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Stress/Aggressiveness-Induced Immune Changes Are Altered in Adult Rats Submitted to Neonatal Malnutrition

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Key Words
Aggressive behavior · Humoral immunity · Malnutrition · Programming · Stress

Abstract
Background/Aims: Neonatal malnutrition induces metabolic and endocrine changes that have beneficial effects on the neonatal in the short term but, in the longer term, these alterations lead to maladaptations. We investigated the effect of neonatal malnutrition on immune responses in adult rats submitted or not to an aggressiveness test. ***Methods:*** Male Wistar rats were distributed to one of two groups according to their mothers' diet during lactation: the well-nourished group (group C, n = 42, receiving 23% of protein) and the malnourished group (group MN, n = 42, receiving 8% of protein). After weaning, all rats received normoproteic diet. Ninety days after birth, each group was subdivided into three subgroups: control rats (n = 14, respectively), aggressive rats (n = 14, respectively) and rats receiving foot shock (FS; n = 14, respectively). Plasma corticosterone concentration was measured after FS sessions. Leukocyte counts and humoral immunity were evaluated. ***Results:*** In neonatal malnourished animals, FS-induced stress reduced plasma corticosterone concentration. Intraspecific aggressiveness induced alterations in leukocyte counts and antibody titers 7 and 15 days after immunization. Neonatal malnourished animals showed no changes in the immune parameters evaluated. ***Conclusions:*** Expression of intraspecific aggressiveness activates the immune system. Neonatal malnutrition seems to have a long-lasting effect on components of both neuroendocrine and immune functions.

Introduction

Expression of aggressiveness is crucial to survival, because aggressive behavior guarantees access to food, reproduction, protection of the young, capacity to confront predators and the participation in territorial defense [1]. On the other hand, stress/aggressiveness can result in trauma, injury and expose to new diseases [2]. Some studies have associated the aggressiveness-like behavior with immune alterations [3–5]. Barreto-Medeiros et al. [3] verified that intraspecific aggressiveness increased the titers of anti-sheep red blood cell (SRBC) antibodies 7 days after immunization in rats. Stefanski [4] has shown a decrease in TCD4+ and TCD8+ cells in rats submitted to stressful social conditions. In a large male cohort, aggres-

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sive behavior was positively associated with helper/inducer and suppressor/cytolytic T lymphocyte and B lymphocyte counts [1]. Thus, stressors can promote alterations in immune responses, characterizing a possible neuroimmunomodulation via activation of the hypothalamus-pituitary-adrenal (HPA) axis and the nervous system [5–7].

There is substantial evidence that nutritional deficits occurring during the critical period of nervous system development can jeopardize the brain and neuronal functions over the long term [8–10]. The immature brain undergoes remarkable changes in organization during fetal and postnatal development, e.g. myelination of pathways, cell differentiation and proliferation, and synaptogenesis [9, 10]. Because brain development is largely determined by the nutrient and oxygen supply, maternal nutrition plays a critical role in fetal growth [10]. Moreover, the imbalance between nutrient demand and intake can result in metabolic and endocrine changes that are beneficial for the neonatal in the short term, but in the longer term, these alterations lead to maladaptations [11].

Although the mechanisms of the endocrine adaptations that underlie these long-term effects of undernutrition are poorly understood, the importance of the HPA axis has been stressed recently [12]. HPA axis function can be adversely affected by a variety of environmental stimuli during the development of an organism [13]. Animal and preliminary human studies have evidenced that events occurring in early life may be a potent cause of a life-long increase in adrenal glucocorticoid secretion [13].

Studies on the effects of aggressiveness-induced immune changes in malnourished rats are scarce. Our recent studies have indicated that the expression of intraspecific aggressiveness seems to trigger the immune system and to raise antigen-specific humoral responses [3]. In the present study, the effect of aggressiveness-induced immune changes was assessed in adult rats submitted to neonatal malnutrition.

Materials and Methods

Animals

Male Wistar rats *(Rattus norvegicus)* were obtained from the Department of Nutrition, Federal University of Pernambuco, Brazil. The rats were maintained at a room temperature of 23 ± 1°C and a 12-hour light-dark cycle (light on 6:00 a.m. to 6:00 p.m.). During the suckling period, the offspring were kept in groups of 6 pups, randomly assigned to each mother. They were distributed into two nutritional groups according to their mothers' diet during lactation: a well-nourished group (group C, n = 42) with mothers receiving a 23% protein diet (Purina chow) and a malnourished group (group MN, n = 42) with mothers receiving an 8% protein diet. After weaning (on the 25th day of age), all rats received standard laboratory chow (52% carbohydrate, 21% protein and 4% lipids; Nuvilab CR1-Nuvitala). Ninety days after birth, each group was subdivided into three subgroups, as follows: groups receiving no foot shock (FS; group C, n = 14, and group MN, n = 14), aggressive response groups (group CA, n = 14, and group MNA, n = 14) composed of animals kept in pairs in the boxes receiving the FS session, and FS groups (group CFS, n = 14, and group MNFS, n = 14) comprising animals that individually received one FS session. The corticosterone concentration in the serum was analyzed following the FS sessions. Immunological measurements were performed in each animal 0, 7 and 15 days following these treatments. The experiment was approved by the Ethical Committee of the Center of Health Science, Federal University of Pernambuco, and followed the Guidelines for the Care and Use of Laboratory Animals.

FS-Induced Stress

Rats were submitted to FS-induced stress in an isolated room, using a box (20 × 20 × 20 cm), with the floor consisting of parallel metallic bars (interbar distance: 1.3 cm), connected to an electric scrambled current source. The test consisted of placing 1 rat in the box, where it received a session of electric stimuli. Each stimulus (an electrical FS) was represented by a 1.6-mA/2-second current pulse [14]. Each session lasted 20 min and consisted of 5 stimuli separated by a 4-min interval [14]. During the first 3 min of this interval, the aggressive response was analyzed. The annotations and the verification of the equipment were done in the last minute of each interval.

Intraspecific Aggressive Response

The aggressiveness test consisted of placing a pair of rats of the same group (matched by weight) in the box, where they received a session of electric stimuli under the above-mentioned conditions. The aggressive response was defined as the presentation of at least one of the two following behaviors: (a) the animals maintained an erect posture, one facing the other, in a threatening attitude but without direct contact, or (b) they maintained evident physical contact (besides scratches, exhibition of the tooth and emission of characteristic vocalization) [3].

Determination of the Plasma Corticosterone Concentration

Blood samples were collected in tubes and centrifuged at 1,000 *g* for 15 min, and the serum was removed and stored at –20°C until corticosterone determination. The plasma concentration of corticosterone, after its extraction with ethyl acetate, was measured by radioimmunoassay using a commercial kit provided by Sigma (St. Louis, Mo., USA) that contains [1,2,6,7-^{3}H]corticosterone as radioligand.

Total and Differential Leukocyte Counts

Total numbers of leukocytes were counted in a hemocytometer. Differential counts were assessed manually using blood smears from each animal. These slides were then stained using a Diff-Quik three-step staining kit (Dade Behring, Newark, Del., USA). Cells were then counted under an optical microscope. At least 100 cells were counted for each sample.

Table 1. Total leukocytes and percentage of differential leukocytes in the blood from the animals

Days after immunization	Group C	Group MN	Group CFS	Group MNFS	Group CA	Group MNA
Day 0						
Total leukocytes, $\times 10^3$	12.3 ± 0.7	10.3 ± 0.6	9.1 ± 0.1*	10.2 ± 0.6	8.6 ± 0.7*	11.0 ± 0.1
Lymphocytes, %	81 ± 1.2	81 ± 2.4	75 ± 1.9*	81 ± 1.3**	72 ± 1.3*	79 ± 2.4***
Neutrophils, %	15 ± 1.2	16 ± 2.2	18 ± 1.7*	16 ± 1.5	25 ± 2.1*	15 ± 1.9
Day 7						
Total leukocytes, $\times 10^3$	10.4 ± 0.8	10.7 ± 0.6	10.9 ± 0.6	12.5 ± 0.8	13.8 ± 0.7*	10.3 ± 0.4
Lymphocytes, %	82 ± 1.3	80 ± 1.5	80 ± 2.4	77 ± 1.5	83 ± 0.9	82 ± 1.4
Neutrophils, %	13 ± 1.2	15 ± 1.4	13 ± 2.0	17 ± 1.1	12 ± 0.6	14 ± 1.4
Day 15						
Total leukocytes, $\times 10^3$	10.8 ± 0.7	10.1 ± 0.7	10.1 ± 0.5	11.0 ± 0.1	11.1 ± 0.8	10.3 ± 0.9
Lymphocytes, %	82 ± 1.5	79 ± 1.5	81 ± 1.5	74 ± 1.7**	74 ± 2.6	79 ± 2.6
Neutrophils, %	12 ± 1.4	15 ± 1.9	12 ± 1.2	21 ± 1.3*, **	15 ± 1.5	15 ± 2.2

Means ± SEM. * $p < 0.05$ vs. group C; ** $p < 0.05$ vs. group CFS; *** $p < 0.05$ vs. group CA .

Determination of Antibody Titers

The animals were immunized immediately after the FS-induced stress or after the aggressive behavior. SRBC were prepared by washing citrated sheep blood thrice in sterile saline. Animals were immunized intraperitoneally with 10^8 cells/ml in a volume of approximately 0.5 ml [15]. Blood samples were collected before immunization (negative control of antibody titer anti-SRBC), immediately after immunization (day 0) and 7 and 15 days later. Subsequently, samples were centrifuged at 200 *g* for 5 min, and the supernatant was collected. Serum complement was then inactivated at 56°C for 30 min and stored at –20°C. Twofold serial dilutions of inactivated serum, saline and a 1% SRBC solution were then mixed in microtiter plates [15]. The highest dilution at which aggregation of SRBCs was still evident was considered to be the antibody titer [15].

Statistical Analysis

The results are presented as means and SEM. Corticosterone concentrations in the nutritional groups were compared by Student's t test. Leukocyte counts and antibody titers were analyzed by two-way ANOVA followed by the Bonferroni test for multiple comparisons. The null hypothesis was rejected when $p \leq 0.05$.

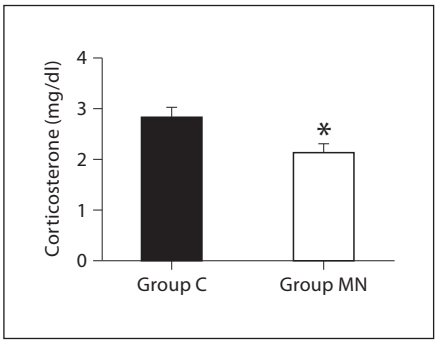

Fig. 1. Blood corticosterone concentrations in the animals after FS sessions. Means ± SEM. * $p < 0.05$ vs. group C.

Results

After FS sessions, plasma concentrations of corticosterone were analyzed. Group MN showed lower concentrations of corticosterone (2.12 ± 0.2) in comparison with group C (2.83 ± 0.2; fig. 1). Compared to group C, MN animals showed no changes in the total and differential leukocyte counts in peripheral blood (table 1). On the other hand, FS animals presented immediate alterations in blood leukocyte counts compared to group C. However, on days 7 and 15, results did not differ after FS. In the control groups (CFS and CA), total leukocyte and lymphocyte counts were decreased, while there were no changes in the MN groups.

On day 15, the percentage of lymphocytes was decreased and the percentage of neutrophils was increased in the MNFS group compared to both control groups (C and CFS groups). The aggressive response group (CA) showed an immediate reduction in the total leukocyte count and in the percentage of lymphocytes, and an increase in the percentage of neutrophils compared with group C. However, 7 days after the aggressive response, an increase in the total number of those cells was observed. There was no difference in the MNA group compared with the CA group except for the percentage of lymphocytes (table 1).

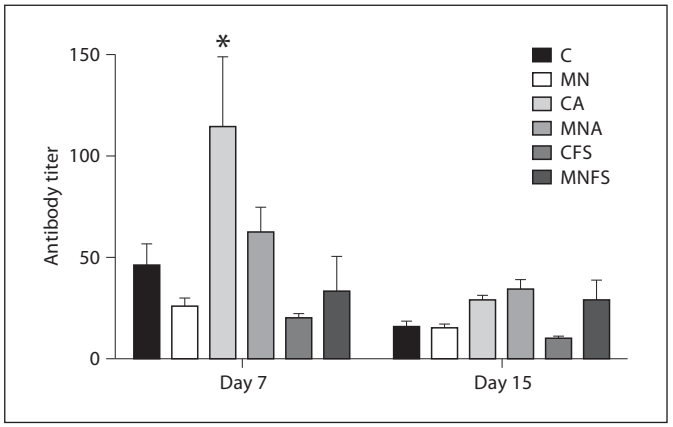

Fig. 2. Antibody titers 7 and 15 days after immunization. Means ± SEM. * $p < 0.05$ vs. group C.

Compared with the control group, the humoral immune response of the animals submitted to neonatal malnutrition did not change (day 7: C = 46 ± 10.6 and MN = 26 ± 3.7; day 15: C = 16 ± 2.5 and MN = 15 ± 1.6). Likewise, C and MN animals subjected to FS sessions showed no alteration in antibody titers (day 7: C = 20 ± 2.2 and MN = 33 ± 17.3; day 15: C = 10 ± 1.0 and MN = 29 ± 9.2). However, intraspecific aggressiveness induced an increase in the titers of anti-SRBC antibodies 7 days after immunization (CA = 114 ± 34.6) compared with the control group. Seven days after immunization, there was no difference between the MNA (62 ± 12.2) and MN groups. Fifteen days after immunization, the humoral immune response did not differ among the groups studied (fig. 2).

Discussion

Environmental factors during development are critical variables in the adaptive capabilities of the adult individual [16, 17]. Various studies have demonstrated that rats exposed to a range of stimuli during neonatal life or the offspring of rat dams subjected to stress during pregnancy or lactation exhibit behavioral and physiological alterations in adulthood [13, 16–18]. The process whereby a stimulus or insult applied at a critical period of development affects a structure or function of the organism, over the long term, has been termed 'programming' [17]. Nutritional insults are one of the most studied programming factors affecting the critical period of development [19]. Depending on the period and duration of the insult, e.g. gestation or lactation, there are different responses [9, 11, 17, 20]. In the present study, animals were submitted to undernutrition during lactation, which seems to be the most crucial period to establish programming in mammals [17].

Our results demonstrated that adult rats submitted to neonatal undernutrition showed a decreased plasma corticosterone concentration after FS sessions. The electrical FS is a classic stress model, well known for promoting increased biosynthetic and secretory activity of chemical messengers in the HPA axis and in the sympathoadrenal system [21]. In previous studies, adult animals whose mothers were submitted to malnutrition during lactation demonstrated endocrine dysfunctions [8, 12, 19]. Although neonatal undernutrition induces increased secretion of corticosterone in the offspring [22], it has an opposite effect on adult corticosterone secretion [23]. An increase in corticosteroid receptors may play a pivotal role in lowering stress-induced corticosterone secretion. Catalani et al. [16] demonstrated that male offspring lactated by stressed mothers have higher hippocampal corticosteroid receptors at 15 months of age and lower hormonal responses to stress. Thus, our findings suggested that neonatal nutritional manipulation, especially during development, can affect HPA axis responses later in life.

In the present study, the expression of the intraspecific aggressive response was accompanied by alterations in the total and differential counts of blood leukocytes and humoral immune responses. Studies on interrelations between aggressive behavior and immune function in mammals are scarce, and those focused on the control of the interference of the stress factor are even rarer. Our data corroborate experimental evidence indicating immunological alterations in animals submitted to acute stress [2, 3, 24, 25]. In fact, the HPA axis as well as other elements of stress responses are related to the expression of aggressive behavior [4, 26]. Trafficking of peripheral cells is transiently altered by glucocorticoids, which rapidly lowers circulating levels of lymphocytes and monocytes, but increases neutrophil levels [25].

Seven days after the aggressive behavior, titers of anti-SRBC antibodies were increased in the aggressive group. The largest production of antibodies, which was found in the aggressive group, reinforces the hypothesis that intraspecific aggressiveness can activate the immune system, increasing its capacity to react against strange antigens [1, 2]. On the other hand, in the present study, pups from malnourished mothers submitted to FS sessions showed no immediate alterations in the immunological parameters evaluated. It is possible that the nutritional deficien-

cy during the neonatal period also had long-lasting effects on the immune system, making it hyporesponsive to the expression of aggressive behavior [1]. Indeed, the ontogeny of the vertebrate immune system requires a strictly regulated sequence of events that includes production of hematopoietic cells, migration of cells through organs, cell-cell interactions during cytodifferentiation and acquisition of definitive functional properties [1, 20, 22, 27, 28]. In spite of the fact that the degree of immunocompetence in early postnatal life varies from species to species, there are similar patterns of immune development during the prenatal and, to a lesser extent, early postnatal periods of mammalian development [29]. Undernutrition during the critical periods of gestation and lactation appears to impair the development and differentiation of the normal immune system [30, 31]. In agreement with our results, Nwankwo et al. [32] observed that malnourished suckling rats infected at 12 days of age with *Staphylococcus aureus* present larger changes in neutrophil counts than their pairs, but a lower ability to deal with infection. Four- to 6-week-old mice fed a low-protein diet in the critical period of development displayed decreased T-cell numbers and responses to phytohemagglutinin [31]. Mice at 4 weeks of age whose mothers were fed with a zinc-deficient diet during lactation displayed retarded thymus and spleen development, and reduced T-cell mitogen responses [33].

In conclusion, the expression of intraspecific aggressiveness before a stressor seems to activate the immune system and raise antigen-specific humoral responses. Consequently, aggressive behavior can increase the capacity of an organism to respond to noxious agents. Malnutrition during the brain growth spurt altered the interrelation between the aggressive behavior and immune responses. Nutritional deficiency in the critical period of development seems to have a long-lasting effect on components of both neuroendocrine and immune functions.

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