

Postnatal Life Events Affect the Severity of Asthmatic Airway Inflammation in the Adult Rat¹

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Genetic and hygienic factors influence susceptibility to asthma. In autoimmune and inflammatory diseases, additional effects of the psychosocial environment have been demonstrated that might also play a role in asthma. In this study, the impact of different early postnatal stressors on an OVA-induced model of asthma was tested in adulthood. Fischer 344 rats were subjected to either repeated handling stimulation (HA), maternal separation (MS), or were left undisturbed in their first 4 wk of life. Behavioral differences were characterized at the age of 4 mo. At 5 mo of age, immunological cellular and serologic changes were investigated and experimental asthma was induced. Results show significantly increased exploratory behavior and reduced anxiety in HA rats compared with MS and controls. Without further behavioral or immunological challenges, HA animals exhibited an increased *ex vivo* NK cell cytotoxicity but no other obvious immunological differences. After induction of asthma, in contrast, MS animals exhibited proinflammatory effects in leukocyte subset composition including increased eosinophil numbers, whereas levels of IgE and the allergy-specific cytokine IL-13 were reduced compared with HA. There was a most remarkable increase of adrenocorticotropin in HA animals, comparing pre- to postchallenge plasma levels. These data demonstrate for the first time that early postnatal stimulative or adverse experiences exert long-lasting changes of the “neuroendocrinoimmune” interface in adulthood, resulting in either protective or aggravating mechanisms in allergic airway disease. Thus, in addition to genetic and hygienic factors, nongenetically acquired individual differences contribute to the pathobiology of asthma. *The Journal of Immunology*, 2008, 180: 3919–3925.

Nongenetic environmental factors influencing the incidence of asthma and allergies have been investigated, along with other risk factors such as family history of atopic diseases (1). In particular, the so-called “hygiene hypothesis” suggests that the decrease of Th1-associated infections such as those caused by mycobacteria or viruses might lead to an overexpression of a Th2-cytokine distribution pattern resulting in an increased susceptibility to atopic diseases (2). Interestingly, the prevalence of hay fever and atopic sensitization increased significantly between 1991 and 1992, and 1995 and 1996, in the eastern part of the country after reunification of western and eastern Germany, being interpreted as supportive for the hygiene hypothesis (3). However, no such changes were found in the prevalence of asthma, asthma-related symptoms, or bronchial hyperresponsiveness. The children investigated were

born about 3 years before reunification and were therefore exposed to western living conditions only after their third birthday. Thus, factors operating very early in life may be particularly important for the acquisition of childhood asthma, whereas the development of atopic sensitization and hay fever may also be affected by environmental factors occurring beyond infancy.

A number of studies have indicated implications of the maternal-neonatal neuroimmune interface for inflammatory and stress-related diseases (4, 5). Rodent models investigating the importance of maternal factors in the development of endocrine and immune regulation have largely used prolonged maternal separation (MS)³/deprivation or short separation (handling stimulation (HA)) paradigms (5). For instance, in experimental allergic encephalomyelitis—an animal model for multiple sclerosis—the clinical signs of the disease increased after MS (6). However, postnatal HA did not alter the susceptibility to another autoimmune condition, adjuvant-induced arthritis (7). Regarding the immense increase in atopic diseases such as allergic asthma (8), it would be of great value to know whether immunobiological evidence for specific adverse experiences very early in life can be elucidated as a pathogenetic factor for the development and course of asthmatic airway disease.

In the current study, we used established rat models integrating two opposing experimental paradigms of postnatal stress (HA, MS), and collected behavioral, neuroendocrinological, and immunological readouts throughout later adult life of the individuals. In the rat asthma model, T cells are known to play a major role in the promotion of airway eosinophilia as well as an isotype switching

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³ Abbreviations used in this paper: MS, maternal stimulation; HA, handling stimulation; CO, control; HPA, hypothalamic-pituitary-adrenal; SI, social interaction; i.t., intratracheal; ACTH, adrenocorticotropin; BAL, bronchoalveolar lavage.

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to IgE via the secretion of several type 2 cytokines, such as IL-4, IL-5, IL-13 (9). In addition, CD26 (dipeptidyl peptidase 4) is, among several other functions, an activation marker of T lymphocytes. In a previous study, we were able to identify CD26 in various rat strains including Fischer 344 (F344) rats as a necessary prerequisite for the induction of allergic airway inflammation (10). This "orchestra" of responsible factors leads to airway narrowing resulting in the typical clinical pattern of asthma symptoms. Our aim was to find out whether postnatal exposure to different stressors, representing experimental paradigms for specific nongenetic factors acting early in life and being different from antigenic hygienic factors, can modulate asthmatic airway inflammation in adult genetically identical inbred rats. Such "artificial life histories" in rodents provide a novel, well-controlled approach to a more comprehensive understanding of the potential role of noninfectious and nongenetic factors that modulate disease susceptibility of the individual and which are extremely difficult to detect in epidemiological studies in humans. The hypothesis is that these postnatal stressors induce long-lasting changes in the adaptive capacity of the individual neuroendocrinimmune network at various levels.

Materials and Methods

Animals and selection of rat strain

Offspring of 18 (experiment 1) and 14 (experiment 2) time-mated primiparous Fischer F344 females were born and kept in a colony room of the Central Animal Laboratory (Hannover Medical School) under pathogen-free conditions under a 12-h light, 12-h dark cycle at 24°C with food and tap water available ad libitum. Only male offspring were used for the present study. Animals were microbiologically monitored according to Federation of European Laboratory Animal Science Association recommendations (11). All research and animal care procedures were approved by the Government of Lower Saxony (Hannover, Germany), and performed in compliance with international animal welfare standards.

Presently, Brown Norway (BN) rats represent the rat strain preferentially used in research using models of experimental asthma. However, because it has been documented that BN rats—depending on the provider—can show granulomatous pneumonia spontaneously, this may greatly modify their predisposition for asthma. Because, furthermore, the recruitment of T cells to the lungs depends on the expression of CD26, F344 rats represent a highly relevant rat strain, in particular because CD26-deficient substrains are available (10, 12). In addition, the F344 strain might be a relevant alternative as their asthmoid-like response has recently been characterized in detail (13), demonstrating hyperreactive airways compared with other strains and exhibiting greater contractile responses along with greater density of airway smooth muscles compared with Lewis rats (10). An important mechanism mediating the effects of postnatal experiences on behavioral and physiological responses in adult rats is found in persistent changes of the hypothalamic-pituitary-adrenal (HPA) axis (4, 5, 14), which exhibits genetically determined differences between rat strains and which is highly responsive in F344 rats in contrast to Lewis rats (6, 15).

Experiments and postnatal manipulations

In experiment 1, we randomly allocated newborn male Fischer F344 rats ($n = 26$) derived from 18 litters to the different experimental conditions (MS, $n = 9$, six litters; HA, $n = 10$, six litters) as well as to the control group (CO, $n = 7$, six litters). These animals were used to characterize behavioral differences at the age of 3–4 mo and susceptibility to experimental asthma in the age of 5 mo. Experiment 2 was conducted in male F344 rats ($n = 23$) kept and reared at identical conditions. These animals were derived from 14 litters (MS, $n = 8$, five litters; HA, $n = 8$, five litters; CO, $n = 7$, four litters). In these animals we screened blood, thymus, and spleen by immunohistology and FACS analysis at the age of 5 mo and also performed assays on blood T cell proliferation and ex vivo splenic NK cell cytotoxicity to study whether these early life experiences primarily affect the immune system of the rats.

During the MS procedure, a dam was placed into a separate cage for 2 h every day, while the pups remained in the nest on their own. This procedure serves as a paradigm for adverse early life experience comprising repeated MS/deprivation (16). In the HA group, each pup, one by one, was placed into a separate cage filled with standard bedding for 5 min every day until being reunited with their mothers. This procedure is regarded as a

paradigm for beneficial stimulation early in life comprising repeated exposure to novelty, tactile, and kinesthetic stimulation of pups (16). MS and HA procedures were performed once daily until weaning on postnatal day 28. In CO, dams and their litters were left undisturbed. All procedures were conducted by the same examiner and at the same time of day.

Behavioral tests of emotionality and anxiety

At the age of 3–4 mo, we subjected the animals from experimental set 1 to a test battery for activity, anxiety, and exploratory-like differences, using established assays (16). Here, only the social interaction (SI) test is described in further detail, because the other behavioral tests revealed consistent results. In the SI test, a single experimental rat was exposed to another individual from the same experimental group (e.g., both rats from HA) but from another home cage to provide an intense social stimulus (16, 17). Tests were separated over several days to avoid cohort removal effects in cages with three animals (17). SI testing was conducted in the light phase of the 24-h cycle using a specially designed 50 × 50 × 40-cm box. The observation period was 10 min. The dependent variable was the time spent in SI such as sniffing, following, crawling, and grooming the partner measured by a stopwatch and counted for pairs of rats as previously described (17, 18). Under these conditions, an increase of SI time is interpreted as an anxiolytic-like response and vice versa.

Sensitization and allergen challenge in the asthma disease model

At 5 mo, the animals (experiment 1) were immunized with OVA in a time frame of 2 wk and consecutively challenged by intratracheal (i.t.) instillation of the same Ag according to (10). In brief, sensitization was performed with 1 mg of OVA (Sigma-Aldrich) and 200 mg of Al(OH)₃ (Sigma-Aldrich) in 1 ml 0.9% (sterile, pyrogen-free) NaCl applied s.c. As second adjuvant, concentrated preparations of 5×10^9 heat-killed *Bordetella pertussis* (donated by the manufacturers, Chiron Behring) in 0.4 ml of 0.9% NaCl were given i.p. at the same time. These injections were repeated 7 days after the first sensitization. After another 7 days, the animals were challenged with 300 μ l of a 0.5% OVA/saline solution. For this i.t. instillation, a short combined isoflurane oxygen inhalation anesthesia was performed, and the rats were suspended in a hanging position by a rubber band fixed to the incisor teeth of the upper jaw (19). The trachea was intubated via the oral cavity, and the lungs were blown up with air before OVA instillation to check the correct position of the tube.

Dissection of animals, and isolation of bronchoalveolar lavage (BAL) and lung tissue leukocytes

The animals were dissected under isoflurane anesthesia 22 ± 2 h after i.t. challenge as described by Kruschinski et al. (10). Briefly, the abdominal wall was opened and the animals were euthanized by aortic exsanguination. A cannula was inserted into the trachea in situ and the lungs were lavaged with portions of 5 ml of cold (4°C) NaCl. The fluid was retrieved by gentle aspiration and this procedure was repeated 10 times. The recovery of fluid was over 90% in all animals. The BAL was pooled, centrifuged ($400 \times g$, 10 min), and the cell pellet was resuspended in 1 ml of PBS (Seromed; containing 1% BSA (Merck) and 0.1% sodium azide (NaN₃; Sigma-Aldrich). For lung cell extraction, a mechanical disaggregation method was used. The trachea, main bronchi, and hilar lymph nodes were removed from the rest of the lung tissue, and the left lung was used for cellular analysis. The complete lung tissue was disaggregated by passing it through a metal sieve with two rounded tweezers and rinsing with 40 ml of PBS. Cells were separated from debris by passing through a 75- μ m nylon mesh and centrifuged ($400 \times g$, 10 min). The cell pellet was finally resuspended in 1 ml of PBS. The right lower lobe was kept at -80°C until use for PCR.

Cell counts, stainings for eosinophils, and FACS analysis

Leukocyte numbers were determined via staining with Türk's solution (Merck) in a Neubauer counting chamber. The eosinophil cell count was assessed on slides prepared by centrifuging 1×10^5 cells in anticoagulant (100 ml of 0.9% NaCl; 100 mg EDTA; 5 g of BSA; pH 7.2) for 8 min at $800 \times g$ in a cytospin centrifuge (Shandon). After a Pappenheim (combined May-Grünwald/Giemsa) staining (Riedel de Haen), eosinophils were identified under the light microscope at $\times 1000$ magnification. At least 1000 cells were differentiated on each slide.

For FACS analysis, cells were transferred to a microtiter plate (Greiner; with $\sim 1 \times 10^6$ cells in each well) and washed twice with 100 μ l of PBS, also including incubation with 50 μ l of human serum (1:10) for 5 min (as described in Ref. 19). For details of the specificity and characteristics of the Abs used, see Ref. 20. A PE-conjugated secondary anti-mouse Ab was used (κ PE; Dianova) to detect the primary Abs: mAb OX61, mAb OX8,

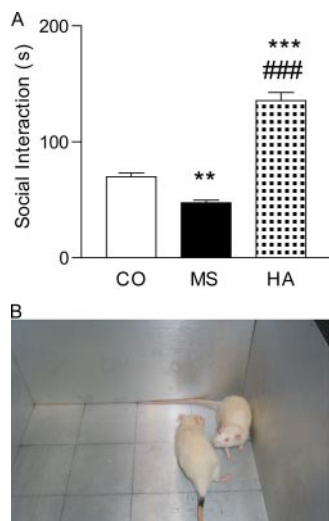


FIGURE 1. SI test of anxiety. Comparison of CO, HA, and MS animals as time spent in social interaction with another rat of the same age from the same treatment group (A). Each bar represents the mean $\pm$ SEM; **, $p < 0.01$; ***, $p < 0.001$ indicate significant differences as compared with CO; ###, $p < 0.001$ significant differences between treatment groups. The procedure is shown in B.

mAb ED1, and mAb RP1. For double and triple stainings, additional mouse anti-rat FITC-conjugated primary mAbs (mAb R73, mAb Ox12, mAb 10/78), and biotinylated Abs (mAb Ox39, mAb W3/25) were used (all Serotec). These Abs were traced with streptavidin Red 670 (Invitrogen Life Technologies). All incubations were done for 30 min at 4°C in 50- μ l portions with alternating washing procedures. Finally, the cells were washed three times, resuspended in 200 μ l of PBS, and analyzed on a FACScan flow cytometer (BD Biosciences) focusing on the mononuclear cell gate. At least 20,000 events were counted in the previously defined cell gate, and the percentage was calculated for each cell subset.

Adrenocorticotropin (ACTH)-, cytokine-, and IgE-ELISA

Blood plasma was obtained at two different time points. At the age of 4 mo (before OVA sensitization), the rats were put under short isoflurane anesthesia for puncture of the retro-orbital sinuses. The second blood specimen was taken from the abdominal aorta at the time point of exsanguination after OVA challenge. EDTA-plasma samples were kept at -80°C until use. ACTH, IL-4, IL-6, IL-10, IL-13, and IgE were measured by means of ELISA using previously described standard techniques for hormones and cytokines according to Straub et al. (21) and for IgE according to Kruschinski et al. (10). These parameters were selected as they represent a rational based cross-section for screening on differences in allergic-like and stress-like responses (10, 13, 16).

Semiquantitative RT-PCR

Total RNA from rat lungs was isolated with TriFast-Reagent (peqLAB) and 3 μ g of RNA was reverse transcribed using Superscript reverse transcriptase (Invitrogen Life Technologies), random primers and deoxynucleoside triphosphates. The reverse transcriptase products were amplified with TaqDNA polymerase (Invitrogen Life Technologies). PCR was performed for IL-1 β (34 cycles, forward primer: 5'-TGT CAC TCA TTG TGG CTG TG-3', reverse primer: 5'-CTG ATG TAC CAG TTG GGG

AAC-3', 526 bp), for IL-6 (30 cycles, forward primer: 5'-TGT TGT TGA CAG CCA CTG GC-3', reverse primer: 5'-TTT CAA GAT GAG TTG GAT GGT C-3', 533 bp), for IL-10 (33 cycles, forward primer: 5'-GCT CAG CAC TGC TAT GTT GAG TG-3', reverse primer: 5'-CAC CTG CTC CAC TGC CTT GC-3', 419 bp), for IL-13 (34 cycles, forward primer: 5'-ATG GCA CTC TGG GTG ACT G-3', reverse primer: 5'-GGC CAT TCA ATA TCC TCT GG-3', 277 bp), for TNF- α (32 cycles, forward primer: 5'-TCC AGT GAC AAG CCT GTA GC-3', reverse primer: 5'-TGG TCC CCC TTC TCC AG-3', 368 bp), and normalized to the expression of 18S-rRNA (19 cycles, forward primer: 5'-CCT GCG GCT TAA TTT GAC TC-3', reverse primer: 5'-GGC CTC ACT AAA CCA TCC AA-3', 510 bp). All oligonucleotides were obtained from MWG-Biotech. PCR products were separated by 1% agarose gel electrophoresis and quantified densitometrically using a Gel Doc image analysis system (Bio-Rad).

FACS analysis and immunohistochemistry of leukocyte subpopulations in blood, thymus, and spleens

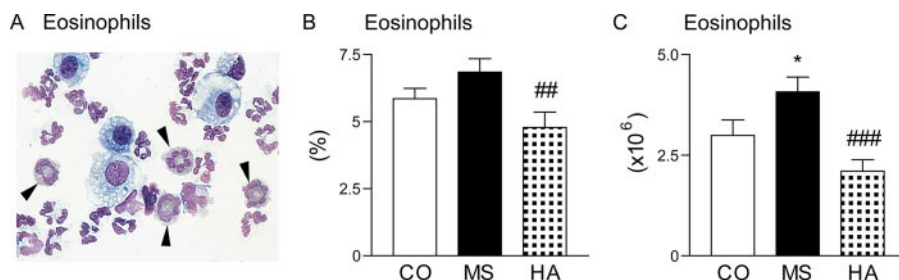
In experiment 2, analysis of leukocyte subpopulations in blood, thymus, and spleens by FACS analysis was conducted as described above (19). In addition, immunohistochemistry was performed using mAbs characterizing NK cells (mAb 3.2.3), CD4 $^{+}$ T cells (mAbs R73 $^{+}$ W3/25 $^{+}$), and CD8 $^{+}$ T cells (mAbs R73 $^{+}$ OX8 $^{+}$), B lymphocytes (mAbs HIS14 or OX33), monocytes (mAb ED1), and granulocytes (mAb RP1). The characterization of these mAbs has previously been summarized (20). For immunohistochemistry, two consecutive alkaline phosphatase antialkaline phosphatase stainings were performed as previously described (22, 23). Immunohistochemistry was used in addition to FACS because this approach provides insights into morphology of organs and local distribution of leukocyte subsets.

NK cell cytotoxicity in splenocytes and blood T cell proliferation assay

In experiment 2, NK cytotoxicity was measured in classical ^{51}Cr -release assays using splenocytes and MADB106 target cells (12, 24), which were derived from standard cell culture conditions, as previously described (23). Leukocyte numbers were determined using a Coulter cell counter and the splenocyte concentrations adjusted to 6×10^6 cells/ml with supplemented RPMI 1640 containing 10% FBS. Splenocytes, prepared through Ficoll-Hypaque gradient, were used as effectors and E:T ratios of 12.5:1, 25:1, 50:1, 100:1 were obtained. Coincubation of effector and target cells was conducted either for 4 h with addition of 1000 U/ml IL-2 (EuroCetus) or for 18 h of incubation, respectively. Control wells containing only labeled targets were also plated to determine the spontaneous release. For determination of maximal possible release, the targets in one set of control wells were lysed with Triton X-100. The spontaneous release was always $<10\%$ of the maximum release. Plates were centrifuged for 4 min before incubation (37°C , 5% CO_2) and again before harvesting 75 μ l of the supernatant for determining ^{51}Cr release in a gamma counter. The specific cytotoxicity was calculated by means of the following formula: ((experimental release - spontaneous release)/(maximal release - spontaneous release)) $\times 100$.

For determination of lymphocyte proliferation, PBMC were isolated from blood via centrifugation on Ficoll gradient (Ficoll Paque Plus; Amersham). Viable cells were counted, washed, and the proliferation assays were conducted in 96-well flat-bottom plates using 2×10^5 cells/well were cultured in the presence of 0.2 μ g of $\alpha\beta$ TCR Ab (plate bound). Cells stimulated with 1 μ g of Con A served as positive controls. After 5 days of incubation, the proliferation rate was quantified by BrdU incorporation and detected with a specific colorimetric BrdU cell proliferation ELISA (Roche Molecular Biochemicals), according to the manufacturer's instructions. BrdU incorporation was measured using an ELISA reader (PowerWave $^{\text{XS}}$ Universal Microplate Spectrophotometer; BioTek Instruments) at 370 and

FIGURE 2. Effects of OVA challenge on the percentage of eosinophils (B) and absolute eosinophil numbers (C) in the BAL of CO, HA, and MS animals. Each bar represents the mean $\pm$ SEM; *, $p < 0.05$ indicate significant differences as compared with CO; ##, $p < 0.01$ and ###, $p < 0.001$ significant differences between treatment groups. Arrowheads in A indicate eosinophils as they present on BAL cytopots.



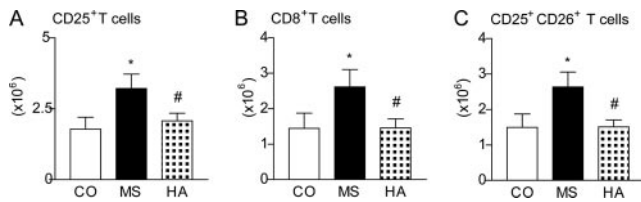


FIGURE 3. Effects of OVA challenge on CD25⁺ T cells (A), CD8⁺ T cells (B), and absolute CD25⁺CD26⁺ T cells (C). Absolute cell counts in the BAL and lung compartment of CO compared with HA and MS animals determined via FACS analysis. Each bar represents the mean $\pm$ SEM; *, $p < 0.05$ indicate significant differences as compared with controls; #, $p < 0.05$ significant differences between treatment groups.

492 nm as reference wavelengths. Results were expressed as absorbance rates ($A_{370\text{ nm}} - A_{492\text{ nm}}$).

Statistics

Statistical analysis was performed using one-way ANOVA with the factor “experience (CO, MS, or HA)” followed by the Fisher-protected least-significant difference test for post-hoc comparison, if appropriate. For the neuroendocrine data and NK cell-mediated-specific cytotoxicity, a two-way ANOVA for repeated measurements was applied. In the figures, asterisks and number signs are used to indicate a statistically significant difference derived from corresponding post-hoc comparisons vs CO (p values: *, <0.05 ; **, <0.01 ; and ***, <0.001) or between MS and HA conditions (p values: #, <0.05 ; ##, <0.01 ; or ###, <0.001). All data are given as arithmetic mean $\pm$ SEM.

Results

Less signs of anxiety in HA

In the SI test of anxiety, HA rats showed increased time spent in SI compared with CO and MS ($p < 0.0001$; Fig. 1A). Furthermore, rats of the MS group showed significantly less time spent in SI compared with CO.

Increased BAL eosinophilia in MS 22 h after OVA challenge

The percentage of eosinophils in the BAL was significantly higher in MS compared with HA ($p = 0.03$; Fig. 2B). Absolute eosinophil numbers showed significantly higher cell counts in MS animals compared with both the CO and the HA group ($p = 0.002$; Fig. 2C). Overall, HA animals showed the lowest level of eosinophil cell counts of all three groups. No other differences were found in leukocytes subsets of the BAL (data not shown).

Increased lung tissue T cell subsets in MS 22 h after OVA challenge

The finding of an allergen-induced eosinophilia in the bronchoalveolar space was verified in the lung via FACS analysis resulting in the same distribution pattern of highest levels in MS animals in all T cell-relevant surface markers. Here, as an example of significant effects, the T cell subtypes CD25⁺ ($p < 0.05$; Fig. 3A),

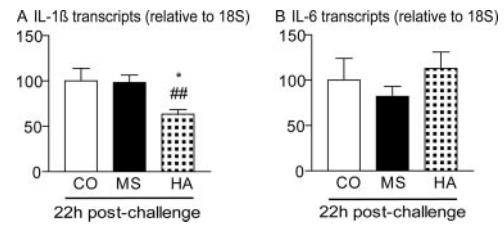


FIGURE 5. Cytokines detected in the lung parenchyma using PCR, demonstrated as the IL-1 β :18s ratio (A) and IL-6:18s ratio (B). Each bar represents the mean $\pm$ SEM; *, $p < 0.05$ indicate significant differences as compared with controls; ##, $p < 0.01$ significant differences between treatment groups.

CD8⁺ ($p < 0.05$; Fig. 3B), and CD25⁺CD26⁺ ($p = 0.04$; Fig. 3C) are shown.

IL and IgE-blood plasma levels in the different postnatal procedures

IL-13 levels in the blood plasma significantly increased 22 h after OVA challenge in HA compared with CO ($p < 0.05$; Fig. 4A). For IL-10, no differences were found (data not shown). IL-6 pre- and postchallenge levels (Fig. 4B) exhibited a decrease over the clinical course ($p < 0.001$): a significant reduction of 26% was seen in CO, and also in MS a 22% reduction was demonstrated. No significant reduction was found in HA. IL-4 levels were not detectable in any of the animals investigated (data not shown), which is consistent with the literature at that early time point (25). Similar to IL-13, significantly higher IgE levels in HA compared with MS were found 22 h after OVA challenge ($p = 0.03$; Fig. 4C).

Lung tissue cytokine transcripts

Cytokine mRNAs measured in the lung parenchyma showed significant differences only for IL-1 β . IL-1 β concentrations were significantly reduced in HA compared with both the CO and the MS group ($p = 0.01$) (Fig. 5A). The other cytokines studied (IL-6, IL-10, IL-13, TNF- α , and IFN- γ), did not reveal significant results between the different experimental groups. However, transcript levels of IL-6, similar to IL-6 measured in the blood plasma, failed to show a significant increase in HA (Fig. 5B).

Lowest basal, but mostly increased ACTH levels in HA after OVA challenge

ANOVA for repeated measurements revealed a significant interaction of both factors ($p = 0.002$), indicating both a “treatment” and a “time” specific effect. At baseline, HA rats showed significantly lowest ACTH levels compared with MS (one-way ANOVA; $p = 0.04$; Fig. 6A) as well as lower levels than CO rats. Comparing pre- to postchallenge, in HA ACTH levels were most

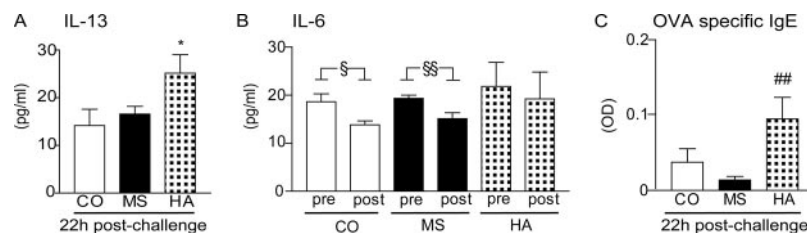


FIGURE 4. Blood plasma levels of IL-13 (A), IL-6 (B), and OVA-specific IgE (C) in asthma dependent on the different postnatal experiences. For IL-6, pre- and postchallenge levels are shown. Each bar represents the mean $\pm$ SEM; *, $p < 0.05$ indicate significant differences as compared with controls; \$, $p < 0.05$ and §§, $p < 0.01$ indicate significant differences between pre- and postchallenge, and ##, $p < 0.01$ significant differences between treatment groups.

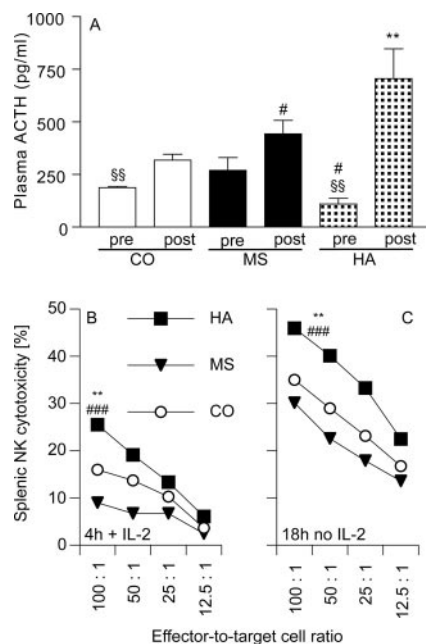


FIGURE 6. ACTH levels pre- and post-OVA challenge comparing CO, HA, and MS (A) and splenic NK cell cytotoxicity after 4 h of coincubation under addition of IL-2 across different E:T (MADB106 tumor cells) ratios (B) as well as 18 h after coincubation (C). Each bar/symbol represents the mean $\pm$ SEM; **, $p < 0.01$ indicate significant differences as compared with controls; #, $p < 0.05$ and ###, $p < 0.001$ significant differences between treatment groups, and §§, $p < 0.01$ significant differences between pre- and postchallenge.

impressively increased. Also in CO, ACTH levels were significantly increased, whereas in MS the increase was not significant. As a result, after OVA challenge, ACTH levels were significantly higher in HA compared with CO as well as MS (one-way ANOVA, $p = 0.02$).

No differences in immunohistology/FACS analysis of blood, thymus, spleen, and lymphocyte proliferation but higher NK cytotoxicity in HA animals

To investigate whether these early life experiences primarily affect the immune system of the rats, we screened blood, thymus, and spleen by immunohistology and FACS analysis and also measured blood T cell proliferation and ex vivo NK cell cytotoxicity in a separate set of animals that were otherwise experimentally naive (i.e., no behavioral assays, immunization, and OVA challenge; experiment 2). We found no differences in immunohistology or composition of leukocyte subsets after screening blood, spleen, and thymus of CO, MS, and HA animals (data not shown). Also, T cell proliferation did not differ among groups (data not shown). Only ex vivo NK cytotoxicity differed between groups with significantly higher NK cytotoxicity in the HA group. NK-specific lysis of MADB106 tumor cells using a classical ex vivo NK cell functional assay is shown in Fig. 6, B and C. Two-way ANOVA showed a significant effect of the factors "postnatal experience" ($p < 0.001$) and "E:T ratio" ($p < 0.05$) in the 4-h (Fig. 6B) and the 18-h (Fig. 6C) assays. Post-hoc analyses revealed in both assays that in the CO and MS animals, NK cell-mediated lysis against MADB106 tumor targets was significantly lower compared with HA rats.

Discussion

Our results from studying artificial biographies in rats with different postnatal experiences demonstrate for the first time that spe-

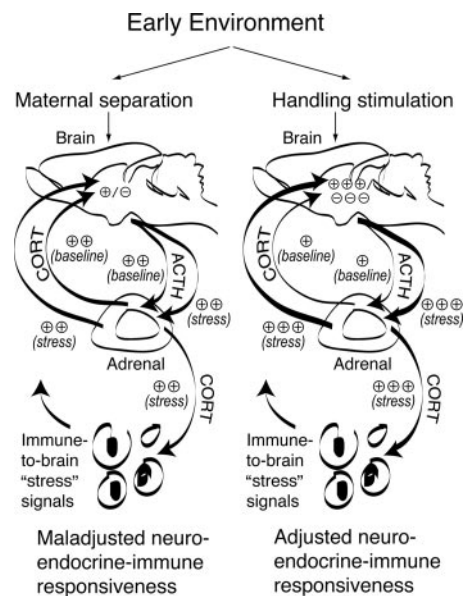


FIGURE 7. HPA axis responsiveness after programming by different postnatal experiences: HPA activity is driven by pulsatile release of corticotropin releasing hormone, vasopressin, and other peptides within the hypothalamus. The secretagogues control the synthesis and release of pituitary corticotropes including the release of pituitary ACTH, which promotes adrenal corticosteroid (CORT) secretion. Basal activity as well as intensity of the stress response is controlled by feedback regulation. CORT hormones feedback via mineralocorticoid receptors maintaining basal HPA activity and via glucocorticoid receptors promoting negative feed back inhibition of stress-induced HPA activation (14). Postnatal stressors specifically modulate central feedback regulation within the brain (hippocampus) resulting in individual differences of HPA responsiveness (4, 5, 28). Maternal separation (left) is believed to deteriorate negative feedback regulation (indicated by "(+)(-)" in the brain, left side) while handling stimulation improves it (indicated by "(+)(+)(+)(-)(-)(-)" in the brain, right side). This results in higher basal but blunted immune-stress-induced ACTH and CORT levels in individuals with a history of maternal separation (indicated by "(+)(+)" at the arrows to/from the brain, adrenal, and immunocytes), while animals with a history of repeated postnatal handling stimulation show lower basal but increased stress-induced ACTH/CORT levels (indicated by "(+)" and "(+)(+)(+)" at the arrows to/from the brain, adrenal, and immunocytes). Identical immune-to-brain "stress" signals (e.g., cytokines) may therefore elicit either adjusted or maladjusted neuro-endocrine-immune responses of the individual including but not limited to the HPA axis responsiveness during asthma.

cific postnatal life events lead to a modification of allergic airway inflammation in adult individuals. Repeated exposure to novelty resulted in decreased signs of cellular inflammatory reactions. In contrast, repeated separation from the mother resulted in increased inflammatory signs. The classical immunological features of allergic asthma, such as eosinophilia, T cell recruitment, and typical cytokine responses, were affected by the different rearing conditions, which per se were also associated with specific changes of the behavioral and neuroendocrine phenotype in adulthood. Thus, in addition to genetic factors and early Ag exposure (2), we provide direct experimental evidence for a specific and opposing role of either adverse or beneficial life events in the early postnatal period as being relevant for the course of asthma in adulthood. The link between these postnatal experiences and the later susceptibility to asthma in adult individual rats is imprinted by life-long persisting alterations of the behavioral and the neuroendocrine stress responsiveness (Fig. 7).

Inflammation is a potent stimulus that elicits HPA activation. The HPA axis becomes activated when neurons in the paraventricular nucleus of the hypothalamus secrete corticotropin-releasing hormone and other mediators. This induces secretions of a pulse of ACTH from the anterior pituitary gland. The ACTH signal is carried through the peripheral circulation to the adrenal glands, which synthesize and release cortisol/corticosterone from the zona fasciculata of the adrenal cortex. In nervous, muscle, lymphoid, and other tissues, corticosterone binds to an intracellular glucocorticoid receptor, which is the starting point for a number of critical signaling pathways. On lymphocytes, glucocorticoid receptor activation regulates expression of IL-4, -5, and -13 following allergen exposures. Basal HPA axis activity as well as intensity of (immune) stress-induced HPA axis activation are regulated by feedback regulation. Corticosteroid hormones feed back via mineralocorticoid receptors maintaining basal HPA activity and via glucocorticoid receptors promoting negative feedback inhibition of stress-induced HPA activation (Fig. 7). Postnatal stressors specifically modulate central feed back regulation within the brain resulting in individual differences of HPA responsiveness (5). Maternal separation is believed to deteriorate negative feedback regulation while handling stimulation improves it. This may result in higher basal but blunted immune stress-induced ACTH and corticosterone levels in individuals with a history of maternal separation, while animals with a history of repeated postnatal handling stimulation show lower basal but increased stress-induced ACTH/corticosterone levels (26–28).

Also in the present study, HA animals exhibit lowest, CO intermediate, and MS highest basal ACTH levels. Following Ag challenge, however, ACTH concentrations were significantly higher in HA, indicating the most intensive activation of the HPA axis. This evidence for a more dynamic responsiveness of the HPA axis could also explain why the inflammatory responses to the allergic airway disease are more tightly controlled in HA. IL-6 is one candidate mediator, which might serve as an immune-to-brain “stress” signal also in the present experiments (Fig. 7). IL-6 is known to be a potent activator of the HPA system and is secreted under stress conditions (29). The IL-6 release from macrophages is furthermore under tight control of the activity of the sympathetic nervous system and related neurotransmitters, which in turn are released by stress (30). However, because IL-6 levels do not differ prechallenge between groups and show under poststress circumstances only a significant decrease in MS and CO groups, these findings are not indicative for an activation of the HPA system by IL-6 but instead may hint to additional differences in the sympathetic response (30) in these animals.

It is a widely accepted hypothesis that severe postnatal or acute experiences of stress can induce a persistent state of hyporesponsiveness of the HPA axis. Similarly, some populations with post-traumatic stress disorder show lower mean basal cortisol levels throughout the circadian cycle (31). Furthermore, a state of stress-induced HPA hyporesponsiveness in some research subjects has been associated with other inflammatory disorders such as atopic dermatitis (32). Similarly, we found no differences between pre- and posttreatment ACTH levels in the MS group, which might indicate a hyporesponsive HPA axis ultimately leading to exacerbations of asthma in MS individuals that underwent repeated postnatal “chronic” stress with a potentially long-lasting and persisting impact on the immune regulatory competence of the individual (Fig. 7).

To study whether these early life experiences primarily affect the immune system of the rats, which then would not develop an appropriate inflammatory response following challenge with OVA, we have performed an additional experiment using an identical

experimental design but omitting behavioral testing as well as immunization and induction of asthma. Screening of blood, thymus, and spleen by immunohistology and FACS analysis as well as T cell proliferation revealed no differences among groups. Only testing *ex vivo* splenic NK cytotoxicity revealed significantly higher NK cytotoxicity in HA animals. As this finding might well be the result of a differential *in vivo* stress response mediated via sympathetic neurotransmitters such as noradrenaline and neuropeptide Y (12, 16, 20, 22–24, 30), to this end, we conclude that the postnatal stressors used in the present study do not primarily affect the immune system of the rats. However, such a conclusion cannot be comprehensive and might differ in the case of other postnatal experiences such as immune “stress” by early postnatal exposure to endotoxin (5, 7, 16). Also, other components of the immune system, which were not studied here, may potentially be affected by postnatal experiences. In terms of the postnatal development of immune regulation, prospective seroepidemiological studies showed that the newborn period is dominated by Th2 reactivity in response to allergens (31). It is also evident that the Th1 memory cells selectively develop shortly after birth (at 3–6 mo of age) and persist into adulthood in nonatopic subjects. For most children who become allergic or asthmatic, the polarization of their immune system into an atopic phenotype probably occurs during early childhood (33). A contributing factor for such a Th1-Th2 shift might be provided by the postnatal environment as exemplified in the present study. Furthermore, in the present study, HA animals were characterized by lower numbers of T cell subtypes such as CD25⁺CD26⁺ T cells. CD25 and CD26 are both known as activation markers of T cells (9, 34, 35). In recent studies, we were able to show that CD26-negative rat strains (12) exhibited a significant reduction of T cell numbers in the BAL compared with CD26-positive wild-type controls (10) and that a dose-dependent recruitment of activated CD4⁺CD25⁺CD26⁺Foxp3[−] T cell subpopulations is evident, which has not yet been described in asthma (13). Therefore, the present study illustrates an association of different postnatal stressor, CD26 expression, and T cell activation.

Interestingly, in the present results, IgE as well as IL-13 levels showed an inverse proportion relative to eosinophils and other leukocyte subsets. IgE concentrations were highest in HA animals that showed the least leukocyte subset changes in the lungs. As shown for ACTH-levels, HA animals exhibited a remarkable ability of quick regulation and control mechanisms, e.g., to contain overwhelming immunoreactions against pathogens that might harm the organism (36). In a study by von Hörsten et al. (37), splenic B cell responses to classical Ags such as sheep RBC were increased in adult postnatally HA rats, whereas postnatal MS suppressed this response in adult individuals. These findings are consistent with the elevated IgE level of the HA animals in the present study and are not necessarily contradictory to a reduced eosinophilia, because Ab production and other components of an allergic immune response might be regulated more or less independently (38).

In conclusion, we provide direct experimental evidence in rats illustrating that susceptibility to asthma is influenced—apart from genetic and recently considered hygienic factors—by the postnatal psychosocial environment. Postnatal stressors representing paradigms of either beneficial or adverse life experience either protect or aggravate the course of experimental asthma in adult animals and are associated with persistent changes in the behavioral and neuroendocrine stress responsiveness as well as recruitment of the activated T cells. These findings will motivate interdisciplinary research on specific factors acting at the neuroendocrinoimmune interface and exerting persistent modulatory effects on the adaptiveness of the individual to allergic challenge.

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Disclosures

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References

- von Mutius, E. 2004. Influences in allergy: epidemiology and the environment. *J. Allergy Clin. Immunol.* 113: 373–379.
- von Hertzen, L., T. Klaukka, H. Mattila, and T. Haahtela. 1999. *Mycobacterium tuberculosis* infection and the subsequent development of asthma and allergic conditions. *J. Allergy Clin. Immunol.* 104: 1211–1214.
- von Mutius, E., S. K. Weiland, C. Fritzsche, H. Duhme, and U. Keil. 1998. Increasing prevalence of hay fever and atopy among children in Leipzig, East Germany. *Lancet* 351: 862–866.
- Liu, D., J. Diorio, B. Tannenbaum, C. Caldji, D. Francis, A. Freedman, S. Sharma, D. Pearson, P. M. Plotsky, and M. J. Meaney. 1997. Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. *Science* 277: 1659–1662.
- Shanks, N., and S. L. Lightman. 2001. The maternal-neonatal neuro-immune interface: are there long-term implications for inflammatory or stress-related disease? *J. Clin. Invest.* 108: 1567–1573.
- Stephan, M., R. H. Straub, T. Breivik, R. Pabst, and S. von Hörsten. 2002. Postnatal maternal deprivation aggravates experimental autoimmune encephalomyelitis in adult Lewis rats: reversal by chronic imipramine treatment. *Int. J. Dev. Neurosci.* 20: 125–132.
- Shanks, N., R. J. Windle, P. A. Perks, M. S. Harbuz, D. S. Jessop, C. D. Ingram, and S. L. Lightman. 2000. Early-life exposure to endotoxin alters hypothalamic-pituitary-adrenal function and predisposition to inflammation. *Proc. Natl. Acad. Sci. USA* 97: 5645–5650.
- Holgate, S. T. 2004. The epidemic of asthma and allergy. *J. R. Soc. Med.* 97: 103–110.
- Larche, M., D. S. Robinson, and A. B. Kay. 2003. The role of T lymphocytes in the pathogenesis of asthma. *J. Allergy Clin. Immunol.* 111: 450–463.
- Kruschinski, C., T. Skripuletz, S. Bedoui, T. Tschernig, R. Pabst, C. Nassenstein, A. Braun, and S. von Hörsten. 2005. CD26 (dipeptidyl-peptidase IV)-dependent recruitment of T cells in a rat asthma model. *Clin. Exp. Immunol.* 139: 17–24.
- Rehbinder, C., P. Baneux, D. Forbes, H. van Herck, W. Nicklas, Z. Rugaya, and G. Winkler. 1996. FELASA recommendations for the health monitoring of mouse, rat, hamster, gerbil, guinea pig and rabbit experimental units: report of the Federation of European Laboratory Animal Science Associations (FELASA) Working Group on Animal Health accepted by the FELASA Board of Management, November 1995. *Lab. Anim.* 30: 193–208.
- Karl, T., W. T. Chwalisz, D. Wedekind, H. J. Hedrich, T. Hoffmann, R. Jacobs, R. Pabst, and S. von Hörsten. 2003. Localization, transmission, spontaneous mutations, and variation of function of the DPPIV (dipeptidyl-peptidase IV; CD26) gene in rats. *Regul. Pept.* 115: 81–90.
- Skripuletz, T., A. Schmiedl, J. Schade, S. Bedoui, T. Glaab, R. Pabst, S. von Hörsten, and M. Stephan. 2007. Dose-dependent recruitment of CD25⁺ and CD26⁺ T cells in a novel F344 rat model of asthma. *Am. J. Physiol.* 292: L1564–L1571.
- De Kloet, E. R., E. Vreugdenhil, M. S. Oitzl, and M. Joels. 1998. Brain corticosteroid receptor balance in health and disease. *Endocrine Rev.* 19: 269–301.
- Stephan, M., F. Helfritz, R. Pabst, and S. von Hörsten. 2002. Postnatally induced differences in adult pain sensitivity depend on genetics, gender and specific experiences: reversal of maternal deprivation effects by additional postnatal tactile stimulation or chronic imipramine treatment. *Behav. Brain Res.* 133: 149–158.
- Breivik, T., M. Stephan, G. E. Brabant, R. H. Straub, R. Pabst, and S. von Hörsten. 2002. Postnatal lipopolysaccharide-induced illness predisposes to periodontal disease in adulthood. *Brain Behav. Immun.* 16: 421–438.
- Kask, A., H. P. Nguyen, R. Pabst, and S. von Hörsten. 2001. Factors influencing behavior of group-housed male rats in social interaction test: focus on cohort removal. *Physiol. Behav.* 74: 277–282.
- File, S. E., and P. Seth. 2003. A review of 25 years of the social interaction test. *Eur. J. Pharmacol.* 463: 35–53.
- Pabst, R., A. Lührmann, I. Steinmetz, and T. Tschernig. 2003. A single intratracheal dose of the growth factor Fms-like tyrosine kinase receptor-3 ligand induces a rapid differential increase of dendritic cells and lymphocyte subsets in lung tissue and bronchoalveolar lavage, resulting in an increased local antibody production. *J. Immunol.* 171: 325–330.
- Bedoui, S., S. Kuhlmann, H. Nave, J. Drube, R. Pabst, and S. von Hörsten. 2001. Differential effects of neuropeptide Y (NPY) on leukocyte subsets in the blood: mobilization of B-1-like B-lymphocytes and activated monocytes. *J. Neuroimmunol.* 117: 125–132.
- Straub, R. H., L. Konecna, S. Hrach, G. Rothe, M. Kreutz, J. Scholmerich, W. Falk, and B. Lang. 1998. Serum dehydroepiandrosterone (DHEA) and DHEA sulfate are negatively correlated with serum interleukin-6 (IL-6), and DHEA inhibits IL-6 secretion from mononuclear cells in man in vitro: possible link between endocrinosenescence and immunosenescence. *J. Clin. Endocrinol. Metab.* 83: 2012–2017.
- von Hörsten, S., A. Helfritz, S. Kuhlmann, H. Nave, T. Tschernig, R. Pabst, S. Ben-Eliyahu, D. Meyer, R. E. Schmidt, and C. Schmitz. 2000. Stereological quantification of carboxyfluorescein-labeled rat lung metastasis: a new method for the assessment of natural killer cell activity and tumor adhesion in vivo and in situ. *J. Immunol. Methods* 239: 25–34.
- Shingu, K., A. Helfritz, S. Kuhlmann, M. Zielinska-Skowronek, R. Jacobs, R. E. Schmidt, R. Pabst, and S. von Hörsten. 2002. Kinetics of the early recruitment of leukocyte subsets at the sites of tumor cells in the lungs: natural killer (NK) cells rapidly attract monocytes but not lymphocytes in the surveillance of micrometastasis. *Int. J. Cancer* 99: 74–81.
- Shingu, K., A. Helfritz, M. Zielinska-Skowronek, D. Meyer-Olson, R. Jacobs, R. E. Schmidt, R. Mentlein, R. Pabst, and S. von Hörsten. 2003. CD26 expression determines lung metastasis in mutant F344 rats: involvement of NK cell function and soluble CD26. *Cancer Immunol. Immunother.* 52: 546–554.
- Underwood, S. L., el-B. Haddad, M. A. Birrell, K. McCluskie, M. Pecoraro, D. Dabrowski, S. E. Webber, M. L. Foster, and M. G. Belvisi. 2002. Functional characterization and biomarker identification in the Brown Norway model of allergic airway inflammation. *Br. J. Pharmacol.* 137: 263–275.
- Cirulli, F., A. Berry, and E. Allea. 2003. Early disruption of the mother-infant relationship: Effects on brain plasticity and implications for psychopathology. *Neurosci. Biobehav. Rev.* 27: 73–82.
- Levine, S. 1957. Infantile experience and resistance to physiological stress. *Science* 126: 405–406.
- Meaney, M. J., J. Diorio, D. Francis, J. Widdowson, P. LaPlante, C. Caldji, S. Sharma, J. R. Seckl, and P. M. Plotsky. 1996. Early environmental regulation of forebrain glucocorticoid receptor gene expression: implications for adrenocortical responses to stress. *Dev. Neurosci.* 18: 49–72.
- Papanicolaou, D. A., and A. N. Vgontzas. 2000. Editorial: interleukin-6: the endocrine cytokine. *J. Clin. Endocrin. Metab.* 85: 1331–1333.
- Straub, R. H., T. Schaller, L. E. Miller, S. von Hörsten, W. Falk, and J. Scholmerich. 2000. Neuropeptide Y cotransmission with norepinephrine in the sympathetic nerve-macrophage interplay. *J. Neurochem.* 75: 2464–2471.
- Wright, R. J., M. Rodriguez, and S. Cohen. 1998. Review of psychosocial stress and asthma: an integrated biopsychosocial approach. *Thorax* 53: 1066–1074.
- Buske-Kirschbaum, A., S. Jobst, A. Wutsmans, C. Kirschbaum, W. Rauh, and D. Hellhammer. 1997. Attenuated free cortisol response to psychosocial stress in children with atopic dermatitis. *Psychosom. Med.* 59: 419–426.
- Yabuhara, A., C. Macaubas, S. I. Prescott, T. J. Venn, B. J. Holt, W. Habre, P. D. Sly, and P. G. Holt. 1997. Th-2-polarised immunological memory to inhaled allergens in atopics is established during infancy and early childhood. *Clin. Exp. Allergy* 27: 1261–1269.
- Herrick, C. A., and K. Bottomly. 2003. To respond or not to respond: T cells in allergic asthma. *Nat. Rev. Immunol.* 3: 405–412.
- Mentlein, R. 1999. Dipeptidyl-peptidase IV (CD26)-role in the inactivation of regulatory peptides. *Regul. Pept.* 85: 9–24.
- Persinger, M. A., and H. Falter. 1992. Infantile stimulation produces mild enhancement in a primary humoral response of adult albino rats. *Psychol. Rep.* 70: 976–978.
- von Hörsten, S., M. Dimitrijevic, B. M. Markovic, and B. D. Jankovic. 1993. Effect of early experience on behavior and immune response in the rat. *Physiol. Behav.* 54: 931–940.
- Hamelmann, E., K. Takeda, J. Schwarze, A. T. Vella, C. G. Irvin, and E. W. Gelfand. 1999. Development of eosinophilic airway inflammation and airway hyperresponsiveness requires interleukin-5 but not immunoglobulin E or B lymphocytes. *Am. J. Respir. Cell Mol. Biol.* 21: 480–489.