Antibody response can be conditioned using electroacupuncture as conditioned stimulus

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To establish a new model of conditioned enhancement of antibody production, electroacupuncture was served as the conditioned stimulus (CS) and an injection of a protein antigen ovalbumin as the unconditioned stimulus (UCS). After a CS/UCS pairing was made, re-exposure of animals to the CS alone resulted in significant conditioned enhancement of anti-ovalbumin antibody production. Even in deep sleep induced by anesthesia, the animals can associate a single CS with UCS and an antibody response can be elicited upon subsequent re-exposure to CS in the absence of exogenous antigen. No effect of electroacupuncture on anti-ovalbumin antibody production was found. *NeuroReport* 15:1475–1478 © 2004 Lippincott Williams & Wilkins.

Key words: Conditioned antibody production; Electroacupuncture; Ovalbumin; Rats

INTRODUCTION

In 1975, Ader and Cohen reported that the immunosuppressive effects of cyclophosphamide on humoral immune responses in rats could be induced by oral saccharin alone after a suitable period of paired exposure to saccharin plus cyclophosphamide, providing insight into the communication between the brain and immune system [1]. After that, the conditioned immunosuppression paradigm developed by Ader and Cohen has been studied and examined extensively [2,3]. However, there are fewer reports about conditioned antibody enhancement paradigm, although the conditioned antibody enhancement can be a more adequate and appropriate model for studying the neural correlates of conditioned immunity, since this procedure by itself does not produce a non-specific alteration of the immune system, as it is caused by the use of cyclophosphamide used in conditioned immunosuppression. Ader *et al.* [4] have demonstrated conditioned immunoenhancement of antibody production but an unwieldy procedure required multiple conditioning trials. Husband *et al.* [5] showed that after pairing a novel tasting solution of saccharin (the conditioned stimulus, CS) with an injection of an antigen ovalbumin (the unconditioned stimulus, UCS), both anti-ovalbumin antibody and ovalbumin-induced T cell proliferative responses *in vitro* could be elevated by re-exposure to the CS alone. Recently, it was further demonstrated that a single conditioning trail was sufficient to produce a dramatic rise in anti-HEL IgG upon re-exposure to the CS alone [6]. However, in these studies, the conditioned stimuli were gustatory such as chocolate milk, saccharine solution [4,6] or olfactory, such as camphor [7]. For consideration of clinical application in human beings, sweet drinks or camphor odor can easily be encountered in daily activities and thus could not be used as conditioned stimulus according to Pavlovian conditioning principle. Thus a question arises as to whether a peripheral electrical signal, a kind of somatosensory stimulation, could be used as conditioned stimulus to enhance immune response. Therefore, this study attempts to determine whether a peripheral electrical signal, a kind of somatosensory stimulation, could be used as conditioned stimulus to enhance immune response.

MATERIALS AND METHODS

Animals: Male Wistar rats weighing 220–300 g obtained from Peking University were used in all experiments following an acclimation period of 1 week to laboratory conditions. The animals were caged individually and maintained under a 12:12 h light:dark schedule (lights on 08:00 h). Food and water were provided *ad lib*.

Electroacupuncture: The rat was placed in a plastic holder with hind legs protruding. Two stainless steel needles of 0.2 mm diameter were inserted perpendicularly about 5 mm into the antirior tibial muscle. To minimize the experimental error, the traditional Chinese acupoint Zusanli (S36, 5 mm lower and lateral to the anterior tubercle of the tibia) was chosen as the stimulation site. Electrical stimulation pulse with duration of 2 ms and frequency of 2 Hz delivered from an EA machine (WQ-6F, Beijing, China) was applied using two outlets via two needles.

Determination of anti-OVA antibodies: Blood samples (0.5 ml) were collected through an incision in the tail 10, 17, 24 and 31 days after test day. The sample sera were taken and stored at –20°C until determination of anti-OVA antibody by enzyme linked immunosorbent assay described previously [8]. Ninety-six well-microtiter plates were coated with 100 µl 1 mg/ml OVA in phosphate buffer (PBS, pH 7.3) overnight at 4°C and washed with PBS and distilled water. Plates were blocked with 1% bovine serum albumin in PBS for 1 h at 37°C. After washing, 1:250 dilutions of sera in blocking buffer were added to wells (100 µl/well) and incubated for 1 h at 37°C. Peroxidase-conjugated rabbit anti-rat IgG immunoglobulins diluted 1:4000 were added to each well and incubated for 1 h at 37°C. Finally, 100 µl enzyme substrate was added to each of the wells. Eight minutes later, H_2SO_4 (2 N, 50 µl each well) was added to end the reaction. The optical density of each well was read at 405 nm, using a microplate reader (BIO-RAD, USA).

Statistical analysis: Results are presented as mean ± s.e.m. Statistical analysis was carried out by ANOVA with repeated measures. To assess group differences, the LSD *post hoc* test was used where appropriate.

Experiment 1: OVA was used as antigen UCS and EA was CS. Animals, therefore, in this experiment were given EA at the time of antigen administration and, after re-exposure to EA, the potential for conditioning of anti-OVA antibody production was examined.

Conditioning procedure: The rat was gently placed in a plastic holder for 10 min. This procedure was used for all rats during the 5 days prior to the exposure to the CS to ensure a familiar environment that would not be a conditional cue since the rat was accustomed to this kind of gentle restraint. On the conditioning day, 57 rats were randomly distributed into five groups. The CS group was given 15 min electro-stimulation followed by an immediate injection of 80 ng OVA (i.p.; Sigma, USA). To investigate whether the stimulation intensity can affect the conditioned antibody production, the conditioned group was divided into two subgroups: the stimulating intensity of CS1 (n=10) group was 2 V and CS2 (n=10) group was 4 V. These two intensities caused slight muscle contraction but no struggling behaviors observed. Thirty days after conditioning, the test day, all above animals received EA again but no antigen injection. Another non-conditioning group was divided into two subgroups, NCS group and NCS0 group: on the conditioning day, the animals of NCS (n=8) group were stimulated with 2 V for 15 min followed by an antigen injection. Thirty days later, these animals were only restrained in the holder. The animals in NCS0 group (n=10) were merely restrained in holder without electric stimulation for 15 min followed by injection of OVA antigen on the conditioning day and the 2 V current was delivered on the test day for 15 min. Animals in the unconditioned group (UCS, n=9) were just restrained in holder both on the conditioning day and on the test day and the antigen was delivered on the conditioning day after 15 min restraint. The conditioning procedure is outlined in Table 1.

Experiment 2: The results obtained in Experiment 1 demonstrate that a robust conditioned immunoenhancement of anti-OVA antibody production can be obtained by the pairing of EA in either the intensity of 4V or 2V with OVA administration. However, the antibody levels of the NCS and NCS0 groups were also somewhat higher than that of the UCS group although there were no significant differences. It seems that, besides the conditioning effects, the other factors such as EA itself or the holder may also have somewhat effect on antibody production. To confirm the conditioning effect induced by EA as CS, a second experiment was conducted to examine the conditioning of anti-OVA antibody production by re-exposure to EA via pairing a single injection of OVA with the EA stimulation under anesthesia. Since Pavlovian conditioning response can take place unconsciously [9,10] it is hypothesized that conditioned anti-OVA antibody production can be also obtained by pairing of EA with OVA administration in anesthetic animals.

Table 1. Procedure for anti-OVA antibody response conditioning.

Group	Conditioning treatment	Test trail	Sample time (day after test)
CSI	EA (2 V) + OVA	EA (2V)	10, 17, 24, 31
CS2	EA (4 V) + OVA	EA (4V)	10, 17, 24, 31
NCS	EA (2 V) + OVA	–[a]	10, 17, 24, 31
NCS0	OVA	EA (2V)	10, 17, 24, 31
UCS	OVA	–[a]	10, 17, 24, 31

[a]Restrained in the holder only.

Conditioning procedure: Because both 2 V and 4 V EA caused similar conditioned elevation of antibody production in Experiment 1, the 2 V current was used in this experiment.

On the conditioning day, thirty-two male Wistar rats (220–300 g) were assigned to one of five different groups. Thirty minutes before conditioning, all animals except the UCS group were deeply anesthetized with 10% chloral hydrate solution (3 ml/kg, i.p.), and the conditioned animals (CS-ANE group, n=8) received EA for 15 min followed immediately 80 ng OVA, i.p. The unpaired conditioning group (CS-up-ANE, n = 7) received EA and an injection of an equivalent volume of saline. The day following conditioning was a rest day [11]. The second day following conditioning, all animals of paired CS group received non-contingent injections of saline, while the unpaired animals received non-contingent injections of OVA antigen. Thus, both groups of animal had exposure to both EA and OVA, with the only difference between groups being whether the two stimuli (CS and UCS) were paired. Because the animals were anesthetized, the holder was not used on the conditioning day. Animals in the non-conditioning group (NCS-ANE, n = 7) were treated the same as the CS group on the conditioning day but received no EA on the test day. In order to see whether the anesthesia itself could affect the primary antibody production, two groups of unconditioned animals were set up: UCS-ANE (n = 5) and UCS (n = 5). On the conditioning day animals in UCS-ANE were injected OVA under anesthesia and animals in UCS were injected OVA without anesthesia. Prior to the test day, all rats were gently placed in the plastic holder for 10 min/day for 5 days. Thirty days after the conditioning, two conditioning groups of animals (CS-ANE, CS-up-ANE) were restrained in the holder and re-exposed to the EA. The other groups

Table 2. Procedure for the anti-OVA antibody response conditioning under anesthesia.

Group	Conditioning treatment	2 days after Conditioning	Test trail	Sample time (days after test)
CS-ANE	EA + OVA (ANE)[b]	Sal	EA	−1, 5, 10, 17, 24
CS-up-ANE	EA + Sal (ANE)	OVA	EA	−1, 5, 10, 17, 24
NCS-ANE	EA + OVA (ANE)	Sal	−[a]	−1, 5, 10, 17, 24
UCS-ANE	OVA (ANE)	Sal	−[a]	−1, 5, 10, 17, 24
UCS	OVA	Sal	−[a]	−1, 5, 10, 17, 24

[a]Restrained in the holder only.
[b]Anesthetized with 10% chloral hydrate solution (3 ml/kg, i.p. injection).

(NCS-ANE, UCS-ANE and UCS) of animals were merely restrained in the holder for 15 min.

Animals were bled one day before and 5, 10, 17, 24 days after the test day and 0.5 ml blood was collected through a small incision in the tip of the tail using a surgical scalpel. The conditioning procedure is outlined in Table 2. Sample sera were analyzed the same as Experiment 1.

RESULTS

The mean anti-OVA antibody levels of different groups in experiment 1 are presented in Fig. 1. ANOVA with repeated measures showed a significant effect between groups ($F(4,42) = 3.449$, $p < 0.01$), throughout days ($F(3,12) = 5.606$, $p < 0.01$), and a group × day interaction ($F(3,126) = 3.034$, $p < 0.01$). *Post hoc* analysis showed that the CS1 group presented higher anti-OVA IgG antibody production compared with the NCS group ($p < 0.01$ both on day 10 and day 17 after the test day), NCS0 group ($p < 0.01$ on day 10 and $p < 0.05$ on day 17 after the test day) and UCS group ($p < 0.01$ on both day 10 and day 17 after the test day). Despite the difference of stimulation intensity, the antibody level of the CS2 group was also higher than that of the NCS group ($p < 0.05$ on day 10 and $p < 0.01$ on day 17 after the test day), NCS0 group ($p < 0.05$ on day 17 after the test day) and UCS group ($p < 0.01$ on day 10 and day 17 after the test day). There were no significant differences among the NCS group, NCS0 group and UCS group.

In experiment 2, ANOVA with repeated measures showed a significant effect between groups ($F(4,27) = 2.785$, $p < 0.05$), difference between days ($F(4,16) = 5.489$, $p < 0.01$), and a group × day interaction ($F(4,108) = 1.87$, $p < 0.05$). Further *post hoc* analysis shows that anti-OVA antibody levels increased significantly in CS-ANE group when compared with all other groups ($p < 0.01$ on day 10 and 17 after the test day). There were no differences among the CS-up-ANE, NCS, UCS-ANE and UCS groups, Fig. 2.

DISCUSSION

The present experiments demonstrate that a robust conditioned immunoenhancement of the antibody response can be obtained by pairing of EA with an antigen administration in one-trial conditioning paradigm. The results confirm previous findings that a conditioning stimulus can be used to develop conditioned enhancement of antibody production [6,12] and further demonstrate for the first time that EA is an effective CS in eliciting conditioned antibody response.

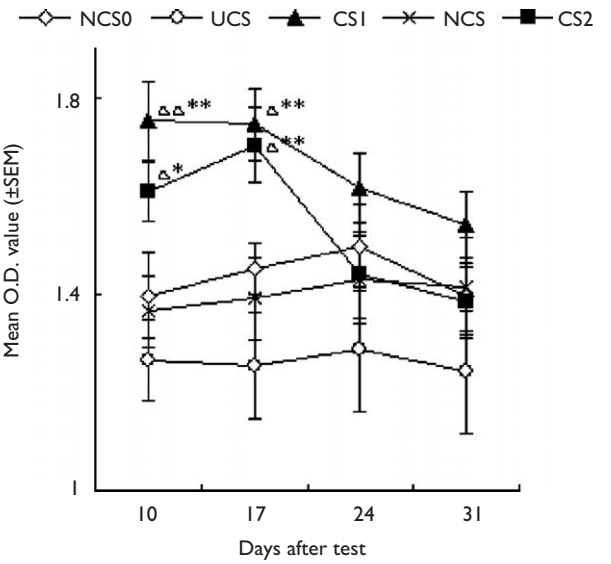

Fig. 1. Mean optic density values (±s.e.m.) of anti-OVA IgG antibody levels of rats from different groups, obtained 10, 17, 24, 31 days after test day, $^{**}p < 0.01$; $\Delta p < 0.05$ (*vs* NCS0 group); $^{\Delta\Delta}p < 0.01$; $^{\Delta}p < 0.05$ (*vs* NCS0 group).

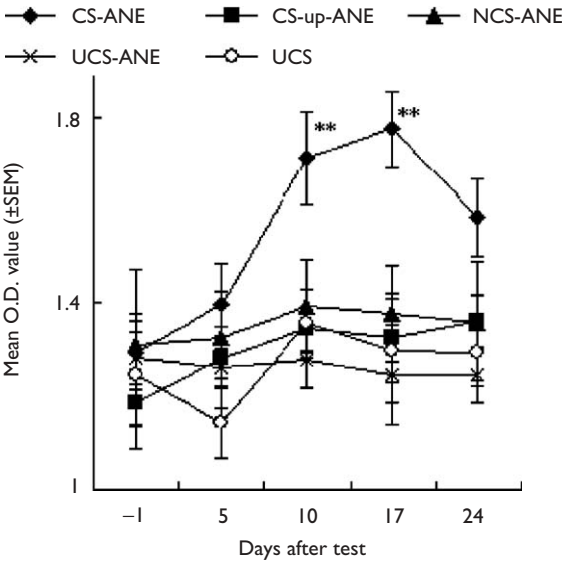

Fig. 2. Mean optic density values (±s.e.m.) of anti-OVA IgG antibody levels of rats from different groups under anesthesia conditioning procedure, obtained 1 day before and 5, 10, 17, 24 days after the test day, $^{**}p < 0.01$ (*vs* CS-up-ANE group).

It was reported that EA at <5 V was ineffective for the elevation of plasma catecholamine levels [13], and that there would be no alteration in catecholamine secretion rate only when the group I and II but not group III and IV afferent fibers were excited [14]. It is, therefore, possible that both 2 V and 4 V EA cause the activation of afferent fibers with group I and II but not group III and IV. This is in agreement with our findings that stimulation at both 2 V and 4 V caused similar conditioned anti-OVA antibody production and no behavioral responses were observed during the EA at either 2 V or 4 V except a slight muscle twitch.

The result that specific immune response can be conditioned using an anesthetic paradigm is in accordance with the studies reported by Hsueh *et al*. [9], in which an anesthetic paradigm was used to recall a conditioned NK cell response.

The significant difference was found between the CS-ANE group and CS-up-ANE group in Experiment 2, suggesting that the contingent manner is essential for the purpose of conditioning. There was no significant difference among the groups of CS-up-ANE, UCS-ANE and UCS, indicating that the EA itself did not influence antibody production. In fact, in the literature, no report has been found that EA could enhance specific antibody productions. Although EA was reported to increase NK cell activity and interleukin-2 (IL-2) production [13,15], but it reduced the production of the antibody against the sheep red blood cell antigen [16] and the anti-collagen antibody level. Our results, together with the others, indicate that the enhanced anti-OVA production in conditioned animals reported in this study is due to the conditioning techniques, not EA itself. In addition, the specificity of anti-OVA antibody production was confirmed in rats exposed to EA but without OVA administration. There was no anti-OVA antibody production seen in such case.

The similar baseline antibody level of the groups of UCS and UCS-ANE in Experiment 2 indicates that anesthetic procedure did not affect the primary antibody production. The slight elevation of antibody production in NCS group of Experiment 1 suggests that there might be an association of UCS with other stimuli, such as restraining holder, during the acquisition. Therefore, when the EA was administered as CS without the plastic holder during conditioning training under anesthesia in Experiment 2, the conditioning effect induced by EA was more evident and no any elevation of antibody production in NCS-ANE group was found.

It is worth noting that in previous studies on conditioned immunomodulation, the most commonly used conditional stimuli are saccharine and camphor. Since the activation model of brain area induced by EA is dissimilar with saccharine or camphor odor [17–20], the conditioning paradigm reported in this study could be used for the mechanism researching and might shed new light on the generalization of conditioned alterations of immunity.

CONCLUSION

We have found that a single trail conditioning, paired a novel conditional stimulus with an antigen, OVA, is capable of eliciting an antibody response. This study is unique and significant in that the EA was served as conditional stimulus. We also show that even in deep sleep induced by anesthesia, animal can process the CS/UCS signal and save the memory about the CS/UCS association.

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