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ANTIGEN CHARACTERISTICS STRONGLY INFLUENCED THE EFFECTS OF STRESS ON THE HUMORAL IMMUNE RESPONSE IN THE RAT

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The objective of the present study was to explore whether the suppressive effect of electric stress (ES) on the immune response in rats was limited to the particular antigen given concomitantly with ES. Therefore, the influence of simultaneous exposure to stress and immunization with an unrelated antigen (keyhole limpet hemocyanin, KLH) on the humoral immune response to bovine serum albumin (BSA) was investigated. Specific anti-KLH antibody levels were also determined in rats exposed to ES and concomitantly immunized with BSA. Five daily sessions of ES or immunization with KLH 2 weeks prior to immunization with BSA did not influence the secondary humoral immune response to BSA, but concomitant exposure to ES and immunization with KLH significantly decreased it. Conversely, the primary humoral immune response to KLH was suppressed by exposure of the animals to ES at the time of immunization with KLH, as well as at the time of the immunization with BSA 2 weeks later. It is suggested that the suppressive effect of ES on the humoral immune response is not specific for a certain antigen. However, the chemical and immunological characteristics of the antigens shaped the profile of stress-induced immune changes with respect to the sensitivity of the primary and secondary immune response and the duration of the effect.

Key words: Electric tail shock stress (ES), bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), humoral immune response, rat

INTRODUCTION

Stress has long been associated with an altered homeostatic state of the organism including behavioral (Faraday, 2002) and endocrine changes (Terrence *et al,* 1997; O'Connor *et al,* 2003). It has been shown that stress also profoundly influences development of an experimentally elicited immune response (Kusnecov and Rabin, 1994; Smith *et al*., 2004), as well as the course of different diseases (Marić *et al*., 1991; Surwit *et al*., 1993). Possible mechanisms involved in the stress-induced immunomodulation comprise specific innervation of the

lymphoid tissue as well as central and peripheral release of different neuroendocrine substances with immunomodulatory properties (Chrousos, 2000).

The effects of stress on the immune response are influenced by numerous variables, such as strain of the animal (Shurin *et al.,* 1995), the type of stressor (Oishi *et al.,* 2003), whether stress exposure is acute or repeated (Silberman *et al.,* 2003) and the temporal proximity of stress exposure and immunization (Fleshner *et al.,* 1992; Zalcman *et al.,* 1988). Interestingly, it was observed that a particular stressor exerted opposite effects on different immune functions in the same animal, e.g. mitogen versus antigen stimulated spleen cell proliferation (Kusnecov and Rabin, 1993), or the innate versus acquired specific humoral immune response (Fleshner *et al.,* 1998). A possible explanation for these discrepancies could be that studies *in vitro*, as well as investigations of a particular immune function, do not necessarily indicate comprehensive immune changes in the organism induced by stress.

The aim of the present study was to investigate the effect of electric stress on the humoral immune response in the rat. The humoral immune response is an integrated physiological process that reflects a well-controlled multistep homeostatic response to antigen challenge and comprises antigen migration to the nearby lymph nodes, processing by the accessory cells, and presentation to T lymphocytes through MHC II surface molecules. Subsequent T and B lymphocyte cooperation leads to the proliferation of B lymphocytes and maturation to plasma cells that secrete antibodies specific for the antigen (Abbas *et al*, 2000.).

We have reported that five daily sessions of electric tail shock stress (ES) delivered at the time of primary immunization with bovine serum albumin (BSA), but not at the time of secondary immunization with the same antigen, suppressed the secondary and tertiary humoral immune responses to BSA in the rat (Stanojević *et al*., 2003). Since long lasting suppression of humoral immunity was observed only if the ES was delivered during immune cell activation and not when memory cells were already formed, it was suggested that early events in the development of the immune response were specially receptive to the inhibitory influence of neuroendocrine mediators released during stress. The question arose whether the suppressive effect of ES was limited to the particular antigen given concomitantly with ES or not. Therefore, we introduced keyhole limpet hemocyanin (KLH) as a second antigen and examined whether simultaneous development of the immune response to KLH applied with or without stress could influence the initial humoral immune response to BSA. Specific anti-KLH antibody levels in rats exposed to ES and immunized with BSA were also determined.

MATERIAL AND METHODS

Animals

Eight to twelve week old male Wistar rats were purchased from the local supplier. The rats were housed in groups of four or five in Plexiglas cages, under 12h:12h light/dark cycles and had free access to a standard diet and tap water.

Upon arrival in our facility, the rats were handled daily for 1 week before the experiment to adapt to the staff. Our Institutional Animal Care and Use Committee approved all experimental procedures involving animals and their care, according to the European Council Directives of 24 November 1986 (86/609/EEC).

Immunization and blood collection

All animals were intraperitoneally (i.p.) immunized with bovine serum albumin (BSA, Sigma, St. Lewis, MO; 1 mg BSA in 0.5 ml saline) on day 0 and day 43. Keyhole limpet hemocyanin (KLH, Pierce, Rockford, Il) was solubilized in phosphate buffered saline (PBS, pH 7.4) overnight at 4 °C, and the animals were immunized i.p. with 0.2 mg KLH in 0.5 ml PBS on day –14. and day 57. Blood was collected by cardiac puncture under light aether anesthesia and the serum removed and stored at –20 °C until antibody levels were determined.

Determination of specific antibodies

Serum levels of anti-BSA IgG antibodies were determined by enzyme-linked immunosorbent assay (ELISA), as previously described in detail (Stanojević *et al.,* 2003). Briefly, the microtiter plates (Corning, NY) were coated overnight at 4 °C with BSA (50 μg/ml in 0.05 M carbonate buffer, pH 9.6) and washed with PBS. Serum samples diluted 1:100 in PBS/ 0.05% Tween 20 were tested in triplicate. Sera (1:1000) pooled from 10 rats subcutaneously immunized with 0.5 mg of BSA in 0.1 ml of complete Freund's adjuvant (6 mg/ml *Mycobacterium bovis*) served as a positive control. Background control wells were filled with PBS/0.05% Tween 20. Plates were incubated for 1h at room temperature, washed with PBS/Tween, and incubated with peroxidase-conjugated goat anti-rat IgG (Sigma, MO; 1:5000) for 1h at room temperature. After washing, the plates were incubated in the dark with chromogen ortho-phenylene-diamine (OPD, Sigma, MO) for 40 min and the reaction was stopped by 2 M H_2SO_4. Optical densities (OD) were determined on a Multiscan reader (Titertek, Flow Lab.) at 492 nm. The results were expressed as OD (mean ± SE). Both within and between assay variability was determined from the OD values for the positive control serum and, if this exceeded 10% between plates/assays, values were corrected according to the mean positive control values.

Serum levels of anti-KLH IgG antibodies were determined by ELISA in a similar manner as anti-BSA antibodies, but with several modifications. The antigen for ELISA was prepared by solubilizing 20 mg of KLH in 2 ml of PBS at 4 °C overnight, centrifuged for 10 min at 14000 rpm and filtered through 0.45 μm apertures. The concentration of KLH in the filtrate was spectrophotometrically determined at 280 nm, based on the KLH extinction coefficient and adjusted to 10 μg/ml in PBS. The microtiter plates were coated with 10 μg/ml of KLH, and blocked with 1% gelatine in PBS/0.05 % Tween 20 for 1 h at 37 °C. Sera samples diluted 1:400 (day 46) or 1:3000 (days 60, 65 and 70) in 0.5% gelatin/PBS/0.05% Tween 20 were tested in triplicate. Sera (1:6400) pooled from four rats i.p. immunized twice within 7 days with 500 μg KLH in 0.5 ml PBS served as a positive

control. Background control wells were filled with 0.5% of gelatine/PBS/0.05% Tween 20.

Preliminary ELISA performed for sera of rats immunized only with BSA or KLH confirmed that there was no cross-reactivity between BSA and KLH.

Stressing Procedure

Electric shock stress (ES) was delivered in a sound-proof room to which the animals were moved prior to each stress session. During the shock sessions, the rats were kept in rectangular Plexiglas boxes (18 x 9.5 x 20 cm) with a semicircular aperture on the lower part of the rear wall that allowed attachment of the electrodes to the rat's tail and prevented movements of the rear paws. Electric shocks were delivered through two silver electrodes separated by 3-4 cm affixed to the tail with electrode paste and adhesive tape. During the experiment, 4 or 5 rats were simultaneously stressed with shocks of the same intensity (55 V, 1 mA). A shock session consisted of eighty 5-s unsignaled shocks (7 electric impulses of 0.3 s duration and 0.6 s pauses) with an average intershock interval of 60 s (range 5-120 s), so the total duration was 80-90 min. After each ES session, the animals were returned to their colony and left undisturbed until the next session. ES sessions were delivered at different times during daylight, in order to avoid anticipatory hormonal and behavioral changes (Ottenweller *et al*., 1989).

Rats of the intact control (IC) group were left in their home cages in the colony. During the stress sessions of the corresponding group, IC animals had no access to food and water to minimize interference of food and water deprivation with the effects of stress in the ES group.

Experimental Design

The experimental design is given in Table 1. In Experiment 1, the influence of electric stress and/or immunization with KLH fourteen days prior to immunization with BSA on the secondary anti-BSA antibody titer was investigated. All animals were immunized with BSA on days 0 and 43. From day -14, rats were either subjected to five daily sessions of ES and treated with PBS (ES-PBS group) or subjected to ES and immunized with KLH (ES-KLH group) or immunized with KLH (IC-KLH) or just treated with PBS (IC-PBS). All rats were bled on Days 46, 51 and 56 for the determination of the specific secondary anti-BSA antibodies.

Experiment 2 was designed to evaluate the influence of electric stress during or 14 days after primary immunization with KLH on the primary and secondary anti-KLH antibody titers. All animals were immunized with BSA on days 0 and 43, and with KLH on days -14 and 57. Rats were subjected to five daily sessions of ES starting on day -14 (ES (-14) group), on day 0 (ES (0) group), or left undisturbed in the colony during immunizations (IC group). All rats were bled on day 46 and on days 60, 65 and 70 for determination of the specific primary and secondary anti-KLH antibodies, respectively.

Table 1. Experimental design

Experiment 1	Days			
Group	-14-10	0	43	46, 51, 56
ES-PBS	ES-PBS*	BSA	BSA	bc
IC-PBS	IC-PBS	BSA	BSA	bc
IC-KLH	IC-KLH	BSA	BSA	bc
ES-KLH	ES-KLH*	BSA	BSA	bc

Experiment 2	Days					
Group	-14-10	0-4	43	46	57	60, 65, 70
ES (-14)	ES-KLH*	IC-BSA	BSA	bc	KLH	bc
IC	IC-KLH	IC-BSA	BSA	bc	KLH	bc
ES (0)	IC-KLH	ES-BSA*	BSA	bc	KLH	bc

IC = intact control
PBS = treatment with 0.5 ml of PBS
KLH = immunization with 0.2mg/0.5 ml KLH
BSA = immunization with 1mg/0.5 ml BSA
* I.p. injections were given after the first session of stress
ES = electric stress (80, 5-s unsignaled shocks, average intershock interval 60 s, total duration 80-90 minute)
bc = blood collection

Statistical Analysis

All biometrical calculations (mean, SE) were performed using a statistical package (STAT View II). Data were analyzed by one-way ANOVA followed by Fischer's protected least-significant difference test for comparisons between independent groups, or by Student's unpaired t-test. Differences were considered significant if $p<0.05$.

RESULTS AND DISCUSSION

In Experiment 1, electric stress applied for five consecutive days from 14 days prior to primary immunization with BSA did not influence the level of anti-BSA antibodies on days 46, 51 and 56, when compared to intact rats treated with PBS on day -14 (Fig. 1A). These results showed that resting immune cells were not receptive to the immunosuppressive influence of ES, confirming the previous concept about the necessity of concomitant immune cell activation for the stress-induced immune changes to occur (Fleshner *et al*., 1998). Additionally, simultaneous development of the immune response induced by immunization with immunologically distinct KLH 14 days prior to primary immunization with BSA did not *per se* affect the formation of specific anti-BSA antibodies (Fig. 2B), which was in agreement with the lack of cross reactivity between BSA and KLH (data not shown). Keyhole limpet hemocyanin is a purified protein derived from the marine

mollusc Megathura crenulata (Dixon *et al*., 1966) and possesses high immunogenic potency probably due to the existence of different cross-reacting epitopes (Frick and Shimbor, 1970; Harris and Markl, 1999). It was interesting that immunization with KLH combined with ES delivery 14 days prior to immunization with BSA decreased anti-BSA antibody level in comparison with rats that were only immunized with KLH (Fig. 1C). Since ES did not interfere with the development of the immune response to BSA (Fig. 1A), it could be speculated that ES influenced immune cells processing KLH, so their secreted products affected nearby resting cells, making them less capable to respond fully to the subsequent challenge with BSA 2 weeks later. It was shown that splenocytes obtained from rats exposed to ES at the time of immunization with KLH produced less interferon - gamma (IFN-γ) and more nitrite than splenocytes from control rats immunized with KLH (Fleshner *et al*., 1998). Since IFN-γ is essential for mounting an immune response (Basham and Merigan, 1983; Finkelman *et al*., 1990) and nitrite can suppress cytokine production and T cell proliferation (Coussons-Read *et al*., 1994; Bingisser *et al*., 1998), these mechanisms could contribute to the decrease in anti-BSA level in rats immunized with KLH and exposed to ES at the same time.

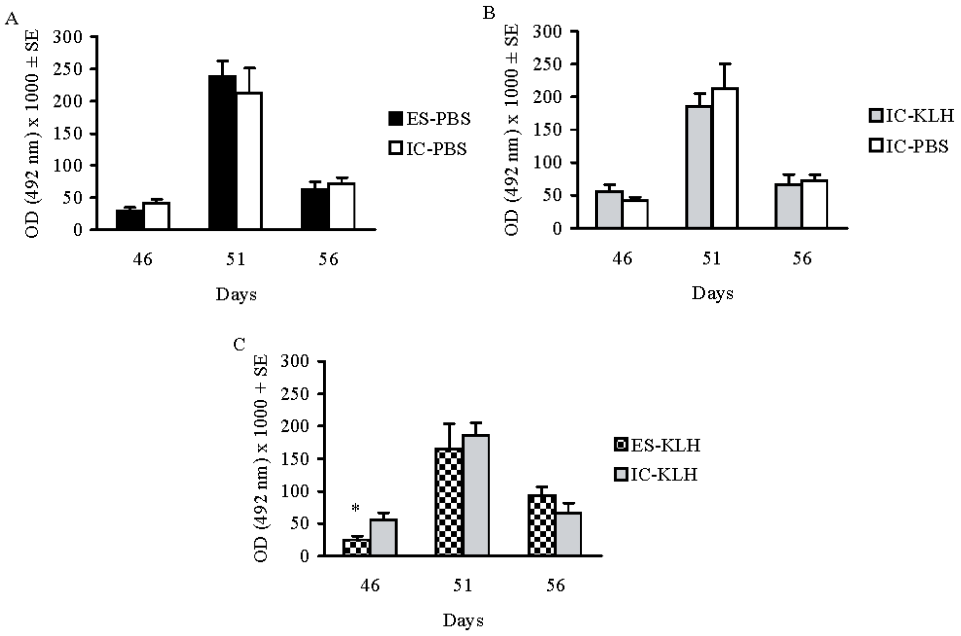

Figure 1. The effects of (A) exposure to electric tail shock (ES-PBS); (B) immunization with keyhole limpet hemocyanin (IC-KLH); or (C) concomitant exposure to ES and immunization with KLH (ES-KLH) on day -14 on the secondary humoral immune response (days 46, 51 and 56) to BSA. IC-PBS = control intact rats treated with PBS. N = 8 rats per group. Values represent mean ± SE. Statistically significant differences: *, $p<0.05$ vs. KLH.

In Experiment 2, the anti-KLH antibody level was decreased in rats that were exposed to ES concomitantly with immunization with KLH on day -14, but also in rats exposed to ES two weeks later, at the time of immunization with BSA on day 0 in comparison with intact KLH-immunized controls (Fig. 2A). However, in both cases ES suppressed the primary humoral immune response to KLH, which contrasted with to the effect of ES on the primary immune response to BSA (Stanojević *et al*., 2003). It must be emphasized that KLH is a very potent immunogen (Dixon *et al*., 1966), so differences in the kinetics of the development of immune responses to KLH and BSA could explain the discrepancies in the effects of ES after primary immunization with those antigens. Decreased anti-KLH antibody level in the ES (-14) group was expected, confirming that stressful experience suppresses the specific antibody level if it coincides with antigen administration (Fleshner *et al*., 1992.), probably influencing early events in the development of the humoral immune response (Laudenslager *et al*., 1988.; Sonnenfeld *et al*., 1992.). However, it was surprising that stress and immunization with a distinct antigen (BSA) performed at the time when the immune response to KLH should be fully developed (day 0), also decreased anti- KLH antibody levels. There is a possibility that neuroendocrine mediators of stress can directly influence lymphocytes involved in production of the specific anti-KLH antibodies, but it could also be possible that ES influenced cells processing BSA so their secreted products interfered with anti-KLH antibody formation. Since KLH-specific T cell proliferation occurs 2-4 days after antigenic challenge (Maier *et al*., 1986; Fleshner *et al*., 1995), 14 days after KLH immunization mediators of stress or suppressive products of BSA processing cells could influence KLH-specific lymphocytes undergoing isotype switch. The animals in our study were immunized with an associated form of KLH (Mw$\sim 10^6$) that mounted a strong primary immune response characterized with sustained existence of both IgM and IgG antibodies for several weeks (Dixon *et al*. 1966). Moreover, specific anti-KLH IgG antibody formation did not limit or terminate IgM antibody synthesis,

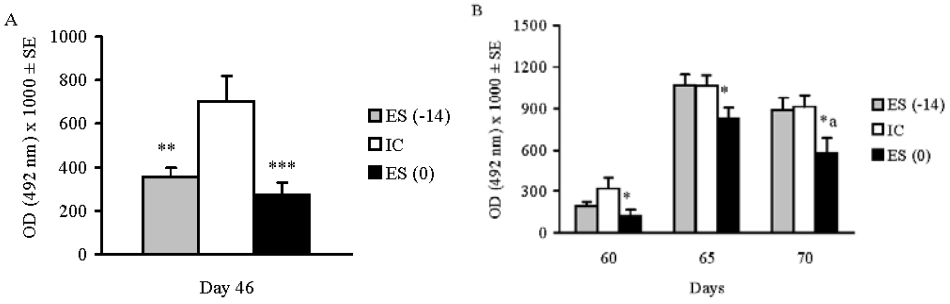

Figure 2. The influence of electric tail shock (ES) delivered during the immunization with KLH on day -14 or ES delivered during the immunization with BSA on day 0 on: (A) primary (day 46) or (B) secondary (days 60, 65 and 70) humoral immune response to KLH. IC = control intact rats. N = 8 rats per group. Values represent mean ± SE. Statistically significant differences:*, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$ vs. IC; [a], $p<0.05$ vs. ES (-14).

hence persistent antibody isotype switch could have been altered by stress delivery 2 weeks after immunization. It has been shown that this stage in antibody formation is very sensitive to hormonal and cytokine fluctuations (Laudenslager *et al*., 1988; Fleshner *et al*., 2001). However, after secondary immunization with KLH the diminished antibody level persisted only in the ES (0) group (Fig. 2B), probably because the interval between stress application and bleeding time was longer in the ES (-14) rats than in the ES (0) group (74 days vs. 60 days, respectively) and the suppressive effect of ES declined after secondary immunization with potent antigen KLH. This was another difference between BSA and KLH, since suppressive effect of ES was still observed after 106 days and three immunizations with BSA (Stanojević *et al*., 2003).

The present study confirmed that resting immune cells were not susceptible to changes induced by stress, signifying the importance of concomitant immune activation for stress to affect the immune response. Additionally, besides central and peripheral release of different neuroendocrine mediators, mechanisms of stress-induced immunomodulation, probably involved soluble factors released from activated immune cells. Although the suppressive effect of electric stress was not specific for a particular antigen, the chemical and immunological characteristics of antigens shaped the profile of stress-induced immune changes with respect to the sensitivity of the primary and secondary immune response and the duration of the effect.

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KARAKTERISTIKE ANTIGENA UTIČU NA EFEKTE STRESA NA HUMORALNI IMUNSKI ODGOVOR U PACOVA

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SADRŽAJ

Cilj rada bio je da se utvrdi da li je supresivni efekat električnog stresa (ES) na imunski odgovor specifičan za antigen kojim su pacovi imunizovani u vreme izlaganja ES. Ispitivan je uticaj istovremene primene stresa i imunizacije sa nesrodnim antigenom (keyhole limpet hemocyanin, KLH) na humoralni imunski odgovor prema govođem serum albuminu (GSA). Takođe su određivana i specifična anti-KLH antitela u serumima pacova koji su bili istovremeno izloženi stresu i imunizovani sa GSA. Rezultati su pokazali da ni petodnevni ES, ni imunizacija sa KLH dve nedelje pre imunizacije sa GSA nisu uticali na nivo anti-GSA antitela, za razliku od istovremenog izlaganja ES i imunizacije sa GSA koji su značajno suprimirali sekundarni humoralni imunski odgovor prema GSA. Nasuprot tome, primarni humoralni imunski odgovor prema KLH je bio suprimiran u pacova koji su bili izloženi stresu tokom imunizacije sa KLH, ali i kod onih koji su bili izloženi stresu dve nedelje kasnije, odnosno tokom imunizacije sa GSA. Naši rezultati ukazuju da supresivni efekat stresa na imunski odgovor nije specifičan za određen antigen, kao i da hemijske i imunološke osobine antigena značajno utiču na kvalitet promena izazvanih stresom u pogledu osetljivosti primarnog i sekundarnog imunskog odgovora na stres i trajanja efekata stresa.