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Estradiol, but Not Dehydroepiandrosterone, Decreases Parasitemia and Increases the Incidence of Cerebral Malaria and the Mortality in *Plasmodium berghei* ANKA-Infected CBA Mice

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Key Words
Cerebral malaria · Dehydroepiandrosterone · Estradiol · Hormones · Nitric oxide synthase

Abstract
Objective: The effect of castration and subsequent replacement of dehydroepiandrosterone (DHEA) or estradiol on parasitemia, mortality and incidence of cerebral malaria (CM) was evaluated in CBA mice infected with *Plasmodium berghei* ANKA. ***Methods:*** Female mice were castrated, and groups of 12–15 animals received daily injections of DHEA, estradiol or saline. Four days after the start of treatment, mice were inoculated with 1×10^6 *P. berghei* ANKA-parasitized erythrocytes. DHEA treatment was continued during the 5 days after infection, and estradiol was administered during the follow-up. Parasitemia was evaluated daily in Giemsa-stained blood smears. Signs of CM were determined by the manifestation of coma, limb paralysis and/or convulsions. Plasma TNF-α levels were evaluated by sandwich ELISA. Nitric oxide synthase (NOS) activity in the brain of moribund mice was measured by the method of Bredt and Snyder. ***Results:*** In non-castrated infected mice, the incidence of CM was 50%, and plasma TNF-α increased and brain NOS activity decreased compared to non-infected controls. Castration had no major effect on the parameters analyzed (parasitemia, mortality, CM incidence, TNF-α levels or NOS activity). Estradiol replacement caused a decrease in parasitemia but resulted in higher CM incidence and faster mortality, with an increase in NOS activity. ***Conclusions:*** Estradiol modulated the immune response of *P. berghei* ANKA-infected CBA mice, decreasing parasitemia and increasing NOS activity, and impacted negatively on survival and CM incidence, showing that neuroimmunoendocrine interactions are important in the physiopathogenesis of malaria infections.

Introduction

Malaria affects between 300 and 500 million people every year, causing more than 2 million deaths, mostly children below 5 years in sub-Saharan Africa, mainly by

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complications such as cerebral malaria and severe anemia. In the past few years, evidence has accumulated that many hormones, especially the sex steroids, can influence the immune system and thus susceptibility for diseases caused by protozoan parasites [1–3]. In areas of high malaria transmission, acquisition of immunity is a slow process, and people remain susceptible to clinical disease from birth to puberty and early adulthood [4], leading to the assumption that many years of exposure to infective bites were necessary to achieve immunity – probably reflecting the need to develop responses to a variety of isolates of this highly polymorphic parasite. However, by studying the acquisition of immunity to malaria in migrants, Baird [5] and Baird et al. [6] showed that adults were able to develop immunity after only a few episodes of malaria, while children took much longer and needed further exposure, suggesting that the developmental stage of the individual influences immunity to malaria. Studies conducted in Kenya have demonstrated that the levels of dehydroepiandrosterone sulfate (DHEAS, a precursor in the synthesis of the androgen testosterone) were significantly associated with decreased parasite density and increased hemoglobin levels in adolescents and young adults, even after correction for age, indicating that pubertal hormones may be responsible for this improved capacity of adults to develop protective immunity [7, 8]. The influence of sex hormones on malaria is also evident during pregnancy. Several studies have demonstrated that pregnancy is a risk factor to malaria, especially in women experiencing the first or second pregnancies [9, 10]. *Plasmodium falciparum*-infected erythrocytes can adhere to the placenta through binding of *var* gene products to chondroitin sulfate A or hyaluronic acid [11], and this can lead to reduced placental perfusion and loss of placental integrity, potentially resulting in fetal suffering, preterm delivery, low birth weight or abortion [12, 13]. These events have been associated with the maternal immunomodulation during pregnancy, largely mediated by hormones such as estrogen, progesterone, cortisol and prolactin [14]. A positive correlation between cortisol levels and parasite load has been shown to exist in *P. falciparum*-infected primiparous pregnant women, and this was also correlated with low natural killer cell cytotoxic activity [15]. Concentrations of prolactin, a stimulator of the immune system, were significantly lower in primiparous as compared to multiparous women in that work.

The effect of hormones on parasite growth has also been demonstrated in experimental studies, using animal and in vitro models. Freilich et al. [16] showed that 16α-bromoepiandrosterone, a DHEA analogue, inhibited *P. falciparum* growth in vitro and *Plasmodium berghei* growth in rats. In the latter, inhibition was similar to that observed in rats treated with chloroquine. Ayi et al. [17] proposed that the antimalarial activity induced by DHEA and 16α-bromoepiandrosterone would be mediated through the induction of phosphatidylserine exposure on the surface of ring-infected erythrocytes, leading to their phagocytosis.

Testosterone and estradiol also influence the immune response to *Plasmodium*. The administration of testosterone or estradiol resulted in 100% lethality in C57BL/10 mice, which otherwise are capable of self-healing *Plasmodium chabaudi* infection [18, 19].

Susceptibility to malaria infection in mice has been shown to be modulated by sex. When γ-interferon-deficient and IL-4-deficient mice were inoculated with *P. chabaudi chabaudi*, mortality was higher and survival time was shorter than in the wild-type mates. However, this was true only for male mice, this difference was not observed in female mice [20]. Castration of male mice eliminated the difference. The authors conclude that the sex hormones modulate the expression of Th1/Th2 cytokines in the spleen, leading to differences in the susceptibility to infection. Indeed, it has previously been reported that normal immune responses are sexually dimorphic, leading to different susceptibilities of males and females to a range of infectious and autoimmune diseases [3].

In experimental malaria studies involving gonadectomy or hormone replacement therapy, the susceptibility of male mice was shown to be dependent on gonadal steroids [21]. Animals submitted to castration prior to infection are more resistant, whereas in female mice testosterone and estrogen administration causes immunosuppression [18, 22–24]. In murine malaria, castration suggests that removal of the gonadal steroids activates cell immunity, but the mechanisms by which hormones may affect T cells are still poorly understood [19, 20]. Taken together, these studies demonstrate that the levels of steroid hormones influence not only the course of *Plasmodium* infection but also the immunological modulation and pathogenesis in malaria.

One of the major complications of *P. falciparum* infection is cerebral malaria (CM) [25]. Although the knowledge on CM has increased, the complete mechanism of this life-threatening complication has not yet been unraveled. Binding of infected erythrocytes to brain endothelial vessels, with potential interference on blood flow and oxygen delivery to tissue, has been reported as a major component in the physiopathogenesis of CM [25]. Im-

munopathology is also a central component of CM, and important roles have been established for CD4+ and CD8+ T cells, macrophage hyperactivation, neutrophils, platelets, cytokines – especially TNF-α – and other mediators, particularly nitric oxide (NO) [26–28]. Given the restrictions imposed on studies in humans, the use of murine models of CM has broadened our understanding of CM and may potentially be transferred to human CM. The most commonly applied murine model of CM, i.e. the infection of CBA mice by the *P. berghei* ANKA strain, shares some characteristics with human CM [29]. Mice develop an acute infection that kills most of the animals in the first 2 weeks with clinical and histopathological signs of neurological involvement. The remaining mice succumb later to other malaria-related complications, e.g. severe anemia and hyperparasitemia.

In the present study, we used the *P. berghei* ANKA/CBA mouse model to evaluate the influence of gonadectomy with or without DHEA or estradiol replacement on the development of CM.

Methods

Animals

CBA mice were obtained from the Central Animal House of Fiocruz, Rio de Janeiro. Mice were transferred to the animal house of the Instituto Evandro Chagas, Belém, and maintained under standard conditions, with free access to standard diet and water. Seven- to 8-week-old female mice were used in the experiments. The experimental protocols were approved by the Ethical Committee of the Instituto Evandro Chagas (CEPAN/IEC Approval Letter No. 001/2002).

Experimental Groups

Mice were divided into six groups: (1) non-castrated infected (NC-Inf); (2) castrated infected (C-Inf); (3) castrated with DHEA replacement and infected (C-DHEA-Inf); (4) castrated with estradiol replacement and infected (C-Estr-Inf); (5) non-castrated non-infected (NC-NInf), and (6) castrated non-infected (C-NInf) mice. Twelve to 15 animals were used per group.

Castration and Hormone Replacement

At 4–6 weeks of age, female mice were anesthetized with 0.02 ml (1 mg) ketamine (Pfizer, 50 mg/ml i.m.). The ovaries were removed by surgical operation under sterilized conditions. After ovariectomy, mice were allowed to recover for 2 weeks before being used in the experiments. DHEA was diluted in dimethylsulfoxide:ethanol and administered at a dose of 120 μg/g/day through subcutaneous injection in the animals' back, starting 4 days before and finishing 5 days after infection. A second group received estradiol cipionate (Rhodia-Mérieux) at a dose of 0.25 μg/g/day i.m. in the back limbs, starting 4 days before infection up to the end of the experiment.

Parasites and Infection

A cryopreserved sample of *P. berghei* ANKA was obtained from the Laboratory of Malaria Research, IOC/Fiocruz, Rio de Janeiro. Five days before infection of the experimental groups, the cryopreserved sample was thawed and inoculated intraperitoneally into a naïve mouse. On the day of infection, parasitemia was evaluated by reading a Giemsa-stained thin blood smear, and red blood cells (RBC) were counted using a Neubauer chamber. The murine blood was then diluted in sterile phosphate-buffered saline to obtain a concentration of 10×10^6 parasitized RBC (pRBC) per milliliter, and 100 μl (1×10^6 pRBC) were inoculated intraperitoneally into each mouse of the experimental group. Parasitemia was daily evaluated in Giemsa-stained thin blood smears. Mice were also examined twice a day to check for clinical signs of CM (limb paralysis, convulsions and coma) and mortality. Moribund mice (experiencing or not clinical CM) were killed under anesthesia, serum was collected for the determination of TNF-α levels, and the brain was excised for measurement of NO synthase (NOS) activity.

Assay of NOS Activity

NOS activity in cerebral tissue was measured in cell lysates by the conversion of ^{3}H-*L*-arginine in ^{3}H-citrulline according to the method of Bredt and Snyder [30]. Cerebrum was removed from moribund mice (experiencing or not clinical CM), and half of the organ was washed with PBS and then sonicated in reaction buffer (pH 7.4, 37°C) containing 50 m*M* HEPES, 1 m*M* valine, 0.1 m*M* dithiothreitol and 2 m*M* $CaCl_2$. After centrifugation of the homogenates (1,000 *g*, 5 min, 4°C), aliquots of 50 μl were incubated with 20 μg/ml calmodulin, 1 m*M* NADPH and 5.8 μCi/ml *L*-[2,3-^{3}H]-arginine (New England) and incubated for 30 min at 37°C in a shaking bath in the presence of 3 m*M* EGTA. The reaction was stopped by adding 1 ml 5% TCA and the mixture transferred to an anion-exchange chromatography column containing Dowex AG 50W-X8. The resin had been saturated with 50 μl of 100 m*M* *L*-citrulline and 2 ml 50 m*M* Tris, 20 m*M* HEPES buffer (pH 7.4) and eluted with 2 ml of distilled water. Concentration of the specifically eluted ^{3}H-citrulline was determined by liquid scintillation counting. The protein level was determined by Lowry's method, and the values were corrected to the amount of protein present in the homogenates and the incubation time (expressed as fmol/mg protein/min).

Detection of TNF-α

To determine TNF-α, sera were collected and tested by a two-site sandwich enzyme-linked immunosorbent assay. Briefly, microtiter plates were coated overnight at 4°C with an anti-TNF-α monoclonal antibody (R&D) at the concentration of 1 mg/well. The samples and standard rMuTNF-α were added in duplicate and incubated at room temperature for 2 h and then for 1 h with a 1/1,000 dilution of a biotinylated rabbit anti-TNF-α antibody (R&D). Alkaline-phosphatase-conjugated goat anti-rabbit IgG antibody, diluted 1,000, was added and incubated for 1 h at room temperature. Finally, *p*-nitrophenyl phosphate substrate was added, and the plates were read at 492 nm. The results were expressed as picograms of TNF-α per milliliter by comparing the optical density with a standard curve prepared using rMuTNF-α.

Statistical Analysis

The results were expressed as means and standard errors of the mean. The Mann-Whitney and Kruskal-Wallis tests were used to

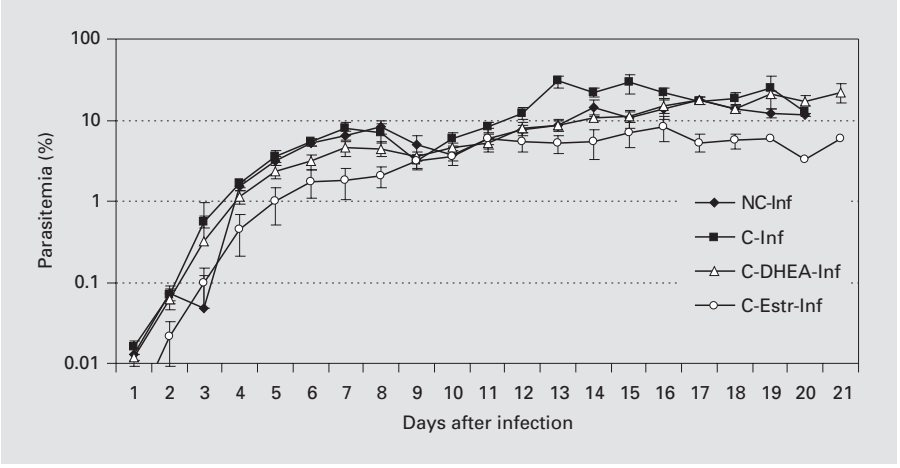

Fig. 1. Course of parasitemia (means ± SE) in CBA mice infected with 1×10^6 *P. berghei* ANKA-parasitized erythrocytes.

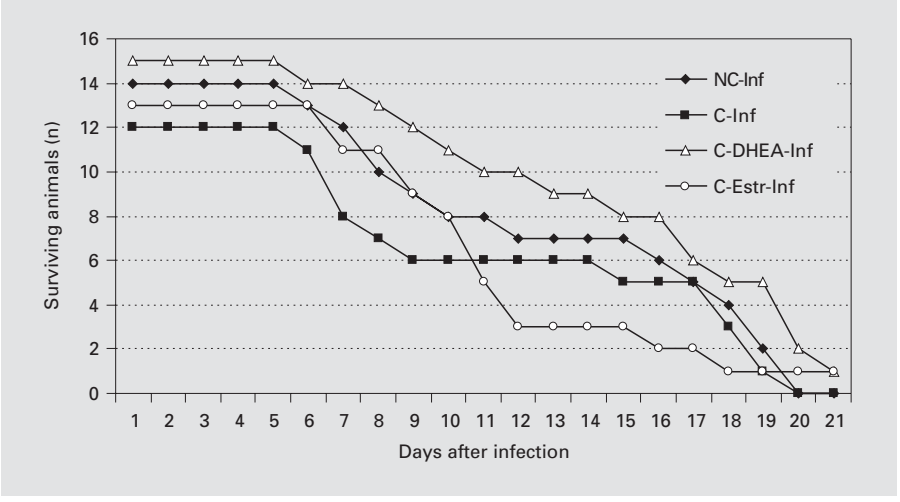

Fig. 2. Survival curve of CBA mice infected with 1×10^6 *P. berghei* ANKA-parasitized erythrocytes.

determine the statistical significance of the intergroup comparisons. A p value less than 0.05 was considered to indicate statistical significance. The log rank test was used to perform the survival analysis, using the program BioEstat 3.0.

Results

Effect of Ovariectomy with or without DHEA or Estradiol Replacement on the Parasitemia, Survival Time and CM-Related Mortality of Mice Infected with P. berghei *ANKA*

Non-castrated *P.-berghei* ANKA-infected mice (NC-Inf) showed an expected course of parasitemia, with exponential growth in the first days but keeping it below 10% until around day 13 of infection, and rising above this value in the next days (fig. 1). Castration (without hormone replacement) had no visible effects on parasitemia in the first days of infection, but from day 13 onwards this group showed parasitemia levels significantly above the other groups ($p < 0.001$). Treatment of animals with DHEA apparently counterbalanced the effect of castration, since the C-DHEA-Inf group showed a course of parasitemia similar to that of non-castrated animals. Treatment with estradiol had the strongest impact on parasitemia. Mice of the C-Estr-Inf group showed levels of parasitemia significantly lower than those observed in all other groups ($p < 0.0001$), mean parasitemia being kept below 10% throughout the follow-up.

Mice of the C-Estr-Inf group presented the shortest, and mice of the C-DHEA-Inf group the longest, survival time (fig. 2). The difference in survival time was significant ($p < 0.01$) between these two groups. However, the differences did not reach statistical significance between the treated groups and the controls (NC-Inf and C-Inf).

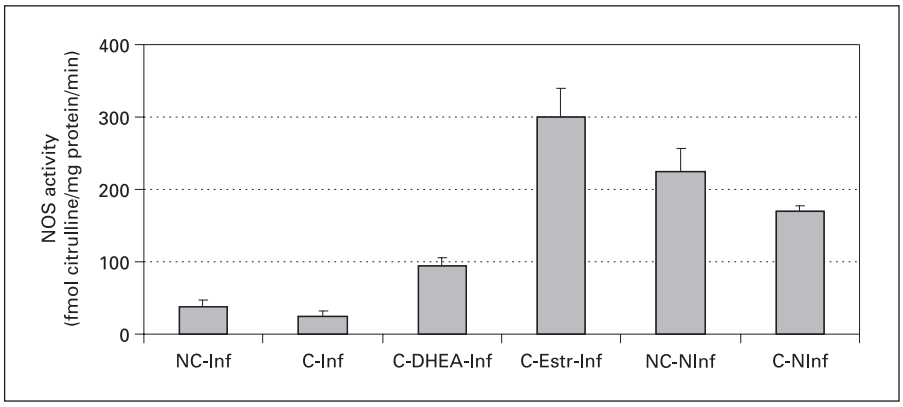

Fig. 3. Activity of NOS in brain tissue of moribund mice infected with 1×10^6 *P. berghei* ANKA-parasitized erythrocytes.

Most mice died between days 6 and 13 of infection showing clinical signs of CM. The incidence of CM was similar in the NC-Inf (50%), C-Inf (50%) and C-DHEA-Inf (40%) groups, and higher in the C-Estr-Inf (77%; $p < 0.01$). Cumulative mortality reached 93–100% in the animals of each group up to day 21 of infection. In groups NC-Inf, C-Inf and C-DHEA-Inf, the remainder of the mice died of non-CM causes, such as severe anemia. Interestingly, 2 of the 3 remnant mice in group C-Estr-Inf died on days 16 and 18, respectively, showing low parasitemia and clinical CM (cumulative mortality by CM reaching 92.3% in this group).

NOS Activity in the Brain of Infected Animals

Infection with *P. berghei* ANKA led to a great decrease in the NOS activity in the brain in non-castrated and in castrated animals (fig. 3). NOS activity was partially recovered by DHEA replacement. With estradiol replacement, NOS activity was not only recovered but greatly increased. Regardless of the group, mice presenting clinical signs of CM had brain NOS activity (fmol of citrulline per mg of protein per minute) significantly higher (216 ± 143.61) than those that died later by non-CM causes (42.5 ± 34.14; $p < 0.05$).

TNF-α Plasma Levels

All infected mice examined presented elevated levels of TNF-α in relation to controls. However, levels were not different between groups, with or without castration, with or without hormone replacement (NC-NInf: 8.76 ± 4.55 pg/ml; NC-Inf: 66.6 ± 37.6 pg/ml; C-Inf: 56 ± 20.6 pg/ml; C-DHEA-Inf: 62.4 ± 31 pg/ml, and C-Estr-Inf: 63.5 ± 37 pg/ml). When comparing mice with or without CM in all groups, the difference in TNF-α levels was not significant.

Spleen/Body Weight Ratios

Infected mice showed intense splenomegaly, measured by the weight of the spleen in relation to the total body weight. The increase in spleen weight was similar in mice of the NC-Inf (0.013 ± 0.005), C-Inf (0.013 ± 0.004) and C-DHEA-Inf (0.014 ± 0.004) groups, as compared to non-infected mice (0.0028 ± 0.0002). Castrated animals treated with estradiol (C-Estr-Inf) had a less marked increase in spleen weight (0.009 ± 0.002), which was significantly different from the two other castrated groups (C-Inf and C-DHEA-Inf).

Discussion

The study of the complex interactions between the immune, nervous and endocrine systems is a growing field of biomedical research, and the understanding of these interactions is crucial for unraveling mechanisms of immunity and pathology in infectious and other diseases. In malaria, a few works have already shown the importance of hormones and the endocrine status on the acquisition of immunity to the blood stage of infection, as well as on its physiopathological consequences, e.g. complications during pregnancy.

In the present work, we show the influence of a male (DHEA) and a female (estradiol) hormone on the course of infection and pathological consequences of malaria in a mouse model of CM. Castration had no apparent major effect on the parameters analyzed (survival time, CM incidence, NOS activity or TNF-α plasma levels), with the exception of an increase in the levels of parasitemia during a limited period of time (days 13–16 of infection in mice surviving CM). DHEA treatment of castrated animals had a limited effect on parasitemia, survival time,

CM incidence and NOS activity, overall ameliorating these parameters. The limited but important effect of DHEA administration is in accordance with reports on human malaria, in which the plasma levels of DHEA were associated with lower parasitemia and protection in hyperendemic areas [7, 8]. The mechanisms through which DHEA improves the immune response against the parasite are not well defined, but some reports have demonstrated a role for androgens in the regulation and modulation of the activity of certain immune cell types, such as T cells, natural killer cells and B cells [3, 15]. In the case of *P. berghei* ANKA infection in CBA mice, cerebral pathology is strongly mediated by the immune system, with a major participation of T cells (CD4+ and CD8+), monocytes/macrophages and cytokines such as TNF-α and IFN-γ [27]. Higher levels of DHEA could possibly help in modulating this response, making it less aggressive.

The most striking findings, however, were recorded in the group of animals treated with estradiol. These animals showed lower parasitemia, higher incidence of CM and died faster. In addition, contrary to the other groups, they had an increase in NOS activity and also presented a less prominent increase in spleen weight during infection. In summary, although some parameters (parasitemia level and spleen weight) showed better performances in relation to other groups, estradiol administration aggravated the severity of infection. This seems also to be in accordance with a number of studies in humans showing that pregnancy is a risk factor for severe malaria [9, 10]. Pregnancy is a physiological state in which female hormones are present in high levels, and pregnant women, especially primiparas, are highly vulnerable to complications. This happens even with women living in hyper- and holoendemic areas and who had already acquired a state of premunition. Pregnancy then breaks down this acquired immunity and exposes the women to severe malaria. The mechanisms mediating these phenomena are not fully understood, but hormone levels seem to be unequivocally involved [15].

A role for NO in malaria immunity and in CM pathogenesis has been proposed for a long time. The first hypothesis incriminating NO in the pathogenesis of CM was proposed by Clark et al. [31]. However, experimental data have provided controversial results, and the overall role for NO in the pathogenesis of CM remains debatable [32]. A major difficulty lies in the measurement of NO, which is usually done indirectly through measuring metabolites related to NO synthesis, and interpretation of results can be confusing. For instance, NO synthesis requires arginine, and hypoargininemia during severe malaria can be interpreted either as an indication that NO synthesis is downregulated or, conversely, as an indication that arginine has been consumed for the production of large amounts of NO [25]. In some studies, increased NO derivatives were found to be associated with coma [33] and poor outcome [34], and NOS expression has been shown to be increased in postmortem tissues of the cerebrum [35, 36]. Other studies revealed no correlation with disease progression [37, 38] or even a protective role for NO [39, 40]. Pino et al. [41] showed that induction of NO via crosslinking of CD23 in lung endothelial cells decreases cytoadherence and mediates killing of *P. falciparum*, suggesting a protective role for NO. Data obtained in mice in general do not support a role for NO in CM pathology. NOS inhibitors, such as *L*-N-monomethylarginine and Nω-nitro-*L*-arginine, even when administered intracranially, did not exert any significant effect on the development of CM in *P.-berghei*-ANKA-infected CBA mice [42, 43], and no increases in urine nitrite secretion were observed in mice with CM. Mice deficient in NOS showed no differences in parasitemia, survival time and expression of CM [44]. Other works even suggest a protective role for NO. Mice rescued from CM by treatment with artemether, chloroquine or clindamycin presented markedly elevated levels of reactive nitrogen intermediates and NOS expression in the spleen [45]. When comparing CM-susceptible (C57BL/6) and -resistant (Balb/c) mice, Hanum et al. [46] showed that resistant mice had higher levels of NO. Macrophages of *P.-berghei*-ANKA-infected mice treated with thalidomide produce higher amounts of NO than non-treated macrophages, and the mortality rate of the former is lower [47]. Our data provide some clues on the difficulty in evaluating the role of NO in CM. Malaria infection apparently inhibited brain NOS activity, since this was much lower in infected (castrated or not) mice than in non-infected controls. This result could, at first glance, imply that NO has no pathogenic role in murine CM, and could even lead to the opposite interpretation, that is, its downregulation precludes its protective action, allowing the development of CM and higher mortality. This view is strengthened by the fact that DHEA administration partially restores NOS activity, decreasing CM incidence and prolonging survival time. However, it must be noticed that NOS activity was assessed in moribund mice, and the low level of activity can also be consequent to its previous high expression with consequent feedback inhibition. Indeed, high NO levels can generate peroxynitrite, which downregulates NOS in a feedback mechanism [48, 49]. This interpretation is con-

sistent with the picture seen in the estradiol-treated mice, whose high NOS activity was associated with a more severe outcome. Interestingly, the high NOS activity was also associated with lower parasitemia, which could be expected from the antiparasitic effects of this mediator [50, 51]. Thus, it seems that the balance in NO production is crucial for the outcome. Elevated – though not very high – levels of NOS activity could lead to a protective effect via the antiparasitic activity of NO. Levels above a certain limit could exert an even stronger antiparasitic activity but also adversely affect CM, i.e. leading to a higher incidence of CM. Estradiol plays a key role in the regulation of pathology, including NOS activity, showing that neuroimmunoendocrine regulation is central in determining outcomes in this specific case and probably in many others.

In malaria, immune response is characterized by hyperreactivity associated with low effectiveness. Polyclonal lymphocyte activation, splenomegaly and hypergammaglobulinemia are all seen in malaria infection, but these phenomena are not only ineffective against the parasite, leading to poor immunity and poor memory responses [52], eventually they can also be associated with pathogenesis, e.g. in murine CM. In the *P. berghei* ANKA/CBA model, the infection causes great changes in lymphoid organs, with thymic atrophy and thymocyte depletion, germinal center architecture disturbance with intense B cell proliferation, poor differentiation and intense apoptosis [Carvalho et al., unpublished data]. Consequently, the major problem in the immune response to malaria is apparently not the activation of the system, but rather its regulation and modulation to generate a balanced, effective and non-pathogenic response. The demonstration here that hormones can either improve or aggravate the scenario indicates that efforts have to be taken to better understand these interactions if preventive or therapeutic interventions, such as vaccines, are to be developed.

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