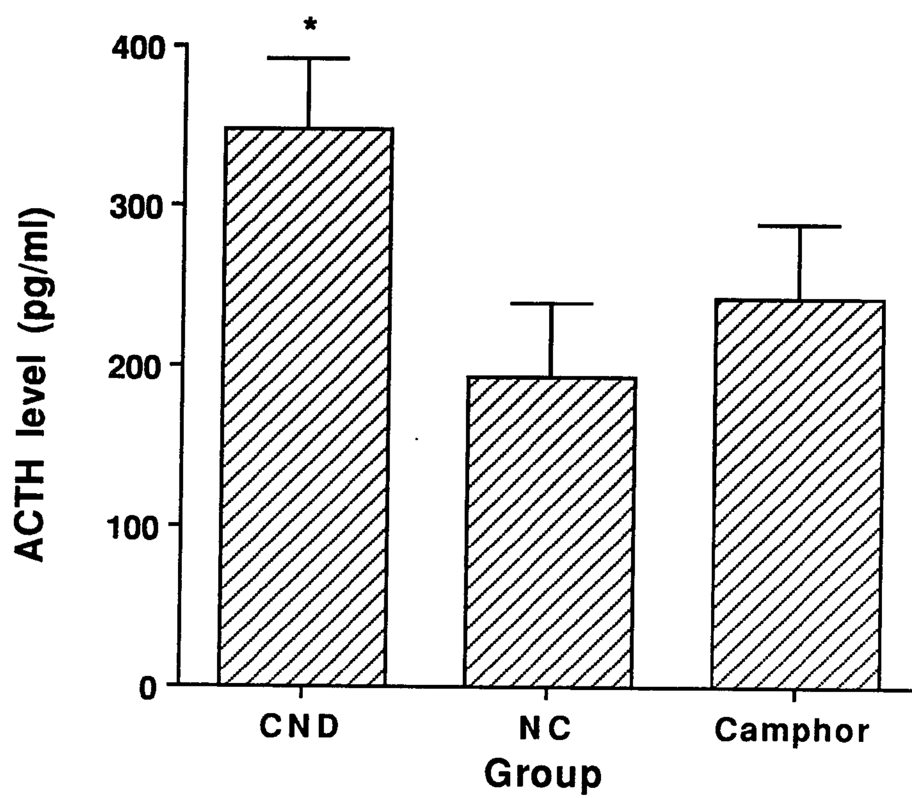

CND
NC
Kb
4.4
2.4
1.4
P 0 2 4 6 0 2 4 6 N
Time (hr)

Figure 5. IFN-β mRNA expression in the conditioned and nonconditioned mice. BALB/c mice were conditioned based on the 3 day paradigm described (Table I). On day 2, cytoplasmic RNA from spleens of CND and NC mice was isolated at various times after the camphor exposure and suboptimal injection of 1 μg poly I:C ip. RNA (30 μg) was separated by electrophoresis and transferred to nylon membrane, which was hybridized with the IFN-β cDNA probe. Arrows represent the IFN-β transcripts (approximately 1.1 Kb)



CND
NC
CND
NC
Kb
Kb
4.4
2.4
1.4
4.4
2.4
1.4
0 2 0 2 4 6 4 6
Time (hr)

Figure 6. Effect of conditioning on the levels of plasma ACTH. Plasma ACTH levels were significantly elevated in the conditioned mice. BALB/c mice were conditioned based on the protocol (Table II). Mice from each group were decapitated on day 2 following exposure to camphor and trunk blood was collected. Plasma was separated from the cells of trunk blood and the amount of ACTH was quantified by RIA. Data represent mean ± SEM of each group (n=6) animals. Significant difference between the CND group and other control groups ($P < 0.05$) was determined by ANOVA and Duncan's multiple range test. There were no differences among the control groups (NC and camphor groups). *Represents the statistical difference between the experimental (CND) and control groups.



*
400
300
200
100
0
ACTH level (pg/ml)
CND
NC
Camphor
Group

Variance procedure followed by Duncan's multiple range test showed that the ACTH levels in conditioned mice were significantly different from that of other control groups ($P < 0.05$). No differences were observed among the two control groups. Although β-End has been reported to be released concomitantly from the pituitary with ACTH, the levels of β-End detected from the same plasma samples were ten times lower than that of ACTH. The β-End levels were found to be distributed randomly among the CND and the two control groups (data not shown). There were no correlations of the β-End level with the ACTH level or NK cell activity. However, plasma ACTH level correlates significantly with NK cell activity ($R = 0.99$; data not shown). Results from the NK assay indicated that the activity of the CND group was significantly higher than that of control groups ($P < 0.05$, Table III).

DISCUSSION

The purpose of the present study was to identify pathways of communication between the CNS and NK cell system and the peripheral signals that are involved in triggering the conditioned enhancement of the NK cell response. The events following the CS recall of the conditioned response were particularly focused on. Our data suggested that IFN-α and ACTH were two of the factors responsible for eliciting the conditioned NK response in the periphery.

Previous results showed that the enhancement of NK cell activity was elicited only in the CND group[28]. The level of NK cell activity showed no significant difference among the control groups (NC, US and Camphor) carried out in parallel. Therefore, either NC or US is used as control for comparison in most of the conditioning studies. The strength of the US and CS plays an important role in determining whether a CS/US learning can or cannot be made. We have shown that CS/US association can be made with doses of 20 or 36 μg of poly I:C but not with suboptimal doses of 1 or 5 μg poly I:C. Since IFN interacts with the CNS during the CS/US association sufficient amounts of this mediator must be produced by the poly I:C stimulation for conditioning to be successful.

TABLE III

The enhancement of NK cell activity was conditioned by the one trial association of odor CS and poly I:C US

a. Values were mean for each group ± SEM. Statistical analysis was performed by repeated measures ANOVA followed by Duncan's multiple range tests with $\alpha = 0.05$. The NK cell activity of CND mice was significantly different from that of control groups ($P < 0.05$). *Represents the significant difference between the experimental (CND) and control groups (NC and Camphor).

		% specific ^{51}Cr released at E:T ratios		
Groups	*n*	*200:1*	*100:1*	*50:1*
CND	8	12.5 ± 0.9[a]*	10.8 ± 0.8*	8.6 ± 0.7*
NC	8	8.7 ± 0.5	7.0 ± 0.5	6.0 ± 0.8
Camphor	8	9.7 ± 1.8	6.4 ± 1.1	4.1 ± 0.8

With regard to the recall of the CR, previous results were unable to show elevation of IFN levels in the sera of conditioned mice[20]. This did not eliminate the possibility that IFN could be produced locally in the vicinity of NK cells by fibroblasts, macrophages or other IFN producing cells. This could effectively raise NK cell activity without ever reaching the circulation in measurable amounts in conditioned mice. Therefore, in the present study, the gene expression of IFN at the transcriptional level was determined.

Poly I:C, a double-stranded synthetic RNA, is a strong IFN-inducer of IFN-α and IFN-β in both humans and mice. The *in vivo* kinetics and dosage effects of poly I:C on the IFN gene expression have not been reported. The results (Figs. 1, 2 and 3) clearly demonstrated that 48 hr after either 20 or 36 μg of poly I:C injection, IFN RNA expression was no longer detectable. Therefore, the IFN RNA levels detected on day 2 at 6 hr after CS exposure in the conditioned animals (Fig. 4) must be induced by signal(s) elicited from the CNS. A suboptimal dose of 1 μg poly I:C was used on the day of recall to activate the NK cell activity to increase their sensitivity to signals from the CNS. The observations (Figs. 2 and 3) showed that 1, 5, and 10 μg of poly I:C did not raise sufficient IFN mRNA to be detectable and 20 μg was the minimal dose of poly I:C that can stimulate the IFN RNA expression to a detectable level.

Figure 1 showed that *in vivo*, 20 μg poly I:C alone can stimulate the IFN gene expression as early as 2 hr post injection. Poly I:C simultaneously stimulated both IFN-α and IFN-β in the spleen. Although the RNA expression of IFN-α and IFN-β peaked 2 hr apart (IFN-α at 4 hr and IFN-β at 2hr) the levels of expression for both remained detectable for 24 hr. Other groups[17,34,51,64] have reported that poly I:C alone does not induce interferon synthesis in murine cells both *in vivo* and *in vitro*. However, Machida *et al.*[40], reported that in mouse L cells poly I:C with DEAE-dextran induced IFN-β and IFN-α at a ratio of about 80:20. IFN from spleen cells also consisted of both species, but IFN-α was reported to be the major component. In these studies, IFN was measured at the protein level with anti-IFN antibodies. No post-transcriptional regulation of murine IFN

synthesis has been reported yet. The data presented in this paper with that of Machida *et al.* suggest post-transcriptional regulation of IFN-β synthesis in spleens of poly I:C treated mice. Further investigation to correlate poly I:C induced IFN protein to its RNA level in the spleen is required to prove this hypothesis.

Synthesis of IFN is usually not detectable in normally growing tissue cultures or in animals, but reach high levels after exposure of cells to inducers. The IFN-α and IFN-β families are nonspliced genes that are clustered on chromosome 9 in human and 4 in mouse[65,66]. Their expressions were originally associated exclusively with viral infection, however, they also can be induced by non-viral stimuli and a complex interplay has been shown to exist among interferons, lymphokines, cytokines and growth-regulatory factors[3,34,35,46]. In the case of IFN-α and IFN-β, induction by virus or poly I:C lead to an accumulation of IFN mRNA due to transient stimulation of transcription[5,34,52]. This transcription is mediated by the 5' end flanking region of the IFN gene. The promoter region contains "inducible elements" which is responsible for the induction of IFN transcription and IFN regulatory factors (IRF-1 and IRF-2), have been reported to play important roles in this region[18,45,67].

Although the mechanism of IFN induction is still unclear, it has been shown that the major type of IFN being produced depends on the type of stimuli and the type of producer cells. In the murine system, induction of type I IFN (-α and -β) seems to be much less cell specific and more inducer specific. For example, in fibroblast cells and spleen cells, IFN-α and IFN-β were induced simultaneously by virus treatment[11], however when poly I:C was the inducer, IFN-α became the major component in spleen cells and IFN-β was the predominant product in the fibroblast cells[40]. Results from this study indicate that signal(s) released during the conditioned recall are more specific to the induction of IFN-α gene in the spleen.

Figure 4 showed that signals produced during the conditioned recall specifically stimulated the IFN-α gene expression in the spleen cells of the conditioned mice but not in

the nonconditioned mice. However, the fragment that hybridized with the IFN-α cDNA probe in the conditioned animal, was a longer transcript than that detected in the poly I:C treated spleen cells (approximate size is 1.2 Kb). This size difference indicates that the signal(s) generated during the CS recall in the conditioned animals is different from poly I:C. Therefore, it regulates the transcription of IFN-α gene differently. Although this transcript has not yet been characterized, it probably represents the read-through transcription of the IFN-α gene into the 3' end flanking region. This assumption was based on the similar results reported for IFN-β and IFN-α genes by Content *et al.*[15] and Raj and Pitha[52].

On the other hand, IFN-β RNA was detected in both the CND and NC groups, and the kinetics of the expression remained the same in both groups (Fig. 5), indicating that IFN-β expression was not specifically induced by the recall signals in conditioned animals. Since camphor alone did not stimulate IFN gene expression the possibility exists that IFN-β gene expression was induced by the influence of handling stress.

Conditioning of the NK cell activity requires communication between the brain (CNS) and the NK cell system. Earlier studies have shown that during the CS/US association, IFN-β but not IFN-α was the afferent signal which linked the US with the CS. Therefore, the release of IFN-β was expected to be conditioned. Surprisingly, IFN-α instead of IFN-β correlated specifically with the conditioned recall of the NK cell activity. Since mouse IFN-α and IFN-β have been shown to share a common receptor[2], it is possible that, during conditioning, that the bidirectional communication between CNS and the immune system might be mediated through such common receptors for IFNs. The effect of blocking the receptors on disruption of the CR remains to be tested to support this speculation.

Previous studies have shown that the HPA axis is involved in the conditioned recall but not in the CS/US association[31]. In light of this observation factor(s) released from the HPA axis is potentially involved in stimulating the NK cell activity and responsible for

recall of the CR. Plasma levels of ACTH instead of β-End were significantly elevated in the CND mice when compared to other control groups (NC and Camphor groups; Fig. 6). The ACTH level correlates significantly with the NK cell activity. The possibility that β-End might be still involved in inducing IFN synthesis has not been completely eliminated. It may be that the kinetics of decay of β-End differs from ACTH. Alternatively, the level of β-End might be low due to possible cross-reaction with β-lipotropin which makes measurements insufficiently definitive. Adrenocorticotropin has the ability to stimulate the NK cell activity *in vivo* [44], but the direct stimulatory effect of ACTH on IFN-α gene expression has not been demonstrated. Gatti *et al.*[19] indicated that ACTH alone cannot stimulate NK cell activity *in vitro*, however, with β-End and other stimulatory factors (IFN-γ, IL-2), ACTH can act synergistically on NK cell activity. It is possible that ACTH affects NK cell activity by interacting with other molecule (such as β-End) and in turn triggers the conditioned enhancement of NK cell response.

Rees *et al.*[53] reported that the immunoreactive plasma ACTH concentration in resting male rat was 23 ± 4 pg/ml in the morning and 63 ± 9 pg/ml in the evening. Results in the present paper showed that the ACTH levels ranged from 195.3 ± 34.2 to 347.2 ± 38.3 pg/ml. In spite of the high ACTH levels in all groups, the conditioned animals were able to release differentially greater amounts of ACTH in response to the CS signal. The high ACTH levels in the control groups might be due to the consequence of the handling and the short time frame of the 3 day paradigm. The β-End levels measured among all the groups ranged from 19.5 ± 3.6 to 38.4 ± 4.8 pg/ml. They were extremely low when compared to the levels of ACTH in the plasma. The basal level of β-End in the resting rat has been reported to be 400 pg/ml[29]. These results suggested that if β-End was released at equimolar ratio with ACTH, the rate of removal from the circulation may have been high or its release was inhibited. Alternatively, these results suggested that the release of both hormones during conditioning might not be concomitant as reported by others[25,63] as there was no correlation between them. Our data show that the regulation of ACTH and β-End

secretion might be dissociated during conditioning. This dissociated regulation also was observed in rats whose arcuate nucleus was destroyed by treatment with monosodium glutamate[14]. In that system, decreased β-End levels were found in the treated rats.

While no correlation can be found between the CR and β-End, there are two reasons why we cannot eliminate the role of β-End in triggering the CR. β-Endorphin has been reported as a stimulator of NK cells. It might act synergistically with ACTH in the conditioned animals to cause the CR. The low level of plasma β-End might have reduced the possibility to distinguish the difference between the levels detected in conditioned and nonconditioned animals. Further investigation on the role of β-End in the CR will be conducted in the future.

In summary, during the CS recall, ACTH is involved in regulating the conditioned enhancement of NK cell response. The mechanism of its effect remains to be determined. Interferon-α appears to be a factor which is induced in the spleen cells of the conditioned mice and might play a direct role in stimulating the elevation of NK cell activity resulting in the CR. However, the possibility that other factors (such as IL-1, IL-2, TNF, etc) might also be involved in the CR is not excluded.

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SUMMARY AND CONCLUSIONS

There are two mechanisms involved in regulating the CR, one afferent and one efferent. The afferent pathway is used during the conditioned association and the efferent pathway is used during the recall. Characterization of the efferent mechanism(s) was the primary concern in the present dissertation study.

A sequence of events which are involved in recall of the conditioned NK cell response have been described. It has been found that not only the conditioned association but also the conditioned recall can be made under the influence of anesthesia, indicating signals involved in the learning and recall of the learned response can be linked and transduced under the reduced awareness caused by anesthesia. Ketamine which was used as one of the anesthetics in this study is a potent N-methyl-D-aspartate (NMDA) antagonist (Trommer & Pasternak, 1990) and supports the view that NMDA receptors are not required in conditioned learning of the NK cell response. Although NMDA receptors are involved in the long-term potentiation of the mammalian CNS (Collingridge & Bliss, 1987) prolonged stimulation of the NMDA receptors often results in the death of most central neurons (Rothman & Olney, 1987). It may be that in the ketamine/rompun anesthetized animals the prevention of overexcitation of the neurons in the CNS might allow the animals to form a better CR. Conditioning and recalling the response under anesthesia provide greater flexibility to focus on mechanisms behind conditioned learning.

The CM is the largest of the subarachnoid spaces, located between the ventral aspect of the cerebellum and the dorsal surface of the medulla oblongata. This space isfilled with cerebrospinal fluid (CSF) and provides an indirect route for access to the ventricular spaces via diffusion of injected material throughout the CSF. Injection of

Met-Enk directly into the CM of the brain stimulated the NK cell activity. The observation shows that a centrally mediated opioid pathway is involved in regulating the NK cell activity. This stimulation was blocked by opiate antagonists, NTX and QNTX. The inability of Des-Tyr-Met-Enk (or DT-Met-Enk) to raise NK cell activity demonstrated that the Met-Enk mediated stimulatory effect was specific and required opioid receptors and the N-terminal tyrosine residue of Met-Enk was responsible for the opioid receptor binding activity. That Met-Enk can mimic the effect of CS exposure given at the conditioned recall supports the suggestion made by Solvason *et al.* (1989) that during the recall, a centrally located, NTX-sensitive, opioid pathway is required in triggering the conditioned enhancement of NK cell response.

The Met-Enk induced enhancement of NK cell response did not mimic in every detail the effector pathway of the CR. While QNTX (a peripheral opiate antagonist) was able to block the enhancement of NK cell activity induced by Met-Enk injected into the CM, it did not block the CR during the recall with the CS. These results indicate that both opioids and non-opioids are peripherally involved in activating NK cell activity of the conditioned animals.

Although CM injection of Met-Enk can mimic the central effect produced during conditioned recall on NK cell activity, other opioid peptides might also be importantly involved in triggering the CR. Therefore, additional specific tests are needed to delineate the type(s) of endogenous opioid peptides induced during the conditioned recall. Blocking the CR with CM injection of specific anti-opioid antibody might be a potentially useful way to achieve this goal. The origin of the endogenous opioid peptide is another important issue which requires investigation. *In situ* hybridization technique could be applied to localize the specific area where the peptide accumulates or is released.

It has been known that opioids have the abilities to modulate the HPA activity (Smriti *et al.*, 1987; Kostas *et al.*, 1987; Odio & Brodish, 1990). The exact role (stimulatory or inhibitory) of the opiate system on the HPA axis under resting or stress

remains uncertain. In conditioned NK model, the centrally induced opioid pathway plays a stimulatory role in regulating the HPA axis. The recall of the CR was blocked by Dex, which clearly indicates that activation of the HPA axis is required for the conditioned enhancement of NK cell response, at recall. Plasma ACTH levels were significantly elevated in the conditioned but not in the nonconditioned animals which supports the view that the HPA axis was activated during the conditioned recall. To further prove that the HPA activity is stimulated by endogenous opioids, the effect of NTX on the release of ACTH from the pituitary gland should be examined. Since NTX blocks the opioid pathway, the plasma ACTH levels of conditioned animals should remain the same as that of nonconditioned mice whose HPA axis are not activated.

Dexamethasone can suppress plasma levels of corticosteroid, ACTH and β-End by negative feedback inhibition of the HPA axis (Kusnecov *et al.*, 1990; Sapolsky *et al.*, 1990; Britton *et al.*, 1985). Dexamethasone is believed to bind to type II corticosteroid receptors which are located in many regions of the brain, and on the pituitary gland and other peripheral tissues (Sheppard *et al.*, 1990). Therefore, the exact blocking site of Dex in the conditioned mice remains to be determined. In general, the feedback inhibition could start at any of the following areas: hippocampus, amygdala, olfactory bulb, hypothalamus, paraventricular nucleus and anterior pituitary gland (Sapolsky *et al.*, 1990; Calogero *et al.*, 1988; Imaki *et al.*, 1991; Sawchenko, 1987; Mercer *et al.*, 1989). It is believed that DEX blocks the activity of HPA axis by interfering the synthesis or release of the CRF (Imaki *et al.*, 1991; Calogero *et al.*, 1988). The fact that Dex did not interfere with the central stimulatory effect of Met-Enk injected into the CM on the NK cell activity, indicates that Met-Enk probably stimulates the HPA axis in a CRF-independent manner as suggested by Nikolarakis *et al.* (1987).

Plasma levels of ACTH were significantly higher in conditioned animals during the CS recall, which indicates that ACTH might be responsible for triggering the CR. However, β-End levels were low and the values were randomly distributed among the

conditioned and control groups. The results indicate that β-End was not released concomitantly with the ACTH as suggested by others (Guillemin *et al.*, 1977; Rossie *et al.*, 1977) or β-End was cleared at a faster rate from the plasma. This dissociated regulation of ACTH and β-End secretion was also observed by other group (Conte-Devolx *et al.*, 1981) in monosodium glutamate treated rats. The means by which ACTH causes a conditioned enhancement of NK cell activity is unclear at this time. Adrenocorticotropin has not been known as an IFN-inducer and it can stimulate the NK cell activity *in vivo* but not *in vitro* (McGlone *et al.*, 1991; Gatti *et al.*, 1990). Nevertheless, when NK cells were exposed to ACTH with other NK stimulators, such as IFN-γ and IL-2, ACTH significantly enhanced the IFN-γ- or IL-2-effect on NK cell activity *in vitro*. Therefore, ACTH probably exerts its effect on NK cells by interacting with other factors during the CR. While there is no correlation between β-End and NK cell activity, there are two reasons why we cannot eliminate the role of β-End in the CR. β-Endorphin has been reported to be a stimulator of NK cells, and it might act synergistically with ACTH in the conditioned animals to initiate the CR. Further investigation on the role of β-End on the conditioned response will be conducted in the future.

A specific correlation between the conditioned recall response and expression of the IFN-α gene was established. Interestingly, a 2.8 Kb IFN-α RNA transcript was detected only in the conditioned animals 6 hr after reexposure to the CS. This suggests that IFN-α might be the factor which directly activates the splenic NK cells in the conditioned animals. Although the average size of the IFN-α gene is approximately 1.2 Kb, the possibility exists that this long transcript might result from the read through transcription into the 3' flanking region of the IFN-α gene. Content et al. demonstrated in their system that a long transcript of human IFN-β resulted from the 3' end read through transcription of the gene (Content *et al.*, 1983). Similar results have been reported for both humans and mice (Kelley & Pitha, 1985; Content *et al.*, 1983). The fact that IFN-β gene expression was observed in both CND and NC groups suggests that IFN-β is not a specific signal for recall of the CR.

In summary, conditioning of the NK cell activity requires communication between the CNS and the NK cell system. It has been demonstrated that IFN-β, an afferent signal generated by Poly I:C is the true US which interacts with the CNS and allows the CS/US association to take place (Solvason *et al.*, 1988, 1991b). During the conditioned recall, ACTH and IFN-α are stimulated and are responsible for eliciting the conditioned recall response. The induction of both factors requires activation of the central opioid pathway and HPA axis. Adrenocorticotropin and IFN-α are the efferent mediators which are triggered by the CS recall. Both ACTH and IFN-α correlate specifically with conditioned enhancement of the NK cell activity. It is also proposed that, during conditioning, the bidirectional communication between CNS and IS might be mediated through common receptors which are shared by IFN-α and IFN-β (Aguet & Blanchard, 1981).

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