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Effects of chronic stress on the immune response to oral human serum albumin-conjugated starch microparticles in rats

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Abstract

Uptake of antigens and bacteria over the follicle-associated epithelium (FAE) is increased after chronic psychological stress. We investigated whether stress affects the immune response to particle-conjugated antigens taken up via the FAE. Rats were submitted to two 10-day periods of water avoidance stress and orally immunized during these periods. Stressed immunized rats displayed altered cell populations and a Th1-skewed immune response within the lymphoid follicles, together with enhanced delayed-type hypersensitivity. We conclude that chronic stress affects the cell-mediated immune response after oral immunization, which may have implications for the understanding of allergic and autoimmune diseases and development of oral vaccines.

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1. Introduction

It is believed that chronic stress can modulate the clinical course and severity of human intestinal disorders (Collins, 2001; Levenstein et al., 2000; Mayer, 2000; Qiu et al., 1999), but mechanisms remain largely unknown. Severe stress episodes are an important risk factor for the development and reactivation of intestinal inflammation. In rodents, chronic stress can initiate or reactivate mucosal inflammation by affecting the intestinal barrier (Ferrier et al., 2003; Santos et al., 2001; Söderholm et al., 2002) and T-cell immunity (Qiu et al., 1999). These findings suggest that stress may affect the immune response to luminal antigens. It is well established that stress affects both the systemic and mucosal immune responses after parenteral immunization in humans

(Burns et al., 2003; Cohen et al., 2001), rats (Fleshner et al., 1989, 1998; Gazda et al., 2003; Laudenslager et al., 1988) and mice (Fukui et al., 1997; Moynihan et al., 1990). The effects, which are both immediate and long-term, are related to the influence of glucocorticoid and adrenal hormones on the lymphoid cells (Cohen et al., 2001; Padgett and Glaser, 2003). Studies on the effects of stress on the immune response after oral immunization are, however, more scarce. Recently, the effects of chronic stress on a food hypersensitivity model (oral antigen + i.p. adjuvant) in rats were investigated (Yang et al., 2006). Stressed rats showed an increased transmucosal uptake of the model antigen, horseradish peroxidase (HRP), and sensitization was demonstrated by both systemic and local responses. This suggests that a stress-induced increase in permeability may alter the immune response to an orally administered antigen.

The Peyer's patches in the mucosal-associated lymphoid tissues (MALT) play a significant role in the development of the immune response after oral immunization. The follicle-associated epithelium (FAE), that covers the Peyer's patches, has unique biochemical properties, facilitating the adherence,

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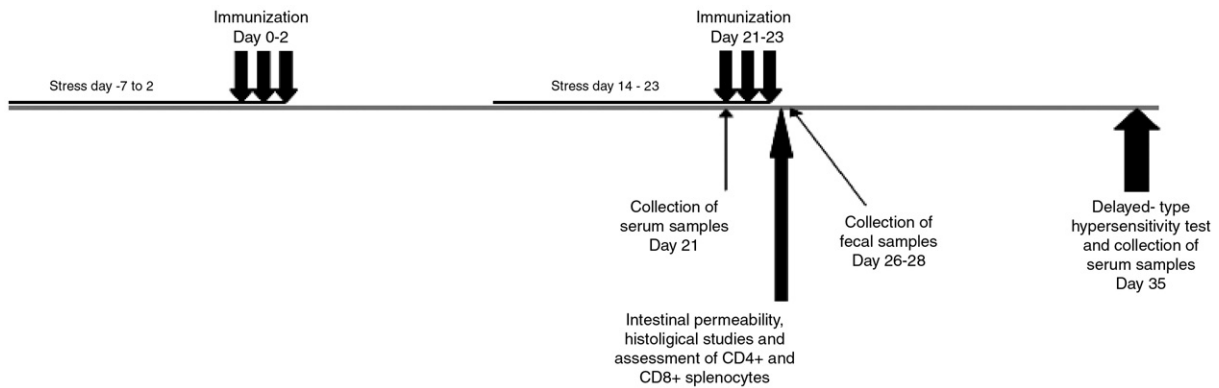


Fig. 1. Outline of the experimental process. Rats were submitted to 1 h of water avoidance stress for two periods of ten consecutive days. On the last three days in each session, the rats were orally immunized. Serum and fecal samples, spleens and intestines were collected for analyses at indicated time points.

uptake and immunological sampling of microorganisms (Neutra et al., 2001). This luminal sampling is believed to be crucial for induction of protective mucosal immune responses, but is also used as a route of entry by various pathogens. Moreover, it is now widely recognized that the induction of effective immune responses for protection of mucosal surfaces requires delivery of oral vaccines to organized MALT (Florence, 1997; Neutra et al., 2001; Norris et al., 1998). Therefore, we require a deeper understanding of the complex mechanisms that maintain the balance between pro-inflammatory and tolerogenic immune reactions for understanding mechanisms of intestinal inflammation and the rational design of mucosal vaccines.

We recently showed that chronic stress in rats modulated the barrier function of FAE, allowing increased uptake of luminal antigens and microorganisms (Velin et al., 2004), and hypothesized that this enhanced uptake could lead to an increased antigen load and presentation in the dome area of the Peyer's patches, thereby initiating an abnormal immune response. The aim of the present study was to elucidate the effects of this stress-induced enhanced antigen uptake on the immune response after oral immunization with a model particulate antigen. For this purpose we used human serum albumin-conjugated starch microparticles (HSA-MP), which are exclusively taken up via the FAE (Larhed et al., 2004). Rats were subjected to chronic stress and orally immunized. Mucosal barrier function and mucosal and systemic immune responses were assessed.

2. Materials and methods

2.1. Animals

Male Wistar rats (B and K Universal AB, Sollentuna, Sweden), bodyweight 150–200 g at arrival, were acclimatized for one week and then handled daily for two weeks before the stress and immunization protocols were initiated. During these weeks and the rest of the experimental period, the animals were kept in pathogen-free conditions with a 12:12 h light:dark cycle and fed ad libitum on a diet of

standard rat pellets (R70, Lactamin AB, Vadstena, Sweden) and tap water. The study was approved by the local committee of animal ethics and the animals were kept under the supervision of a licensed veterinarian.

2.2. Stress and immunization protocols

Twenty-four rats were divided into four groups: I) non-stress and non-immunization II) stress and non-immunization III) stress and immunization IV) non-stress and immunization. The experimental process is outlined in Fig. 1. Rats in groups II and III were submitted to water avoidance stress (WAS) for ten consecutive days, one hour/day, as previously (Velin et al., 2004). The rats were placed on a platform ($h=8$ cm, $d=6$ cm) in a plastic container ($h=56$ cm, $d=50$ cm) holding 25 °C water at a level 1 cm below the platform. To minimize the effects of circadian rhythm, the procedures were started between 9 and 10 a.m. each day. The rats were weighed before each session and the number of fecal pellets were counted after each session as an indirect index of changes in colonic propulsive activity (Barone et al., 1990). The rats in groups I and IV were kept in their home cages as controls, and weighed daily. Directly after the stress session on day eight, the rats were anaesthetized by isofluran inhalation. The rats in groups III and IV were orally immunized with 1 ml of 6 mg/ml HSA-MP in physiological saline while those in groups I and II were given 1 ml physiological saline. Immunization was repeated on days nine and ten of the stress period. The rats were then left in their cages and weighed daily. After ten days, the stress protocol was repeated with immunization on days 21–23. After the last immunization, two to three rats per group were anaesthetised and used for permeability, flow cytometry, histology and cytokine studies. Starting 5 cm proximal to the ileocecal valve, 15 cm segments from the distal ileum were dissected and immediately transported to the laboratory in ice-cold oxygenated Krebs buffer (115 mM NaCl, 1.25 mM CaCl_2 , 1.2 mM MgCl_2 , 2 mM KH_2PO_4 and 25 mM NaHCO_3 , pH 7.35). Spleens were aseptically removed and kept on ice-cold phosphate-buffered saline

(PBS) until prepared for flow cytometry analyses (see below). The remaining rats were left in their cages and used for delayed-type hypersensitivity tests and antibody analyses.

2.3. Preparation of HSA-MP

The microparticles were prepared from acryloylated starch (maltodextrin, MD6, Stalex AB, Malmö, Sweden) in a w/o emulsion by radical polymerization (Artursson et al., 1984; Laakso et al., 1986). Briefly, the acryloylated starch (500 mg) was dissolved in 5 mL of a 0.2 M sodium phosphate buffer, at pH 7.5, containing 1 mM EDTA. Ammonium peroxodisulfate was added to give a final concentration of 0.8 M in the aqueous phase, which was homogenized in 300 mL toluene:chloroform (4:1) with 0.08 g Pluronic® F68 as an emulsifier. *N,N,N',N'*-tetramethylethylenediamine (TEMED, Sigma, St. Louis, MO) was used to initiate the polymerization.

The model antigen HSA (Aventis Behring, Germany) was conjugated to the microparticles using the carbodiimide method of Bethell et al., as described previously (Stertman et al., 2004). The amount of HSA conjugated to the microparticles was determined by amino-acid analysis and was found to be 43.6 µg HSA per mg starch. The amount of microparticles/mL was determined by dry weight. The size-number distribution of the microparticles was $2.00 \mu\text{m} \pm 1.42$ (mean $\pm$ SD), as determined by laser diffractometry (LS 230, Coulter, USA).

2.4. Ussing chamber experiments

While immersed in ice-cold oxygenated Krebs, the external muscle and myenteric plexus were stripped of the distal ileal specimens. Sections of FAE, identified macroscopically and subsequently verified by light microscopy, were cut into appropriate sizes and mounted in modified Ussing chambers (Harvard Apparatus Inc., Holliston, MA) (Grass and Sweetana, 1988). When mounting FAE, the segments were carefully adjusted so that the exposed tissue surface covered the entire 0.096 cm^2 surface area of the chamber. Before placing the chambers in the 37°C system, the serosal compartments were filled with 1.5 mL of cold 10 mM glucose in Krebs buffer, and the mucosal compartments were filled with 10 mM mannitol in Krebs buffer to maintain equal osmolality. Both sides were continuously oxygenated with 95% O_2 /5% CO_2 ; the buffer was circulated by the gas flow. The transepithelial potential difference, short-circuit current and electrical resistance across the tissue were monitored throughout the experiments. The chambers were equilibrated for 40 min, with replacement of the buffer at 20 min, to achieve steady-state conditions. Three FAE segments from each rat were mounted in chambers for permeability studies. Tissues for histology were collected and further processed as described below.

The 45 kDa protein antigen HRP (type VI, Sigma Chemical Co., St. Louis, MO) was used for transcellular studies and the 384 D inert probe ^{51}Cr -EDTA (Perkin Elmer, Boston, MA)

was used for paracellular studies. After equilibration, 300 µL samples were collected from the serosal side and replaced with glucose buffer. HRP and ^{51}Cr -EDTA were added to the mucosal side to a final concentration of 10^{-5} M and 34 µCi/mL, respectively. Serosal samples were collected at 30, 60 and 90 min from the start. An aliquot from each sample was saved for HRP analysis and the remainder was placed in a gamma-counter (1282 Compugamma, LKB, Bromma, Sweden) for ^{51}Cr -EDTA measurements. HRP was assayed using the QuantaBlu™ Fluorogenic Peroxidase Substrate Kit (Pierce, Rockford, USA) according to a protocol developed at our laboratory (Velin et al., 2004). The absorbance of the color reaction product was measured at excitation wavelength 325 nm and emission wavelength 420 nm using a fluorimeter (Cary Eclipse, Varian, Victoria, Australia). Blanks were included in each analysis and all samples were run in duplicate and measured against a standard curve.

2.5. Immunohistochemistry

Segments of FAE from three rats per group were frozen in liquid nitrogen, embedded in mounting medium and cryosectioned at 5 µm. Coded slides were fixed in acetone for 10 min, briefly rinsed in PBS and blocked with 2% goat serum for 10 min. Primary mouse anti-rat antibodies for CD4 (OX4), CD8a (OX8) and dendritic cells (DCs) (OX-62) (BD Pharmingen, Sweden) were diluted 1:50 and applied to individual sections. After 1 h of room temperature incubation in a humid chamber, the slides were rinsed in PBS, a biotinylated goat anti-mouse Ig antibody (BD Pharmingen) diluted 1:50 was added and the slides were incubated for 30 min. After rinsing in PBS, a prediluted streptavidin-conjugated HRP solution (BD Pharmingen) was added and the slides were incubated for 30 min. After repeated washing, sections were incubated with DAB solution (BD Pharmingen) for 1–2 min, rinsed in water and stained with haematoxylin before being mounted and examined by light microscopy (Leica MZ50, Leica Microsystems, Sweden).

2.6. Preparation of serum and fecal samples

Blood and fecal samples were collected for antibody analysis. Blood samples were taken via cardiac puncture (day 21, $n=3$ from each group) or from the hind leg vein (day 35, $n=2-4$) and serum was prepared and stored at -20°C . Freshly voided feces were collected on days 3–5 after the booster, frozen and freeze dried. After the net dry weights were determined, extracts of the feces were prepared on ice. Briefly, the fecal pellets were reduced and PBS containing 5% non-fat dry milk, 0.1 mg soybean trypsin inhibitor/mL (Sigma) and 2 mM phenylmethylsulphonylfluoride (Sigma) was added at a concentration of 20 µL solution/mg of dry feces. Solid matter was suspended by rigorous vortexing and separated by centrifugation at $13,000 \times g$ for 10 min at 4°C . The supernatants were then frozen at -20°C until analyzed for antigen-specific and total IgA (see below).

2.7. Analysis of fecal IgA antibodies

A sandwich-type ELISA developed by Hau et al. (Hau et al., 2001) was used to determine the total amount of IgA in fecal samples. Briefly, Nunc Immunoplate Maxisorb F96 plates were coated with goat anti-rat IgA (Fc)/7S antiserum (Nordic, Tilburg, The Netherlands) diluted in carbonate buffer (pH 9.6) overnight at 4 °C. To avoid nonspecific binding, the plates were blocked with 1% tryptophan in PBS. Fecal samples, added in series of two-fold dilutions, or rat IgA standard solution (BD Pharmingen) diluted in PBS with 0.05 % Tween© 20. HRP-conjugated goat anti-rat IgA (Fc)/7s antiserum (Nordic, Tilburg) was used with *o*-phenylenediamine substrate, SIGMA FAST™ OPD (Sigma), for detection. The reaction was stopped with 2 M H₂SO₄ and the absorbance was measured at 492 nm.

2.8. Quantification of cytokine gene expression

The FAE specimens (3 mm² from each rat, 3 rats from each group) were collected at animal sacrifice and kept at –70 °C. Total RNA extraction was performed using combined TRIzol® Reagent (Life Technologies, NY, USA) and RNeasy® Mini Kit (Qiagen, CA), as recently described (Monstein et al., 2004). cDNA synthesis was performed using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Groningen, The Netherlands). The amount of 1.5 µg of purified total RNA with RNeasy®

MinElute™ Cleanup Kit (Qiagen) was used to produce first-strand cDNA with an oligo (dT) primer.

The gene expressions of IFN-γ, IL-4, IL-10 and TNF-α, were determined by quantitative real-time PCR with β-actin as endogenous control in Smart Cycler® (Cepheid, CA), using QuantiTect™ SYBR® Green PCR Kit (Qiagen). The PCR primers were derived from previous studies (Crabtree et al., 2004; Krook et al., 2002; Matsubara et al., 2004). The sizes of the PCR amplicons were 73 bp, 71 bp, 351 bp, 85 bp and 105 bp for β-actin, IFN-γ, IL-4, IL-10 and TNF-α respectively. In Smart Cycler® the amplification was performed for 45 cycles of 95 °C for 40 s, 58 °C for 40 s, and 72 °C for 1 min, followed by the melt curve analysis which can confirm the specificity of the amplification. Up to 500 ng single-strand cDNA was used as template in quantitative PCR amplification. The level of gene expression was expressed as 2^{–ΔΔC_t} after being normalized to beta-actin and relative to a calibrator (Applied Biosystems, User Bulletin #2 ABI Prism 7700 Sequence Detection System).

2.9. Flow cytometry assay of splenocytes

The spleens were punctured with a syringe and cells were squeezed out into the PBS buffer. The cell suspensions were centrifuged, resuspended in TRIS buffer with ammonium chloride and incubated on ice for 10 min. After two washings in RPMI and filtration through a 70 µm nylon filter (Cell Strainer, Becton Dickinson, USA), the cells were resuspended

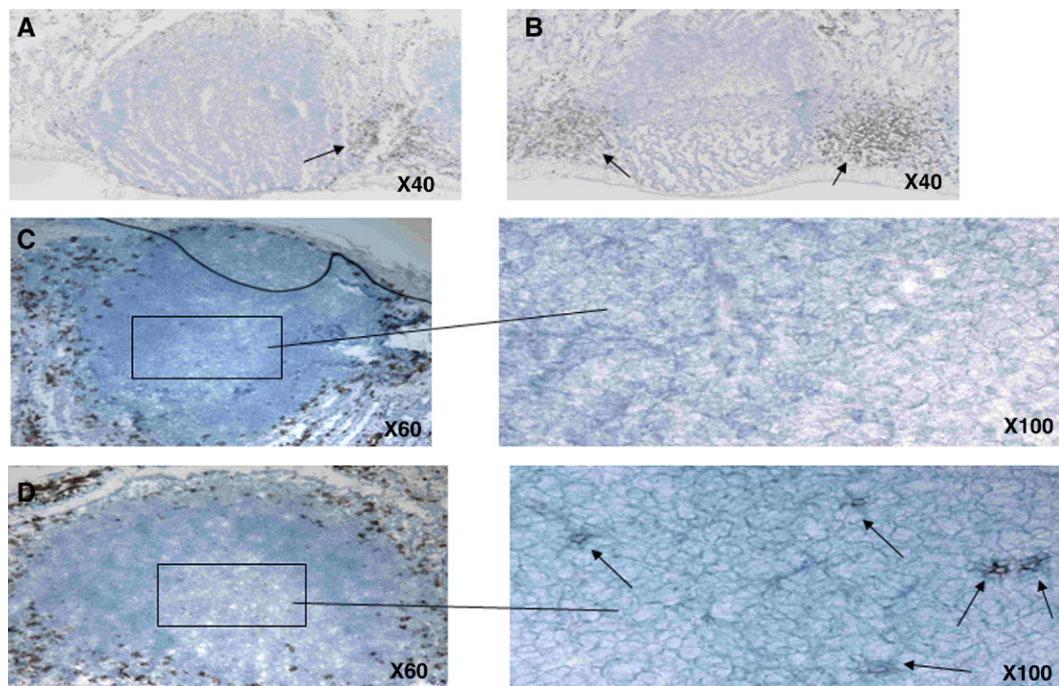


Fig. 2. Immunohistochemistry of CD8 (OX-8) and dendritic cells (OX-62) in rat Peyer's patches. Rats were submitted to water avoidance stress and immunized with human serum albumin conjugated to starch microparticles (A) Arrows indicate the expression of CD8 in stressed immunized rats. (B) Arrows indicate the expression of CD8 in non-immunized non-stressed rats. (C) There was no expression of dendritic cells within the follicles of non-immunized non-stressed rats. (D) There was substantial expression of intra-follicular dendritic cells in stressed immunized rats (arrows).

in culture medium (RPMI 1640 containing 10% fetal calf serum, 0.5% gentamycin and 1% 200 mM L-glutamine) and counted. For labeling, the cells were resuspended in 0.5% BSA in PBS and 200 μ L of each cell suspension was dispensed into vials (3×10^5 cells/vial). Five μ L each of phycoerythrin (PE)-conjugated CD4 monoclonal antibody and peridinin chlorophyll- α protein (PerCP)-conjugated CD8a monoclonal antibody (BD Pharmingen) were added to the tubes and incubated for 20 min in the dark. Isotype-matched fluorochrome-conjugated irrelevant immunoglobulin (BD Pharmingen) was used at the same concentration for assessment of nonspecific binding. Finally, the cells were washed with PBS containing 0.5% BSA and analyzed within 24 h.

2.10. Delayed-type hypersensitivity tests

After the second stress and immunization period, four rats from groups III and IV and two rats from groups I and II were left in their cages. Two weeks later, these rats were intradermally challenged in their right ear with 10 μ L of 1 mg/ml HSA in physiological saline. The left ear was injected with physiological saline as an internal negative control. Ear thickness was measured by a person blinded for the groups, before the injection ($t=0$) and during the following three days ($t=24$, 48 and 72 h). The relative increase in ear thickness was then calculated.

2.11. Analyses of serum antigen-specific antibodies

Serum HSA-specific antibodies were determined by enzyme-linked immunosorbent assay (ELISA) using the protocol as previously described (Stertman et al., 2004). In the present study, the following secondary antibodies were used: alkaline phosphatase-conjugated goat anti-rat IgG (Sigma) 0 diluted 1:1000 and biotin-conjugated mouse anti-rat IgM, IgG1, IgG2a, IgG2b or IgG2c (BD Pharmingen) diluted 1:250. A sandwich-type ELISA was developed to determine the IgG subclasses in sera against relevant standard curves with capturing and standard antibodies from BD Pharmingen. In each sample, the final concentration of antigen-specific IgG1, IgG2a, IgG2b or IgG2c was determined from pooled standard curves by calculating the mean concentration from three dilutions of a sample. Pooled negative serum was added to each plate as a negative control. Titers are given as $\log_2$ (dilution $\times 10$). A positive sample (serum from rats immunized intraperitoneally with Freund's adjuvant) was included as a standard on all plates.

2.12. Statistics

Parametric values are presented as means $\pm$ SEM. Comparisons between two groups were made using Student's t test; comparisons among more than two groups were made using one-way analysis of variance (ANOVA). Two-way or 3-way ANOVA was used to analyze significant differences in response to immunization and stress. Nonparametric values

are given as medians (25–75th interquartile range). Comparisons between two groups were made using the Mann–Whitney U test and those between more than two groups

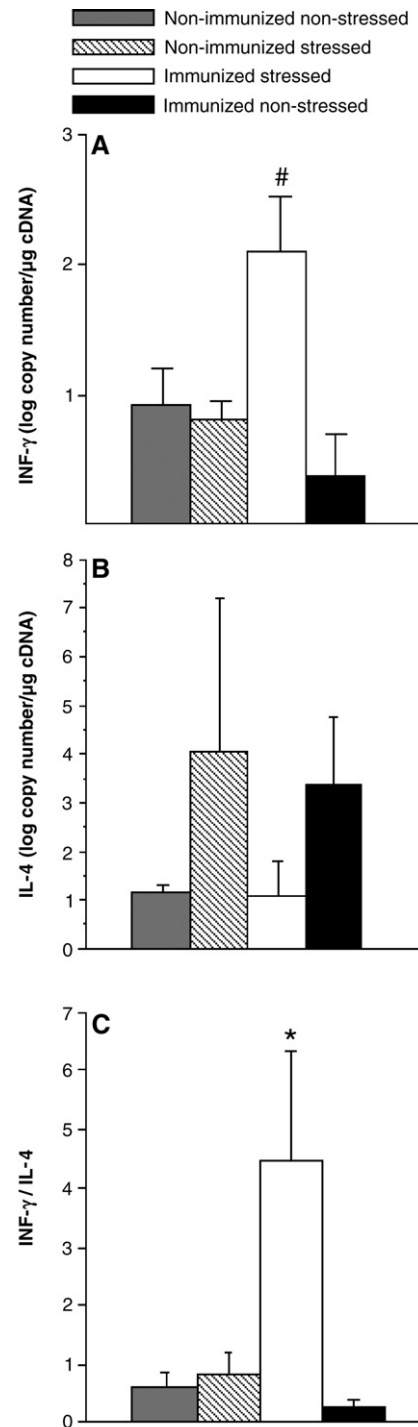


Fig. 3. Cytokine gene expression in rat FAE after stress and immunization. (A) The expression of INF- γ was increased in immunized stressed rats compared to immunized non-stressed, [#] $P=0.056$. (B) Expression of IL-4 was equal in all groups (C) The ratio INF- γ /IL-4 was increased in stressed immunized animals compared to all groups, ^{*} $P<0.05$. Values are given as means $\pm$ SEM.

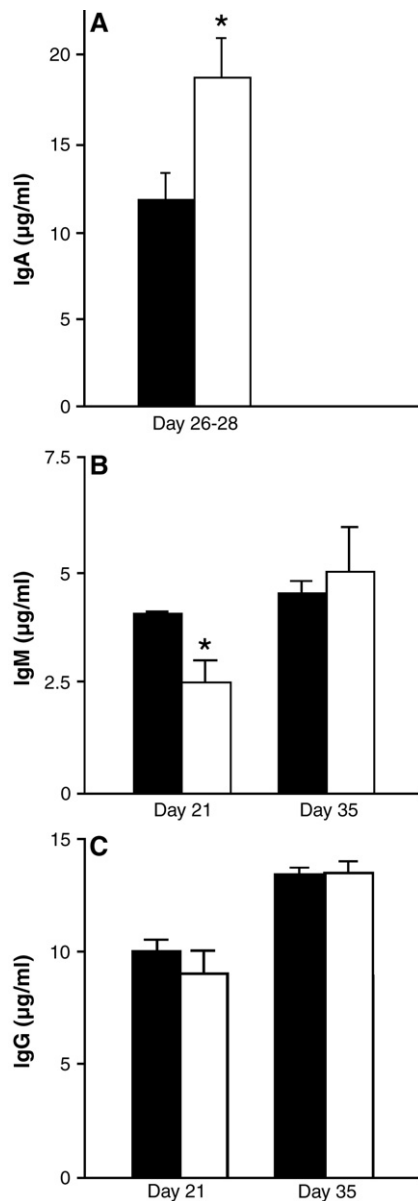


Fig. 4. Humoral immune responses in rat after stress and immunization. (A) The fecal total s-IgA response (B) The anti-human serum albumin IgM and (C) the IgG responses. The total s-IgA response in feces was measured on three consecutive days after booster (day 26–28). The humoral responses in serum were measured three weeks after primary immunization (day 21) and two weeks after the booster immunization (day 35). Bars represent means for the responding rats (2–4) ± SEM, * = $P < 0.05$. Filled bars = non-stressed immunized rats and open bars = stressed immunized rats.

used the Kruskal–Wallis one-way analysis of variance. Differences with $P < 0.05$ were considered significant.

3. Results

3.1. Expected physiological changes induced by chronic stress

As in our previous study (Velin et al., 2004), the chronic stress protocol induced changes in physiology and corticosterone. During the first experimental period stressed rats

gained less weight (mean weight 319 ± 5 g on day one and 334 ± 6 g on day 10) than non-stressed (328 ± 5 g on day one and 366 ± 5 g on day 10), irrespective of immunization ($P < 0.005$). All rats gained weight equally during the ten days between the first and second stress periods but, during the second period of stress, the stressed rats once again gained significantly less weight (356 ± 7 g to 389 ± 7 g vs. 383 ± 5 g to 434 ± 5 g; $P < 0.0001$).

Chronic stress also increased the defecation rate during each stress session. The rate was also consistently higher during each daily stress over the 10-day stress periods. The defecation during stress was 10 ± 3 pellets/h as compared to 1.5 ± 1 pellets/h in non-stressed.

Studied in Ussing chambers, FAE segments from rats exposed to chronic stress showed a significant increase in short-circuit current (active ion transport) across FAE. Electrical conductance (paracellular ion transport) was unchanged by stress, and FAE permeability to the paracellular marker ^{51}Cr -EDTA was also equal in stressed and non-stressed animals whether immunized (7.0 ± 1.9 vs. 7.2 ± 1.8 10^{-6} cm/s) or not (4.5 ± 0.9 vs. 6.2 ± 1.4). However, uptake of the protein antigen, HRP, across FAE was substantially higher after stress than that in non-stressed, in immunized rats (333 ± 33 vs. 47 ± 13 pmol/h/cm²) as well as in non-immunized (252 ± 38 vs. 50 ± 14), $P < 0.05$ for both comparisons.

3.2. Chronic stress affected mucosal immune responses

3.2.1. Mucosal dome area cell populations

Numerous CD4⁺ cells were found in the follicle area of all rats and there were no differences in expression among any of the groups (data not shown). On the other hand, the number of CD8⁺ cells was lower in the inter-follicular regions of stressed immunized rats than in the same regions of non-immunized non-stressed (Fig. 2A and B). There was also a reduction in the number of CD8⁺ splenocytes in stressed animals (see below). In all groups, scattered DCs (OX-62⁺) surrounded the follicles (Fig. 2C). Interestingly, in rats that were both stressed and immunized, DCs were also found inside the centre of the follicles (Fig. 2D). These central DCs were not found in any of the other three groups (Fig. 2C).

3.2.2. Mucosal dome area cytokine mRNA expression

The change in dome area cell populations was accompanied by changes in cytokine expression. IFN- γ was increased

Table 1

Flow cytometry analysis of CD4⁺ and CD8⁺ splenocytes

Group		CD4 ⁺	CD8 ⁺
I	Non-stress and non-immunization	29.0 ± 2.0	19.7 ± 1.6
II	Stress and non-immunization	35.8 ± 2.2	14.6 ± 1.3
III	Stress and immunization	49.7 ± 3.4	14.1 ± 1.4
IV	Non-stress and immunization	44.3 ± 2.8	18.8 ± 1.9

^aSplenocytes were prepared from spleens collected after the last stress session and labeled as summarized in the Materials and methods. Immunization increased the % of CD4⁺ cells, $P < 0.001$, and stress decreased the % of CD8⁺ cells, $P < 0.05$. Values are presented as mean ± SEM, % of gate.

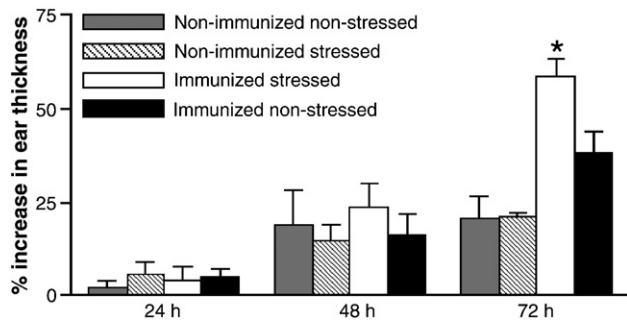


Fig. 5. The delayed-type hypersensitivity response was measured as the increase in ear thickness after injection of human serum albumin antigen in the ear two weeks after the booster immunization. The ear thickness was measured before and 24, 48 and 72 h after injection. Bars represent means $\pm$ SEM, * $P < 0.05$ vs. non-immunized, non-stressed rats.

in the FAE area of stressed immunized rats compared to non-stressed immunized (Fig. 3A), whereas there were no significant differences between the groups in IL-4 expression (Fig. 3B). The IFN- γ /IL-4 ratio was significantly increased in FAE of stressed immunized rats as compared to all other groups (Fig. 3C), suggesting a Th1-predominant cytokine response. There were no significant differences between the groups in the expression of IL-10 and TNF- α (data not shown).

3.2.3. Mucosal humoral response

There was no HSA-specific secretory IgA(s-IgA) in any of the fecal samples. However, the total s-IgA level was higher in stressed rats during the three days analyzed than in the controls (Fig. 4A). A 3-way ANOVA with the treatment (immunized, non-immunized), stress condition (stressed, non-stressed) and days after treatment as factors was performed. Because of the unbalanced design, the analysis was done by the general linear model. No interaction terms were included in the analysis. The result of the statistical analysis showed statistical significance between the levels of stress condition, $P < 0.02$, while the factors treatment and days after treatment did not show any statistical differences between their levels, $P = 0.094$ and $P = 0.38$, respectively.

3.3. Systemic immune responses altered by chronic stress

3.3.1. CD4⁺ and CD8⁺ lymphocytes in the spleen

As shown in Table 1, stress significantly decreased the percentage of CD8⁺ splenocytes ($P < 0.05$) and increased the percentage of CD4⁺ splenocytes ($P = 0.05$). Immunization significantly increased the proportion of CD4⁺ cells ($P < 0.001$) but had no effect on the proportion of CD8⁺ splenocytes.

3.3.2. Delayed-type hypersensitivity test

After intradermal injection of HSA in the ear on day 35 (14 days after the second immunization), ear thickness increased with time, indicating a local cellular response (Fig. 5). At 72 h, the response, measured as % increase in ear

thickness, was significantly higher in immunized stressed rats compared to non-immunized non-stressed (59.0 ± 4.3 vs. 20.7 ± 3.8 % increase, $P < 0.05$).

3.4. The antigen-specific systemic humoral immune response

Immunized rats showed high anti-HSA IgM and IgG responses irrespective of stress protocol. There was a significantly decreased anti-HSA IgM response on day 21 in stressed immunized rats compared to non-stressed, however, at 35 days the responses were equal (Fig. 4B). There were no differences in IgG responses between the groups (Fig. 4C). HSA-specific IgG1, IgG2a and IgG2b were detected in both of the immunized groups, indicating a mixed Th1/Th2 profile. No HSA-specific IgG2c was detected in any of the samples. The ratio of antigen-specific IgG1 to the sum of antigen-specific IgG2a and IgG2b (a measure of the Th1/Th2 profile) was calculated for each individual rat, however, no significant differences between the mean group ratios were seen (data not shown).

4. Discussion

We have previously shown that the uptake of bacteria and other antigens over the FAE is increased in response to chronic psychological stress (Velinet al., 2004). From these findings it was postulated that, as a result, the immune response to particulate luminal antigens may be affected. To study this hypothesis, we used HSA-conjugated starch microparticles as a model particulate antigen. Starch microparticles are effective as an oral carrier/adjuvant in mice (Rydell and Sjöholm, 2004; Strindeliuss et al., 2004a,b, 2002; Wikingsson and Sjöholm, 2002) and HSA-conjugated starch microparticles are taken up specifically in the intestinal mucosa through the FAE (Larhed et al., 2004). This particle system is thus a useful model for studies of the consequences of stress-induced antigen transport in the FAE on the immune response. In the present study, chronic stress induced the expected effects on bodyweight and intestinal physiology. Moreover, stress substantially increased the rate of antigen transport in the FAE, suggesting that the antigen load in the Peyer's patches was increased. This was accompanied by altered cell populations and a Th1-skewed cytokine response, and a subsequently exaggerated cell-mediated immune response after oral immunization with particulate antigens in stressed rats.

Our data also provide some clues as to the mechanisms involved. We found that stress increased the number of DCs in the centre of the follicles of the Peyer's patches. Intra-follicular DCs present antigens to B cells within the follicles and switch off apoptosis of B cells with affinity to this antigen, thereby playing an important role in the formation of memory B cells (van Nierop and de Groot, 2002). Our findings support a study showing that psychological stress before immunization exerted an adjuvant-like effect on skin DCs, which increased their migration and enhanced delayed-

type hypersensitivity after parenteral immunization (Saint-Mezard et al., 2003). It can thus be speculated that the stress-induced increase in FAE antigen uptake increases the activity and migration of DCs, and subsequently enhances both non-specific activity and antigen-specific responses. In line with this, infectious inflammation has been associated with an increased number of follicular DCs in mammalian ileal lymphoid follicles (Aleksandersen et al., 2002; Teichmann et al., 2000). We also found a reduced number of CD8⁺ cells in the Peyer's patches of stressed rats, together with a significantly decreased proportion of CD8⁺ splenocytes. A striking decrease in the number of CD8⁺ cells in Peyer's patches following restraint stress was also found by Sudo et al. (Sudo et al., 2001).

Other studies have also shown a reduction in the total number of T lymphocytes (Dhabhar et al., 1994, 1996), as well as in the CD8⁺ subpopulation (Stefanski et al., 2001; Sudo et al., 1997), in blood following stress in rodents, representing a redistribution of the lymphocytes from blood to organs such as bone marrow, lymph nodes and skin as a result of acute stress (Dhabhar and McEwen, 1996). It has been proposed that trafficking of CD8⁺ lymphocytes to the skin is part of the enhanced cell-mediated response to acute stress (Saint-Mezard et al., 2003). However, previous studies of the effects of chronic stress on the cell-mediated immune response give conflicting results (Dhabhar and McEwen, 1997), which seem to depend on the duration and type of stress employed. Moreover, there may be differences in the effects of stress on the cell-mediated response between oral and parenteral immunization routes; a stress-induced increase in the uptake of particulate antigen could therefore be an important modulator of the immune response.

Several studies have shown a negative correlation between both acute (Fukui et al., 1997; Gazda et al., 2003) and chronic (Fleshner et al., 1989; Laudenslager et al., 1988; Moynihan et al., 1990) stress events and the antibody response after parenteral immunization. In the present study of chronic stress with oral immunization, there were only minor changes in the systemic antibody response. This may be due to differences in the development of the immune response after oral and parenteral immunization (Bouvet et al., 2002). Moreover, there is only weak evidence supporting a stress-induced reduction of the systemic antibody response for primary immune responses (Cohen et al., 2001), as was the case in our study. M-cell-mediated antigen uptake is, via isotype switching of B cells to IgA-producing plasma cells, predominantly associated with a response involving mucosal secretion of s-IgA (Kraehenbuhl and Neutra, 2000; Ogra et al., 2001), and we found higher levels of total s-IgA in the feces of stressed vs. non-stressed rats, suggesting a more pronounced local mucosal response. On the other hand, stress has previously been shown to reduce salivary s-IgA secretion in humans (Stone et al., 1987, 1993) and animals (Guhad and Hau, 1996). The higher levels of intestinal total s-IgA could thus be a consequence of non-specific stress-induced barrier

dysfunction and an inflammatory response (Soderholm et al., 2002).

The enhanced immune response seen in the cellular arm but not in the humoral arm of the immune response to HSA may also be an effect of differences in the propensity to develop Th1 and Th2 responses, as a number of host and antigen factors influence the Th1/Th2-balance (Liew, 2002). In our study the Th1/Th2 profile (based on the IgG subclass profile in serum) did not show any influence of stress, whereas the increased IFN- γ /IL-4 ratio in FAE may suggest skewing toward a Th1 cell profile. The differences seen between the cellular and humoral arms may, thus, reflect different compartments of the immune system: mucosa and skin vs. blood. However, there is a large inter-animal difference in the stress response, which makes it difficult to draw strong conclusions. Also, a previous study (Yang et al., 2006) on oral immunization with a soluble antigen together with i.p. adjuvant treatment, sensitization with a Th2-profile was seen: production of antigen-specific IgE-antibodies, recruitment of mast cells and eosinophils, increased expression of IL-4. The differences between these studies may be explained by different chemical characteristics and nature of the antigens used (soluble antigen vs. particle-conjugated antigen) and different routes of uptake (villous epithelium vs. FAE) (Larhed et al., 2004), and thereby different immunological response patterns. Further studies are needed to fully elucidate stress-induced changes in the mucosal immune response patterns to luminal antigens.

In conclusion, this study showed that chronic stress affected the cell-mediated immune response after oral immunization with particulate antigens. This could be due to the increased antigen uptake in the FAE altering the numbers of DCs within the lymphoid follicles, the redistribution of CD8⁺ lymphocytes and the Th1-skewing of the local immune response. Our findings may have implications for disturbances in the induction of tolerance in allergic and inflammatory diseases, as well as for the designing of oral vaccines.

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