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Stress Applied During Primary Immunization Affects the Secondary Humoral Immune Response in the Rat: Involvement of Opioid Peptides

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The effect of unpredictable, inescapable and uncontrollable electric tail shocks (ES) on the humoral immune response to bovine serum albumin (BSA) was investigated in the rat. Contributions of the procedures that accompany shock delivery, such as witnessing the ES procedure (stress witnessing, SW) and exposure to the apparatus for shock delivery (apparatus control, AC) to the changes in specific immunity induced by ES were also tested. All procedures were applied during primary and/or secondary immunization. It was demonstrated that exposure to ES during primary immunization with BSA significantly suppressed specific anti-BSA antibody production after secondary and tertiary immunization with the same antigen. Exposure to the SW procedure during primary immunization with BSA enhanced the specific antibody level after secondary immunization, while exposure to the apparatus alone did not influence the development of either the primary or secondary humoral immune response to BSA. Both ES-induced suppression and SW-induced potentiation of the humoral immune response were partially inhibited by prior treatment with the opioid receptor antagonist naloxone. Additionally, treatments with the opioid peptides methionine- and leucine-enkephalin decreased anti-BSA antibody level, mimicking to some extent the effects of ES. It is suggested that ES and endogenous opioid peptides had long-term effects on humoral immunity through mechanisms involving immunologic memory.

Keywords: Antibodies; Apparatus control; Electric shock stress; Opioid peptides; Stress witnessing

INTRODUCTION

Stress has been defined as a real or perceived threat to the maintenance of body homeostasis (Chrousos and Gold, 1992). Stress is also regarded as a condition where expectations do not match the current or anticipated perceptions of the internal or external environment, leading to patterned compensatory responses (Goldstein and McEwen, 2002), and is considered to be beneficial for the organism's well-being if adaptive and controlled, or detrimental if maladaptive and uncontrolled (Dom and Chrousos, 1993). It is well established that exposure to uncontrollable and inescapable stressors has profound effects on behavior and physiological functions. After inescapable footshocks rats perform poorly in aversively and appetitively motivated tasks (Jackson *et al.*, 1980), show reduced aggression and social dominance (Drugan and Maier, 1982) and express associative learning deficits (Lee and Maier, 1988).

Pertinent to the physiological response to stress, both suppression (Zhang *et al.*, 1998) and potentiation (Lysle *et al.*, 1990; Wood *et al.*, 1993; Dhabhar and McEwen, 1996) of immune responses have been reported. The effects of stress on the immune response vary with the strain of the animal (Shurin *et al.*, 1995; Bukilica *et al.*, 1996; Stanojević *et al.*, 2002), the type and the intensity of stressor (Croiset *et al.*, 1987; Rinner *et al.*, 1992), the dose and the route of antigen administration (Moynihan *et al.*, 1990; Fleshner *et al.*, 1992), the time between immunization and stress (Zalcman *et al.*, 1988), whether stress exposure is acute or repeated (Odio *et al.*, 1986; Fleshner *et al.*, 1998), and the particular immune function tested (Kusnecov and Rabin, 1993; Zalcman and Anisman, 1993; Fleshner *et al.*, 1998). The way in

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which electric shocks are delivered, that is intermittently or continuously, also determines the outcome of the effect of this stressor on the immune response (Sacerdote *et al.*, 1994). In line with this, inescapable and uncontrollable stress, applied in a series of intermittent shocks, consistently suppresses immune function (Laudenslager *et al.*, 1988; Mormede *et al.*, 1988; Fleshner *et al.*, 1995). Endogenous opioid peptides mediate footshock stress-induced analgesia (Altier and Stewart, 1996), uncontrollable/inescapable stress-induced suppression of natural killer cell cytotoxicity (Shavit *et al.*, 1984), and the decrease caused by intermittent footshock stress in the survival time of rats inoculated with mammary ascites tumor cells (Lewis *et al.*, 1985).

The aim of the present study was to investigate the effect of electric shock stress on humoral immune responses in the rat. The humoral immune response 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).

The design of our study took into account previous findings that a single electric shock session fails to significantly alter natural killer cell activity while subacute shock consisting of 4 daily sessions suppresses it (Irwin *et al.*, 1990), and that suppression of the antibody response is observed in rats only if they were exposed to a single electric shock session 72 h after immunization (Zalcman *et al.*, 1988). The study design was further based on findings that 80, but not 5–40, intermittent tail shocks augments the release of opioid peptides (Maier *et al.*, 1983; Sutton *et al.*, 1994). Because endogenous opioid peptides have been suggested as possible mediators of the effects of stress, their involvement in the stress-induced changes in immune responses were tested by treatment with the opioid receptor antagonist naloxone or the δ opioid receptor ligands methionine-enkephalin (Met-Enk) and leucine-enkephalin (Leu-Enk; Parsons *et al.*, 1990).

MATERIALS AND METHODS

Animals

Eight to twelve week old male Wistar rats weighing 196 ± 1.4 g (range 191–200 g) were purchased from the Medical Military Academy, Belgrade. Depending on the experiment, rats were housed in groups of 4 or 5 in Plexiglas cages under 12 h:12 h light/dark cycles. Rats had free access to standard food diet and tap water, unless otherwise stated. Upon arrival in our facility, rats were allowed to acclimatise for 1 week before experiment. During that period, the rats were handled daily to adapt to the experimenters. 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).

Antigen and Drugs

Bovine serum albumin (BSA) and the non-selective opioid receptor antagonist naloxone ((5α)-4,5-epoxy-3,14-dihydro-17-(2-propenyl) morphinan-6-one)·HCl)) were purchased from Sigma (St. Louis, MO). The opioid pentapeptides Met-Enk (Tyr-Gly-Gly-Phe-Met) and Leu-Enk (Tyr-Gly-Gly-Phe-Leu) were obtained from Serva (Heidelberg, Germany). All drugs were dissolved in 0.9% saline.

Immunization and Blood Collection

All rats were immunized intraperitoneally with 0.5 ml of BSA solution (2 mg/ml in saline). Immunizations were performed after the first session of stress, or 1/2 h after treatment with opioid peptides. Rats were bled by cardiac puncture between 08:00 and 09:00 h under light ether anesthesia. The blood (1 ml) was allowed to clot for 1 h at room temperature and for another hour at 4°C, and sera were removed and stored at −20°C until antibody levels were determined.

Determination of Specific Anti-BSA Antibodies

Serum levels of specific IgG anti-BSA antibodies were determined by enzyme-linked immunosorbent assay (ELISA). The wells of 96 well flat bottom microtiter plates (Corning, NY) were coated overnight with 50 μl of BSA solution (50 μg/ml in 0.05 M carbonate buffer, pH 9.6), and the following day washed three times with phosphate buffered saline (PBS, pH 7.4). Sera diluted 1:25, 1:50 or 1:100 in PBS/Tween (PBS with 0.05% Tween 20) to a final volume of 50 μl were plated in triplicate in the wells of the microtiter plates. Positive control serum, pooled from 10 rats subcutaneously immunized with 0.5 mg of BSA in 0.1 ml of emulsion containing complete Freund's adjuvant (CFA, 1.7 ml Arlacel A, 60 mg *Mycobacterium bovis*, 8.5 ml mineral oil Bayol F in 10 ml CFA), was diluted 1:1000 in PBS/Tween. Background control wells were filled with PBS/Tween. Plates were incubated for 1 h at room temperature, washed three times with PBS/Tween and 50 μl of peroxidase-conjugated goat anti-rat IgG (Sigma, MO) diluted 1:5000 in PBS/Tween was added. After 1 h of incubation at room temperature, plates were washed three times with PBS/Tween and 50 μl of chromogen ortho-phenilen-diamine (Sigma) was added to each of the wells. Color was allowed to develop in the dark for 40 min and the reaction was stopped by the addition of 25 μl of 2 M H_2SO_4. Optical densities (OD) were determined on a Multiscan reader

(Titertek, Flow Lab.) at 492 nm. Results were expressed as OD (mean ± SE).

To avoid interference by possible binding of secondary antibodies to the antigen on the plate, background control values were subtracted from OD for all sera samples. Both within and between assays variability were determined with OD values for positive control serum and, if this exceeded 10% between plates/assays, values were corrected according to the mean positive control values.

Stressing Procedures

Electric Shock Stress (ES)

Previous studies have determined electric shock protocols that effectively alter antibody responses (Zalcman *et al.*, 1988; Irwin *et al.*, 1990) and increase opioid peptide release (Maier *et al.*, 1983; Sutton *et al.*, 1994). Accordingly, in our study rats were subjected to five consecutive daily sessions of 80 unpredictable, inescapable and uncontrollable electric tail shocks through the early phases of the development of the primary immune response, and/or to three daily sessions of the same shocks during the initial phases of the development of the secondary immune response.

ES was delivered in the sound-proof room to which the rats were moved prior to each stress session. During the shock sessions, the rats were kept in rectangular Plexiglas boxes ($18 \times 9.5 \times 20\,cm^3$) 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. Depending on 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, rats 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).

Stress Witnessing (SW)

Because animal handling and other manipulations inevitable during experimental stress procedures can affect immune responses (Brenner *et al.*, 1990), we also investigated the influence of witnessing the ES procedure in controls, and of exposure to the apparatus for shock delivery alone on antibody production.

The SW procedure consisted of witnessing the procedure of shock delivery to the rats of the ES group. The SW rats were moved to the sound-proof room prior to each stress session along with the rats of the ES group and remained there during the ES procedure. Specifically, rats of the SW group were kept in the Plexiglas boxes in the identical manner as used for ES, but received no shocks and witnessed the ES procedure during the 80–90 min that the ES rats received shocks. After each ES session, rats of the SW group were returned to their colony and left undisturbed until the next session.

Apparatus Control, AC

Rats of the AC group were moved to the sound-proof room, kept in Plexiglas boxes for 80–90 min in an identical manner as used for ES and then returned to their colony. These rats received no shocks and were devoid of any contact with rats of the ES group.

Intact Control (IC)

Rats of the IC group were left in their home cages in the colony. During the stress sessions of the corresponding groups, IC animals were food and water deprived.

EXPERIMENTAL DESIGN (TABLE I)

In *Experiment 1*, the primary and secondary humoral immune responses to BSA were investigated in the rats of the ES, SW and AC groups. All rats were weighed on Days −3 and 7 of the experiment and the results were expressed as relative increase of body weight (bw) (%).

Rats were immunized with BSA on Day 0, and on Days 0–4 subjected to five consecutive daily exposures to electric shock stress (ES), or the stress witnessing procedure (SW) or exposed to the apparatus for shock delivery (AC). Intact rats (IC) were left undisturbed in

TABLE I Experimental design

Days	0	0–4	7, 16	43	43–45	46, 51, 56	86	89, 94, 99, 106
Exp. 1	BSA	ES, SW, AC	bc	BSA	ES, SW, AC	bc	–	–
Exp. 2	BSA	ES	–	BSA	–	bc (d.51)	BSA	bc
Exp. 3	BSA	Nx+ES, Nx+SW	–	BSA	–	bc	–	–
Exp. 4	BSA	Met-Enk, Leu-Enk	–	BSA	Met-Enk, Leu-Enk	bc	–	–

BSA, immunization with 0.5 ml of bovine serum albumin (2 mg/ml in saline); ES, electric shock stress (80, 5-s unsignaled shocks, average intershock interval 60 s, total duration 80–90 min) delivered in the apparatus; SW, stress witnessing procedure with no shock received in the apparatus; AC, apparatus control, exposure to the apparatus only; bc, blood collection; Nx + ES, Nx + SW, i.p. treatment with 10 mg/kg bw of naloxone prior to each ES and SW; Met-Enk, Leu-Enk, i.p. treatment with 0.2 mg/kg bw of methionine-enkephalin or leucine-enkephalin.

the colony and food and water deprived for 80–90 min daily. Each group consisted of 15 rats. Rats were bled on Days 7 and 16 for the determination of the primary humoral immune response to BSA. Secondary immunization with BSA was performed on Day 43, and rats were additionally subjected to three consecutive daily sessions (Days 43–45) of the appropriate stress model (ES, SW), exposed to the apparatus (AC) or left in the colony and food and water deprived for 80–90 min daily (IC). Groups consisted of 7–10 rats. According to results from a pilot experiment dealing with kinetics of the humoral immune response to BSA, rats were bled on Days 46, 51 and 56 and sera collected for antibody determination after secondary immune challenge.

In *Experiment 2*, the duration of the effect of electric shock stress delivered during primary immunization on humoral immunity was investigated in the rats of the ES group by giving a third immunization with the same antigen. Rats were subjected to five consecutive daily exposures to electric shock stress (ES) on Days 0–4 or left undisturbed in the colony and food and water deprived for 80–90 min daily (IC). Number of rats per group was 17–20. All rats were immunized with BSA on Days 0, 43 and 86 and left undisturbed in the colony during the secondary and tertiary immunizations. Half of the rats from the ES and IC groups were bled on Day 46 for the determination of the secondary humoral immune response, and all rats were bled on Days 89, 94, 99 and 106 for the determination of the humoral immune response after tertiary immunization.

Experiment 3 was performed to investigate whether release of endogenous opioid peptides mediated the suppressive and potentiating effects of electric shock stress (ES) and the stress witnessing procedures (SW) on the secondary humoral immune response, respectively. Thirty minutes prior to each of five daily stress sessions on Days 0–4 rats were treated intraperitoneally with 10 mg/kg bw of naloxone (ESNx and SWNx groups) or with 0.3 ml of saline (ESSal and SWSal groups). Control rats were also treated with naloxone or saline (ICNx and ICSal groups). All rats were immunized with BSA on Days 0 and 43, left undisturbed in the colony during the secondary immunization, and bled for the determination of the secondary humoral immune response on Days 46, 51 and 56. Each group consisted of 8–10 rats.

Experiment 4 was designed to evaluate the effects of exogenously applied opioid peptides on anti-BSA antibody production. Thirty minutes prior to primary immunization with BSA and then for 4 consecutive days (Days 0–4) and/or 30 min prior to secondary immune challenge with BSA and then for 3 consecutive days (Days 43–45), rats were treated intraperitoneally with 0.2 mg/kg bw of Met-Enk or Leu-Enk. Control rats were treated with 0.4 ml of saline (Sal). According to the treatment during primary and secondary immunization, the following groups were formed: MetEnk-MetEnk, MetEnk-Sal, Sal-Sal and Sal-MetEnk (and similarly for the treatments with Leu-Enk). Each group consisted of 9–10 rats. To mimic the stress procedures, all experimental groups were food and water deprived for 80 min daily, on Days 0–4 and 43–45. All rats were bled on Days 46, 51 and 56 for the determination of the specific secondary humoral immune response to BSA.

STATISTICAL ANALYSIS

All biometrical calculations (mean, SE) were performed using the statistical package STAT View II. Data were analyzed by two-way and one-way ANOVA followed by Fischer's protected least-significant difference test for comparisons between independent groups. Differences were considered significant if $p < 0.05$.

RESULTS

Experiment 1. There were no differences in the level of the specific anti-BSA antibodies in rats that were subjected to five daily sessions of electric shock stress (ES), or the stress witnessing procedure (SW) or apparatus exposure (AC) when compared to the intact rats (IC) on Days 7 and 16 after primary immunization (Fig. 1A–C). The relative increase of bw was lower in the rats of the ES, SW and AC groups on Day 7 when compared to rats from the IC group ($F_{3,56} = 9$; $p = 0.0003$; respectively 33.3% ± 1.1, 33% ± 1.2, 32.8% ± 1.2 vs. 40% ± 1.5; $p < 0.01$).

After secondary immune challenge, rats of the ES group had significantly lower anti-BSA antibody levels on Day 51 when compared to anti-BSA antibody levels in sera of rats that were undisturbed in the colony (IC group; Fig. 2A). In contrast, a significantly higher level of specific antibodies was observed on Day 51 in sera of animals that witnessed delivery of electric shocks (SW group) when compared to the samples obtained from rats of the IC group (Fig. 2B). Two-way ANOVA with factors Immunization (primary or secondary) and Procedure (stress or no stress) revealed that the suppressive effect of ES ($p < 0.001$), as well as the potentiating effect of SW ($p < 0.001$) were associated with presentation of stress during primary but not during secondary immunization.

Accordingly, one-way ANOVA ($F_{3,26} = 32.7$; $p = 0.0001$) showed that rats that were exposed to electric shock stress during primary immunization (ESIC) as well as rats exposed to electric shocks during both immunizations (ESES) had lower anti-BSA antibody levels on Day 51 in sera in comparison with intact rats, and also in comparison with rats stressed only during secondary immunization (ICES; Fig. 2A). There were no differences among experimental groups on Day 46 ($F_{3,26} = 0.4$; $p = 0.7762$) and on Day 56 ($F_{3,24} = 1.7$; $p = 0.2039$). Rats that witnessed electric shock stress during primary immunization (SWIC) exhibited a significantly higher level of the specific anti-BSA antibodies on Day 51 when compared to both intact rats (ICIC) and rats of the ICSW group that were subjected to the stress witnessing procedure only during secondary immunization

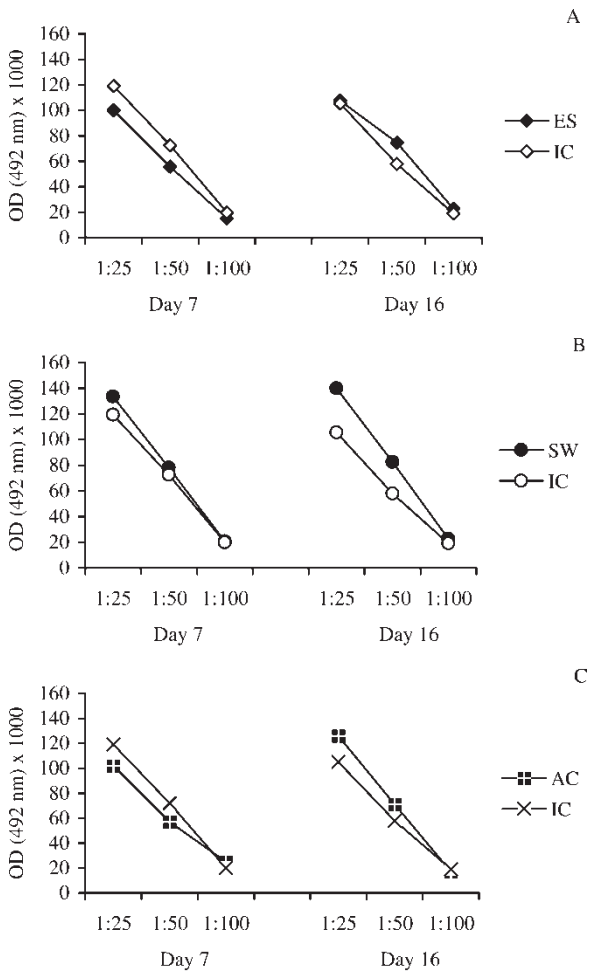

FIGURE 1 Effects during primary immunization with BSA on the primary humoral immune response to BSA of: (A) exposure to electric shocks (ES); (B) stress witnessing procedure (SW) and (C) exposure to the apparatus for shock delivery (apparatus control, AC). Intact control rats (IC) were undisturbed. $N = 15$ rats per group. Values represent means. There were no statistically significant differences.

($F_{3,26} = 8.9$; $p = 0.0003$; Fig. 2B). Again, there were no differences among experimental groups on Day 46 ($F_{3,27} = 0.4$; $p = 0.4401$) and on Day 56 ($F_{3,25} = 2$; $p = 0.1418$). Figure 2C shows that daily exposures to the apparatus during primary (ACIC), secondary (ICAC) or both immunizations (ACAC) did not influence the secondary humoral immune response to BSA in the rats on Day 46 ($F_{3,30} = 2.5$; $p = 0.0771$), Day 51 ($F_{3,28} = 1.3$; $p = 0.2859$) and Day 56 ($F_{3,25} = 1.3$; $p = 0.2882$). These results suggested that placement of rats into the boxes and subsequent daily exposures to the apparatus that was inevitable during the electric shock and stress witnessing protocols did not contribute to the suppressive and potentiating effects of ES and SW, respectively, on anti-BSA antibody production.

In *Experiment 2*, rats were subjected to immunizations with BSA on Days 0, 43 and 86 and exposed to five sessions of electric shocks only during primary immunization (ES). The suppressive effect of ES delivered during primary immunization was retained during the tertiary immune response to BSA, without further electric shocks. Differences in the levels of the specific antibodies in sera of rats from the ES and IC groups were observed on Days 99 and 106 ($F_{1,35} = 30.3$; $p = 0.0001$ and $F_{1,35} = 11.2$; $p = 0.002$, respectively; Fig. 3).

Experiment 3. To investigate the possible involvement of endogenous opioid peptides in the electric shock-induced decrease in antibody levels, rats were injected with the non-selective opioid receptor antagonist naloxone or saline prior to each stress exposure during primary immunization. In this experiment, the suppression of the secondary immune response to BSA by electric shock was more profound than in Experiment 1, as revealed by significant decreases in anti-BSA antibody levels on Days 46, 51 and 56 (Fig. 4A). This finding could be due to an additional interaction between injection and stress. Two-way ANOVA with factors Procedure (stress or no stress) and Treatment (naloxone or saline) revealed a significant effect of electric shocks in decreasing the immune response on Day 46 ($p < 0.001$) and Day 51 ($p < 0.001$). These results were confirmed by one-way ANOVA on Day 46 ($F_{3,28} = 9.5$; $p = 0.0002$) and Day 51 ($F_{3,28} = 13.7$; $p = 0.0001$), showing that antibody levels in sera of rats exposed to electric shocks and treated with saline (ESSal), as well antibody levels in sera of electrically stressed rats treated with naloxone (ESNx), were significantly different from intact rats treated with saline (ICSal; Fig. 4A). These results showed that naloxone did not antagonize the effect of electric shock on the specific humoral immune response on Days 46 and 51 (Fig. 4A). However, two-way ANOVA revealed that both electric shocks ($p < 0.001$) and treatment with naloxone ($p < 0.01$) contributed to the overall effect on the immune response observed on Day 56. One-way ANOVA showed that naloxone partially prevented the suppressive effect of electric shocks on Day 56 (Fig. 4A), as development of anti-BSA antibodies was not suppressed in rats treated with naloxone prior to stress exposure (ESNx), while inhibition by electric shocks was still present in rats treated with saline ($F_{3,28} = 13.9$; $p = 0.0001$; Fig. 4A). Injection of naloxone in intact rats did not affect the secondary immune response to BSA (Fig. 4A). These results suggested that endogenous opioid peptides partly mediated the effect of electric shocks on humoral immunity, but only on day 56.

In *Experiment 3*, it was also investigated whether endogenous opioid peptides participate in the enhancement of anti-BSA antibody level induced by witnessing stress. Rats were injected with naloxone or saline prior to every stress witnessing procedure during primary immunization. Two-way ANOVA with factors Procedure (stress or no stress) and Treatment (naloxone or saline) revealed significant effects of both witnessing stress ($p < 0.001$) and treatment with naloxone ($p < 0.05$) for the changes in the immune response observed on Day 51 (Fig. 4B). However, one-way ANOVA showed that rats that witnessed the ES procedure during primary immunization (SWSal) exhibited a significantly higher level of the specific anti-BSA antibodies when compared to intact rats treated with saline (ICSal) and intact rats

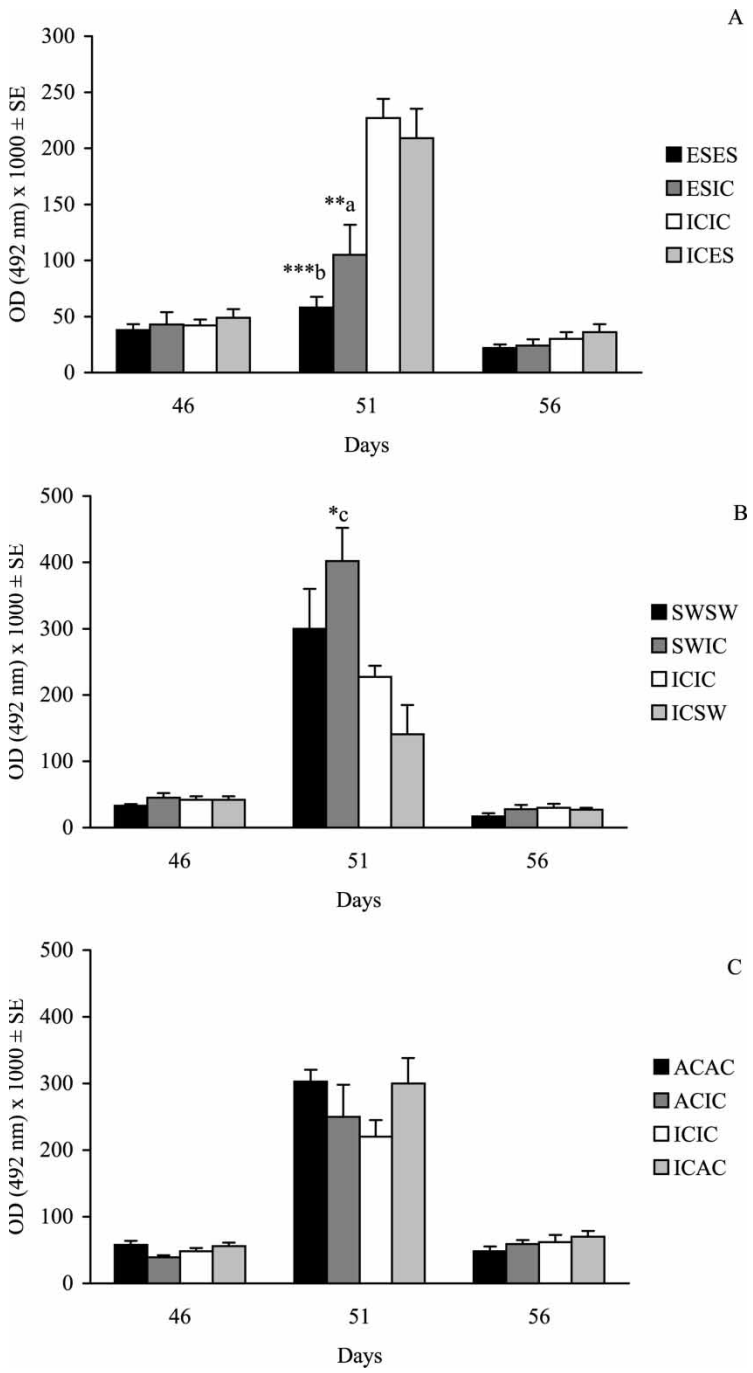

FIGURE 2 Effects on secondary humoral immune response to BSA of: (A) electric shocks applied during primary (ESIC), secondary (ICES) or both immunizations (ESES); (B) stress witnessing procedure applied during primary (SWIC), secondary (ICSW) or both immunizations (SWSW) and (C) exposure to the apparatus for shock delivery during primary (ACIC), secondary (ICAC) or both immunizations (ACAC). Intact control rats (ICIC) were undisturbed. $N = 7–10$ rats per group. Values represent mean ± SE. Statistically significant differences: $^{*}p < 0.05$, $^{**}p < 0.001$ and $^{***}p < 0.0001$ vs. ICIC; $^{a}p < 0.01$ and $^{b}p < 0.0001$ vs. ICES; $^{c}p < 0.01$ vs. ICSW.

treated with naloxone (ICNx), but no differences were observed among intact rats that were treated with saline or naloxone ($F_{3,34} = 7.1$; $p = 0.0008$; Fig. 4B). These results disclosed a partial inhibitory influence of naloxone on the increase in the humoral immune response induced by witnessing stress, and hence the possible involvement of endogenous opioid peptides in its immunoenhancing effects. No differences in the level of anti-BSA antibodies were observed among groups on Day 46 ($F_{3,31} = 2.1$; $p = 0.1122$) and Day 56 ($F_{3,33} = 0.5$; $p = 0.6691$; Fig. 4B).

*Experiment 4.*The effects of exogenous opioid peptides on anti-BSA antibody production were investigated. Rats immunized with BSA on Days 0 and 43 were treated daily with Met-Enk, Leu-Enk or saline on Days 0–4 and/or on Days 43–45. Two-way ANOVA with factors Immunization (primary or secondary) and Treatment (Met-Enk or saline) revealed interaction of factors on Day 46

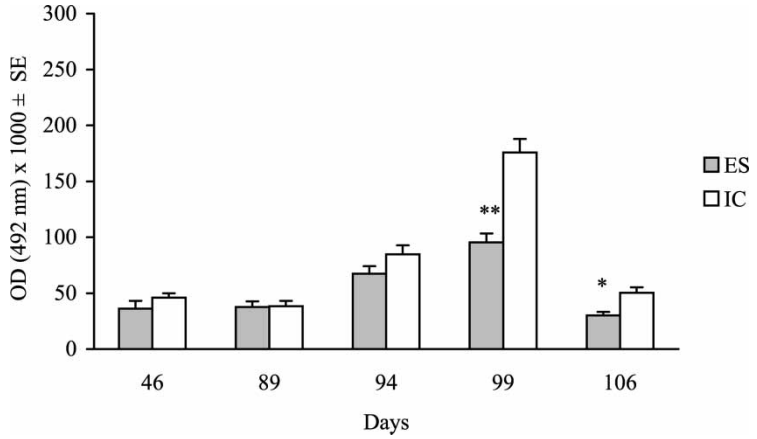

FIGURE 3 Effects of electric shocks (ES) applied during primary immunization with BSA on the humoral immune response to BSA after secondary and tertiary immunizations. Intact control rats (IC) were undisturbed. $N = 17-20$ rats per group. Values represent mean ± SE. Statistically significant differences: $^{*}p < 0.01$ and $^{**}p < 0.0001$ vs. IC.

($p < 0.05$). Rats treated with Met-Enk during both immunizations (MetEnk-MetEnk) had significantly lower anti-BSA antibody levels on Day 46 in comparison with saline-only treated rats (Sal-Sal) and rats treated with Met-Enk during secondary immunization (Sal-MetEnk) ($F_{3,33} = 3.9$; $p = 0.0176$; Fig. 5A). There were no differences among groups on Day 51 ($F_{3,35} = 0.6$; $p = 0.6419$) and Day 56 ($F_{3,34} = 0.3$; $p = 0.8428$; Fig. 5A).

Two-way ANOVA also showed that treatment with Leu-Enk at the time of primary immunization decreased the secondary immune response to BSA on Day 46 ($p < 0.001$). Antibody level in animals treated with Leu-Enk during the primary (LeuEnk-Sal) or both

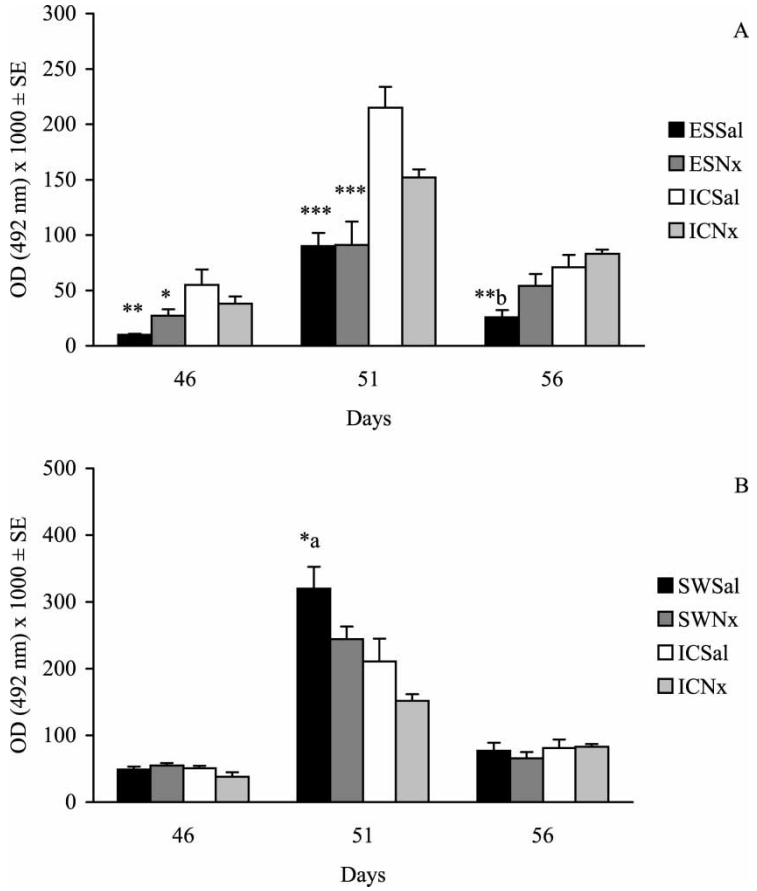

FIGURE 4 Effects during primary immunization with BSA on the secondary humoral immune response to BSA of: (A) treatment with naloxone (ESNx) and saline (ESSal) prior to electric shocks; and (B) treatment with naloxone (SWNx) and saline (SWSal) prior to stress witnessing. Intact control rats were treated with naloxone (ICNx) and saline (ICSal). $N = 8-10$ rats per group. Values represent mean ± SE. Statistically significant differences: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. ICSal; $^{a}p < 0.01$ and $^{b}p < 0.001$ vs. ICNx.

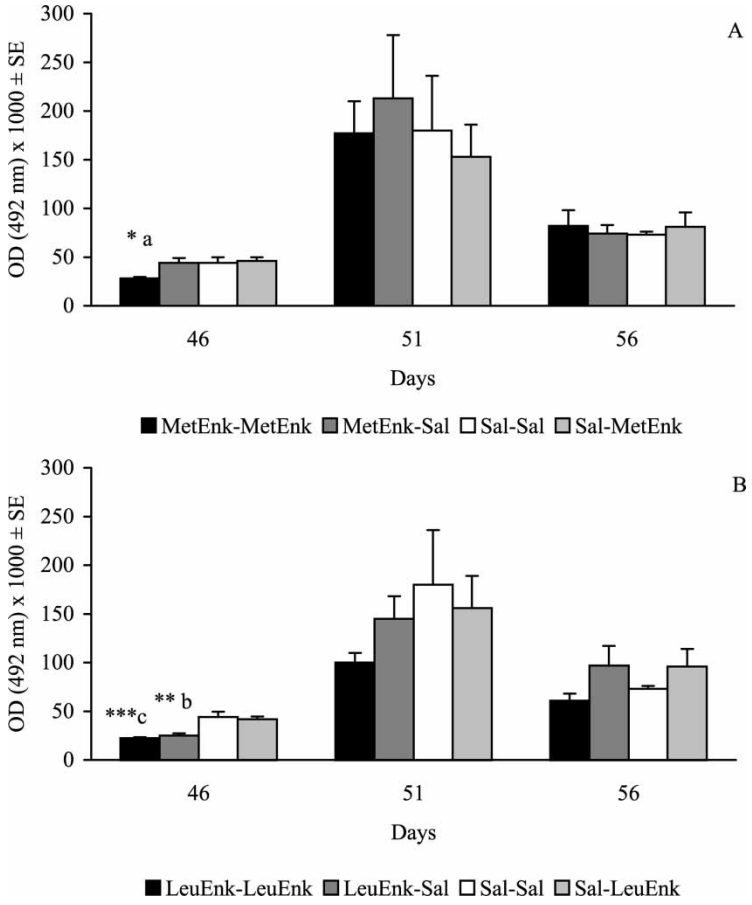

FIGURE 5 Effects on the secondary humoral immune response to BSA of: (A) treatment with Met-Enk during primary (MetEnk-Sal), secondary (Sal-MetEnk) or both immunizations (MetEnk-MetEnk) and (B) treatment with Leu-Enk during primary (LeuEnk-Sal), secondary (Sal-LeuEnk) or both immunizations (LeuEnk-LeuEnk). Control rats were treated with saline during both immunizations with BSA (Sal-Sal). $N = 9-10$ rats per group. Values represent mean ± SE. Statistically significant differences: $^{*}p < 0.05$, $^{**}p < 0.001$ and $^{***}p < 0.0001$ vs. Sal-Sal; $^{a}p < 0.01$ vs. Sal-MetEnk; $^{b}p < 0.001$ and $^{c}p < 0.0001$ vs. Sal-LeuEnk.

immunizations (LeuEnk-LeuEnk) was significantly different on Day 46 in comparison with saline-treated control rats (Sal-Sal) as well as in comparison with animals treated with Leu-Enk only during secondary immunization (Sal-LeuEnk) ($F_{3,35} = 12.3$; $p = 0.0001$; Fig. 5B). No differences were observed among groups on Day 51 ($F_{3,34} = 1.9$; $p = 0.1553$) and Day 56 ($F_{3,34} = 2.5$; $p = 0.0761$; Fig. 5B).

DISCUSSION

In the present study, we demonstrated that exposure to stressors during primary immunization profoundly affects the humoral immune response upon subsequent antigen challenge.

As food deprivation alters serum corticosterone concentration and spleen cell number (Tarcic *et al.*, 1995), which might influence immune responses, interference by this factor with effects of stress was avoided by food-depriving all rats, including the intact controls, during the stress procedures. Rats that were subjected to five consecutive daily exposures to electric shocks (ES), the stress witnessing procedure (SW) or to apparatus alone (AC) gained less weight than the untreated (IC) group, suggesting that the stressful nature of the procedures rather than food restriction itself led to the decreased weight gain. Likewise, Ottenweller *et al.* (1989) described that during 10 days of witnessing stress or electric shocks food consumption was reduced. Additionally, the decrease in bw gain after stress may be a consequence of increased energy expenditure or changes in digestive physiology (Ottenweller *et al.*, 1989; Meerlo *et al.*, 1996).

Exposure to electric shocks (ES) or witnessing stress (SW) during primary immunization did not influence the primary anti-BSA antibody response (Fig. 1A,B), but secondary anti-BSA antibody levels were decreased and increased, respectively (Fig. 2A,B). Our results corroborate previous findings of long-lasting immune changes after cessation of chronic stress (Odio *et al.*, 1986), but for the first time it is shown that a short period of repetitive stress exposure could result in pronounced suppression of humoral immunity. The lack of a stress effect during primary immunization is in accordance with other studies (Laudenslager *et al.*, 1988) demonstrating that the impact of stress on antibody level becomes significant only after IgG antibody levels rise. Since on Days 7 and 16 many of the plasma cells have not switched to IgG production

(Laudenslager *et al.*, 1988), it could be speculated that those plasma cells that underwent successful early isotype switch before Day 7 were not sensitive to the influence of electric stress and the stress witnessing procedure. Other studies also pointed to the early events in the development of humoral immune response as targets for immunomodulation induced by stress. Thus, stress decreases MHC II expression by splenic monocytes (Sonnenfeld *et al.*, 1992) and alters macrophage number and/or functional state (Fleshner *et al.*, 1995) that could affect antigen processing leading to a decline in antibody production. These possibilities cannot be ruled out in our experiments. However, antigen processing is required during all immunizations. Thus, on the basis of the long-term effects of stress after primary challenge and lack of effects after secondary immunization, it seems more likely that the cellular target for the stress-induced immunomodulation could be memory lymphocytes that were formed during the first encounter with the antigen and responded differently after the second challenge.

The apparatus control group (AC) was a control for manipulations inevitable during the delivery of electric shocks and the stress witnessing procedures. This control procedure, involving transport to the sound-proof room, placing the animals into the test boxes with exposure of rats to the apparatus over 5 days had no effects on antibody production (Figs. 1C and 2C). Although restraint can induce numerous immunological disturbances (Zwilling *et al.*, 1990; Feng *et al.*, 1991; Hermann *et al.*, 1994; Zhang *et al.*, 1998), the restraining method used in our experiments (plexiglass boxes allowing movement of the front paws and tail fixation) was probably less harmful than procedures usually employed for "restraint stress" (e.g. fixation of paws prohibiting animal movements or confinement in a conical tube), although weight gain was decreased even though immunity was not affected. Consequently, it is concluded that electric shocks and the stress witnessing procedure were responsible for the observed suppression and potentiation of antibody production after the second immune challenge, respectively.

A number of endocrine mediators can contribute to the electric shock-induced changes in humoral immunity, such increased release of corticotropin-releasing factor (Jain *et al.*, 1991), ACTH (Dhabhar *et al.*, 1995), glucocorticoids (Fleshner *et al.*, 1996) and catecholamines (Sonnenfeld *et al.*, 1992). As our experimental model comprised exposure of rats to unpredictable, inescapable and uncontrollable electric shocks, known to release endogenous opioid peptides (Maier *et al.*, 1983; Shavit *et al.*, 1984), we tested the possible contribution of opioids to electric stress-induced suppression of antibodies by pretreatment of stressed rats with the non-selective opioid receptor antagonist naloxone (Simon, 1986). The dose of naloxone of 10 mg/kg bw was chosen with the aim of fully occupying available opioid binding sites and to prevent binding of endogenous enkephalins released after stress. In view of the partial inhibition of antibody suppression in the naloxone-pretreated stressed rats, our results indicated the involvement of opioid peptides in electric shock-induced changes of the humoral immune response.

Interestingly, the stress witnessing procedure enhanced the specific secondary humoral immune response (Fig. 2B). Our SW model was complex, consisting of placing the animals in the apparatus for electric shock delivery and exposure of rats to pheromones and vocalization from stressed rats during the 5 days of testing. Rats signal their affective state to other rats via ultrasonic vocalizations (Knutson *et al.*, 2002). Odours of stressed rats also seem to be important signals. Exposure to odour from stressed animals induces behavioral changes (Zalaquett and Thiessen, 1991), opioid-mediated analgesia (Fanselow, 1985; Fanselow and Sigmundi, 1986) and enhancement of humoral immune response (Cocke *et al.*, 1993) in the non-stressed conspecifics. Moynihan *et al.* (1994) described that pheromones from footshock-stressed mice enhanced antigen-specific antibody production and interleukin (IL)-4 production in non-stressed BALB/c mice. Both effects were antagonized by naltrexone (Moynihan *et al.*, 2000), suggesting that opioids could also mediate these changes. In our experimental model, naloxone partially inhibited the increase in anti-BSA antibody level on Day 51 from witnessing stress (Fig. 4B). Therefore, the release of endogenous opioid peptides may mediate the immune changes observed after 5 days of exposure to noises and smells of stressed rats. Naloxone slightly, though not significantly, reduced the antibody response in intact rats on Day 51 (Fig. 4A,B), suggesting that endogenous opioid peptides might tonically regulate immune responses (Radulović and Janković, 1994).

Enkephalins are ligands for δ opioid receptors that are widely distributed in the central (Mansour *et al.*, 1988; Pan *et al.*, 2002) and peripheral nervous system (Stein *et al.*, 1990) and are involved in numerous nervous functions (Solbrig *et al.*, 2002). After stress, the content of messenger ribonucleic acid encoding enkephalin precursor significantly increases in discrete brain areas (Larsen and Mau, 1994; Wiedenmayer *et al.*, 2002) and the concentration of enkephalins increases in the periphery (Parsons *et al.*, 1990), suggesting involvement of δ opioid receptor ligands in stress-induced physiological changes. Immunomodulatory properties of enkephalins are provided through central mechanisms, including binding to central opioid receptors (Janković and Radulović, 1992; Dimitrijević *et al.*, 2000) and modulation of transmitter systems in brain areas involved in immunoregulation (Basu and Dasgupta, 2000), but also through direct effect on immune cells by enkephalins released from peripheral sites, or following injection (Vujić-Redžić *et al.*, 2000). Opioid receptors on immune cells are structurally and biochemically identical to those found in neural tissue (Gavériaux *et al.*, 1995; Sedqui *et al.*, 1996), while immune cells constitutively or upon induction synthesize and/or release enkephalins (Schäfer *et al.*, 1994; Ovadia *et al.*, 1996; Cabot *et al.*, 2001). In the present study, five

consecutive daily injections of Met-Enk and Leu-Enk decreased the antibody response (Fig. 5A,B), mimicking in part the effects of electric shocks. The daily dose of 0.2 mg/kg bw of Met-Enk and Leu-Enk used in our experiments (approximately 50 μg or 10^{-7} moles per whole rat), taking into account distribution and degradation after intraperitoneal injection (Hambrook *et al.*, 1976; Janak *et al.*, 1992), is considered likely to reach the immune compartments in the range that mimics the levels of endogenous enkephalins available to the cells under stress conditions (Pierzchala and Van Loon, 1990; Dziedzicka-Wasylewska *et al.*, 2002). It was shown previously that lower doses of Met-Enk potentiated, but higher doses suppressed numerous immunological functions under *in vivo* (Janković and Marić, 1987a,b,c; Gabrilovac and Marotti, 2000) and *in vitro* conditions (Radulović *et al.*, 1995). However, the dose of Met-Enk (0.2 mg/kg) that was previously shown to enhance IgM production (Janković and Marić, 1991), suppressed IgG levels in the present experiments. Therefore, it can be proposed that the opioid peptides Met-Enk and Leu-Enk interfere with antibody formation and immunological memory at the level of isotype switching, impairing normal development from class IgM to IgG antibodies.

The finding that naloxone also partially inhibited the immunopotentiation induced by witnessing stress suggests that opioid peptides other than δ-opioid receptor-preferring enkephalins were released during stress witnessing. Dynorphin A primarily activate κ opioid receptors (Ishihara *et al.*, 2001), while beta-endorphin, previously classified as μ/δ ligand, binds to specific ϵ opioid receptors (Nock *et al.*, 1993; Tseng, 2001). These opioid peptides could act through μ (Stefano *et al.*, 1996) and κ (Ignatowski and Bidlack, 1999; Suzuki *et al.*, 2001; Kirst *et al.*, 2002) opioid receptors expressed on immunocytes, providing a link for the stress-induced opioid-mediated immunomodulation.

In conclusion, our results showed that exposure to unpredictable, inescapable, uncontrollable electric shock exerted suppression, whereas exposure to signals (presumably vocalisation and odours) from rats receiving electric shocks potentiated the specific secondary humoral immune response. Endogenous opioid peptides partly contributed to both effects. Importantly, the effects were observed only when stressors were presented during primary but not secondary immunization, suggesting that mechanisms involved in immunological memory might be particularly susceptible to stress responses.

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