Corticosterone Regulation of the Effector Function of Malarial Immunity During Pregnancy

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In the experimental *Plasmodium berghei* mouse model, as in human malaria, reduced maternal responsiveness and even loss of immunity were observed during pregnancy. Loss of immunity in the second half of pregnancy occurred during a period of elevated plasma corticoid levels. Further analysis showed that plasma corticoid levels were significantly higher in immunodepressed mice than in mice that remained immune throughout pregnancy. Plasma corticosterone levels differed increasingly from those in mice with persistent immunity towards recrudescence. In nonimmune infected controls, however, only a slight increase in plasma corticosterone, already present during the subpatent period, was measured. Blocking of maternal corticoid production by adrenalectomy delayed the increase of plasma corticosterone (fetoplacental origin) and reduced the number of mice that lost immunity during pregnancy considerably. The role of various plasma corticoid levels in the regulation of effector function of immunity during pregnancy is discussed.

Reduced resistance during pregnancy resulting in more severe infections and increased mortality rates has been reported for viral, parasitic, and fungal infections, as well as for tumors (1, 4, 23, 33, 39, 42, 53). Among these is malaria with a higher incidence and severity in pregnant women, possibly causing prematurity and low birth weight (9, 11, 13, 34, 35).

As in human malaria, loss of immunity is also observed in a considerable proportion of mice previously immune to the virulent parasite *Plasmodium berghei* (50, 51).

A decreased maternal immunoreactivity during pregnancy, contributing to tolerance of the fetal allograft, has been suggested frequently (6). Humoral immunity is either not affected or even slightly stimulated during pregnancy (12, 22, 32). The decreased mixed-lymphocyte reaction, phytohemagglutinin stimulation, and lymphocyte cytotoxicity, as well as delayed rejection of cardiac allografts, during pregnancy reflect impairment of T-cell function in vitro and in vivo (3, 8, 15, 16, 28, 42). Moreover, macrophage activity is reduced or increased in different periods of pregnancy (25, 38). Since it is frequently assumed that T-cells and macrophages play an important role in malaria immunity (26, 29, 41, 44), a pregnancy-associated depression of their functions may lead to loss of immunity.

Several factors have been implicated in pregnancy-associated immunodepression, such as alpha-fetoprotein (37, 46) or changes in serum hormone levels (e.g., progesterone, human cho-

ronic gonadotropin, and glucocorticoids [27, 45], which either directly inhibit T-cell activity or generate suppressor T-cells (7, 24). The inhibiting effect of glucocorticoids on the immunological functioning of T-cells and macrophages is well known (2).

Experiments described in this paper show a relation between the level of plasma corticosterone and loss of malaria immunity. Mice that lost immunity during pregnancy exhibited higher plasma corticosterone levels than those with persisting immunity, whereas adrenalectomy before pregnancy not only blocked maternal corticosterone production, but also prevented loss of immunity, suggesting regulation of the effector function by plasma corticosterone.

MATERIALS AND METHODS

Mice. Random-bred Swiss mice, obtained from the animal facilities of the University of Nijmegen or from T.N.O., Zeist, The Netherlands, were kept in plastic cages with food and drinking water ad libitum.

Pregnant mice. Two or three female mice, aged 12 to 20 weeks, were housed with one male. Every morning for the next 4 days mice were examined for the presence of a vaginal plug. Females not mated after 4 nights were removed from the male; mated females were removed 3 days later. The day of finding the vaginal plug was day 1 of pregnancy. After delivery the litter was removed from the mother. All pregnancy-related experiments concerned primigravid mice.

Parasite. *P. berghei* strain K173 was maintained by intraperitoneal subinoculation of infected blood (10⁵

parasitized erythrocytes) into female Swiss mice weekly. The course of a primary infection, which is always lethal, has been described previously (21).

Immune mice. The immunization procedure (19) was as follows. Mice were infected with 10^7 parasitized erythrocytes and treated with sulfadiazine (30 mg/liter of drinking water) from 2 to 33 days thereafter to prevent patency. Immunity was assessed by resistance to challenge (10^5 parasitized erythrocytes) given 35 days after the immunizing infection. Only mice with an established immunity were used in the experiments. Immunity is of the premunition type; i.e., small, subpatent numbers of parasites persist in immune animals for different periods of time (20). Spontaneous recrudescences were rare (18). Immune mice did not develop patency after any challenge.

Recrudescence. A pregnancy-associated recrudescence (parasitemia higher than 5% infected cells) was determined from thin smears prepared from tail blood and stained with May-Grünwald-Giemsa solutions. Since recrudescence can only occur when parasites are present, immune mice were given an extra challenge (10^5 parasitized erythrocytes) 3 days before mating.

Isodiagnosis. The presence of parasites in immune mice was determined by subinoculation of 0.02 ml of tail blood, diluted with 0.2 ml of saline containing heparin (5 U/ml), into a clean animal, which was subsequently checked for patency.

Adrenalectomy. Each adrenal gland was removed through a lateral incision, separation of the muscle wall, and blunt preparation of the organ. Operation was performed under ether anesthesia. Adrenalectomized mice were kept at 28°C for 1 week and received seven daily injections of 1 ml of saline subcutaneously, supplemented with 0.9% NaCl solution as drinking water throughout the experimental period. The time between adrenalectomy and mating was at least 2 weeks.

Plasma samples. Blood (100 to 150 μ l) was obtained from the retro-orbital plexus of anesthetized (ether) mice, using heparinized disposable micropipettes. In this way animals could be sampled longitudinally. Blood samples were centrifuged and the plasma was frozen (-20°C). To avoid interference by diurnal changes of the corticosterone level, all samples were taken between 11:00 and 11:30 a.m.

Corticosterone. Plasma corticosterone was measured by radioimmunoassay, using an antiserum raised in sheep against corticosterone-21-hemisuccinate-bovine serum albumin (gift from T. Benraad, Department of Experimental and Chemical Endocrinology, University of Nijmegen). Plasma samples were diluted (100-fold) in distilled water with 0.2% ethylene glycol, incubated overnight at 4°C with [3 H]corticosterone (specific activity, 57 Ci/mmol; Amersham International Limited), and extracted with toluene. The organic phase was evaporated, and corticosterone was separated from cross-reacting substances by paper chromatography (Whatman no. 1), using a modified Bush B5 system (1 volume of toluene, 0.5 volume of methanol, and 0.5 volume of water). Paper was scanned for radioactivity (Packard radiochromatogram scanner, model 7201), and the radioactive zone was eluted with ethylene-glycol-water. Recoveries after extraction and chromatography were $24 \pm 8\%$ (standard deviation; $n = 46$). The procedure for the radioimmunoassay of

corticosterone in the eluate is the same as that described for aldosterone (17). The antiserum dilution was 225,000, and corticosterone (Steraloids) concentrations of 25 to 2,000 pg were used for the standard curve. The interassay coefficient of variation was 14% ($n = 46$).

Statistical evaluation. Significance of differences between plasma corticosterone levels in groups of mice was assessed by the Kruskal and Wallis test and the Wilcoxon test.

RESULTS

Recrudescence during pregnancy. A high percentage of mice, immune to *P. berghei*, lose their immunity during pregnancy and suffer a frequently lethal infection (51). The cumulative proportion of recrudescence mice in relation to the day of pregnancy is depicted in Fig. 1. Recrudescences are almost only found in the last trimester of pregnancy. Some mice exhibited recrudescence after parturition.

Plasma corticosterone and thymus involution during pregnancy in normal and adrenalectomized Swiss mice. During a malaria infection there is reduced immune reactivity which is associated with thymus involution (21), and this in turn could be taken as an indirect marker for immunodepressive changes in T-cell areas. Therefore, changes in thymus weight during pregnancy were recorded and compared with the pattern of recrudescences. Moreover, since corticosterone, the physiologically important glucocorticoid of mice, is well known for its immunosuppressive properties, serum plasma levels were determined during pregnancy and compared with changes in thymus weight. The effect of plasma corticosterone on thymus invo-

FIG. 1. Cumulative pattern of recrudescence mice ($n = 98$) in relation to the period of pregnancy.

lution was also determined in adrenalectomized mice, i.e., in the absence of maternal corticosterone production. The results of these experiments are given in Fig. 2a to d.

Progressive thymus involution was observed in intact pregnant mice from day 14 onwards (Fig. 2a), and this correlated with an increase in plasma corticosterone levels starting as early as day 12 of pregnancy (Fig. 2b). In adrenalectomized mice thymus involution was not significant until parturition (Fig. 2c), and this was several days after an increase in plasma corticosterone level (Fig. 2d), which was apparently produced by the fetoplacental unit, since the increase in plasma corticosterone in adrenalectomized mice was proportional to the number of

fetuses (data not shown). In intact and adrenalectomized mice increase in plasma corticosterone preceded thymus involution, but a reduction in corticoid level was not closely followed by thymus regeneration.

An approximately 10-fold increase of plasma corticosterone was observed during pregnancy, with peak values on day 18 and return to "non-pregnant" values after parturition (Fig. 2b and d). Individual plasma levels varied strongly, especially during the period of peak values.

Plasma corticosterone and loss of immunity in intact pregnant mice. Increased plasma corticosterone levels coincided with recrudescence during pregnancy. This prompted analysis of plasma corticosterone levels in mice developing

FIG. 2. Changes in thymus weight and plasma corticosterone in normal and adrenalectomized pregnant Swiss mice. Average thymus weight ($\pm$ standard deviation) was determined in groups of three to six mice, and average plasma corticosterone values ($\pm$ standard deviation) were determined in groups of 4 to 11 animals. Data for nonpregnant controls (open symbols) were obtained from a control group sacrificed on different days of pregnancy together with experimental mice (closed symbols).

recrudescence during pregnancy in comparison with mice that remained immune throughout.

Female mice with an established immunity were allowed to mate. Blood samples were taken on days 10, 14, 16, and 18 or 19 of pregnancy, and plasma corticosterone concentrations were determined. Thin smears were prepared each time to determine recrudescence. The blood of each mouse found to be smear negative in the period between days 14 and 18 was subinoculated into control mice, and all mice negative by isodiagnosis were taken out of the experiment, because without parasites no recrudescence could occur.

To relate plasma corticosterone with loss of immunity, mice were divided into three categories on each day of sampling: (i) mice actually having a recrudescence; (ii) mice developing recrudescence later in pregnancy; and (iii) mice without recrudescence throughout pregnancy. The combined results of two independent experiments are depicted in Fig. 3. Plasma corticosterone concentrations of mice in the three categories differed significantly on days 14 and 16 of pregnancy ($P < 0.05$, Kruskal and Wallis test). Pairwise comparison to explore this difference shows significantly higher corticosterone levels in the group of mice with recrudescence compared with the levels in one of the other groups ($P < 0.05$, Wilcoxon test). Results on days 18 and 19 were not clear, because the number of mice involved was too small, which was due to the fact that either recrudescence mice died earlier or had to be eliminated due to prematurity or abortion (50), which caused an acute drop of the corticosterone level. Nevertheless, the results seem to indicate a higher corticosterone level in recrudescence mice (day 18, $P = 0.05$; day 19, $P = 0.10$).

In the period of days 14 and 19 the average corticosterone concentration, as well as the magnitude of the standard deviation of mice developing recrudescence later (group 2), shifted from the value found in the group with no recrudescence (group 3) to that in the group actually having a recrudescence (group 1). The significantly higher corticosterone values in recrudescence mice and the growing significance of increase of plasma corticosterone concentrations towards recrudescence could suggest a direct relation between plasma corticosterone levels and parasitemia. Figure 4 shows plasma corticosterone in relation to primary infection. A small increase in plasma corticosterone was already observed in the prepatent period, was not related to the magnitude of the parasitemia, and was very small in comparison with values observed during a pregnancy-related recrudescence.

Plasma corticosterone and loss of immunity in

adrenalectomized pregnant mice. The close correlation between high levels of plasma corticosterone and recrudescence suggests a causal relationship. This implies that blocking and corticosterone production should prevent loss of immunity and recrudescence. Maternal production of corticosterone can be blocked by adrenalectomy.

Immune mice were adrenalectomized and plasma corticosterone levels were determined 2 weeks later. Then, mice were challenged with 10^5 parasitized erythrocytes to assure the presence of parasites, as much as possible, and allowed to mate 3 days later. On days 14, 16, 18 or 19, and 20 of pregnancy, as well as 1 week after parturition, plasma corticosterone levels were determined. On the same days blood smears were made to assess a possible recrudescence, and isodiagnosis was performed on smear-negative mice. Finally, mice were sacrificed and examined for the presence of adrenal tissue, which was eventually verified by light microscopy. Mice with remnants of adrenal tissues and isodiagnosis-negative mice were taken out of the experiment.

As in normal adrenalectomized mice (Fig. 2d), plasma corticosterone levels were only elevated from day 16 on (Fig. 5). With comparatively low values until day 18, there is a considerable

FIG. 3. Plasma corticosterone concentrations during pregnancy. Of 37 mice, 24 (65%) developed recrudescence. Plotted corticosterone levels are mean values ($\pm$ standard deviation) of each group of mice. On day 10 of pregnancy the results were pooled since corticosterone levels of the groups were the same. Symbols: ●, mice actually having recrudescence, +, mice developing recrudescence later in pregnancy; ○, mice without recrudescence throughout pregnancy.

FIG. 4. Mice ($n = 10$) were longitudinally sampled (see text) 1 day before (control values) and 2, 5, 7, and 9 days after infection. At the same time thin smears were made to determine parasitemia. Plotted data are the mean ($\pm$ standard deviation). Nine mice survived until day 7 and three mice survived until day 9 of infection. PE, Parasitized erythrocytes.

reduction in plasma corticosterone compared with intact immune mice (Fig. 3). Of 22 animals, only 3 (14%) developed recrudescence during pregnancy, exhibiting higher plasma corticosterone levels than the others on day 16 of pregnancy.

DISCUSSION

A considerable proportion (approximately 50%) of mice with an established immunity relapsed during their first pregnancy (50). In Swiss mice approximately 70% of these recrudescence mice develop lethal infection (51), implying complete loss of a previously existing immunity during pregnancy. Considering that spontaneous recrudescences are rare in immune nonpregnant mice (18), these experiments revealed a remarkable pregnancy-associated suppression of effector function of immunity. Pregnancy-associated immunosuppression has also been observed with regard to human malaria (9, 11, 13, 34, 35), viral infections (e.g., poliomyelitis [33], influenza [42], herpes simplex virus [53], hepatitis [39], and encephalomyocarditis [23]), the protozoan infection amoebic colitis (1), and the fungal infection coccidioidomycosis (42). Also, tumor development can be enhanced during pregnancy, e.g., Burkitt's lymphoma (4). The *P. berghei* mouse model described in this paper may be an adequate experimental model for the analysis of the mechanisms of immunodepression during pregnancy.

An important result of the experiments was that mice that lost immunity during pregnancy had significantly higher plasma corticosterone levels than mice with persistent immunity (Fig. 3). Analysis of changes during pregnancy revealed that loss of immunity as marked by

recrudescence (Fig. 1) was observed in a period of progressive thymic involution (Fig. 2a) and increasing plasma corticosterone levels (Fig. 2b). Recrudescence and thymic involution chronologically followed elevation of plasma corticosterone levels (Fig. 1 and 2). Combined with the well-known immunosuppressive properties of glucocorticoids, this suggested a causal relationship among elevation of plasma corticoid levels, thymus involution (30, 36, 40), and impaired immune reactivity (recrudescence). In combination with the scattering of plasma corticosterone levels, particularly during peak periods (Fig. 2), this led to the hypothesis that mice developing plasma corticosterone values belonging to the upper part of the distribution during the peak period (Fig. 2b) were prone to lose immunity during pregnancy. Two observations supported this hypothesis: first, the significantly higher corticosterone levels in mice that lost immunity during pregnancy in comparison with those with persistent immunity throughout (Fig. 3); and second, absence of recrudescence when early increase of plasma corticosterone was prevented by adrenalectomy (Fig. 5). Some additional remarks have to be made. The scattering of corticoid levels in recrudescence mice (Fig. 3) indicates that part of these mice exhibited higher corticosterone levels than nonimmune controls (Fig. 2b), suggesting extra corticosterone production. Moreover, the plasma corticosterone level in the group of mice developing recrudescence some time after sampling increased progressively towards the end of pregnancy in comparison with the group with persisting immunity

FIG. 5. Plasma corticosterone concentrations in adrenalectomized pregnant mice ($n = 22$). Plotted values are the mean ($\pm$ standard deviation) of mice without recrudescence, whereas values of recrudescence mice are plotted individually. Symbols: ●, mice actually having recrudescence; +, mice developing recrudescence later in pregnancy; ○, mice without recrudescence throughout pregnancy.

(Fig. 3). It can be assumed that there is an increasing chance that recrudescence and increased corticosterone values occur shortly after sampling towards the end of pregnancy. Together, these observations suggest that development of recrudescence leads to increased corticosterone production, but, on the other hand, the plasma corticosterone level was only minimally changed during infection (Fig. 4) and appeared to be independent of parasitemia. This makes it unlikely that stress associated with disease was responsible for the impressive increase in recrudescence in mice, but leaves unexplained the apparent excessive production of plasma corticosterone in some recrudescence mice.

Maximum plasma corticosterone levels in adrenalectomized mice (Fig. 2d) were not markedly different from those in intact controls (Fig. 2b); only the rise was delayed. The increased plasma corticosterone levels towards the end of pregnancy in adrenalectomized mice is assumed to depend on production in the fetoplacental unit (5), a view that is supported by the observation that the level was related to the number of fetuses. The delayed increase in plasma corticosterone in adrenalectomized mice also delayed thymic involution. In view of the impressive reduction of the recrudescence rate in adrenalectomized mice, this may suggest that plasma corticosterone levels in adrenalectomized mice can be high enough to suppress the effector mechanism of immunity, but that the period of sufficiently high, immunosuppressive levels starts too late and is too short to cause recrudescence. The observation that plasma corticosterone values in recrudescence adrenalectomized mice were high already on day 16 of pregnancy (Fig. 5) is in line with this assumption.

The number of recrudescences after parturition was small (Fig. 1), and since recrudescence was scored when parasitemia passed 5% infected cells, the actual breakdown of immunity occurred even before parturition. However, after parturition recrudescence infections are suppressed spontaneously (50), suggesting that the immunosuppression phased out around parturition, correlating nicely with the reduction in plasma corticosterone shortly before parturition (Fig. 2 and 3).

In humans, too, elevated plasma cortisol levels coincide with loss of immunity during pregnancy (9, 10). Moreover, an exogenous supply of corticoid could break malaria immunity (W. Eling and A. van Zon, *Parasitology* 77:ii, 1978). Also, immunosuppression related to elevated corticoid levels was suggested in cases of amoebic colitis (1, 31). These observations support the hypothesis that plasma corticoid levels regulate the effector part of immunity against several

pathogens, although the mechanism of this regulation remains to be clarified. T-cells and macrophages are important in malaria immunity (19, 26, 41, 44, 52), and changes in activity of these cells during pregnancy could lead to loss of immunity. It is well known that elevated plasma corticoid levels decrease the number as well as the activity of circulating lymphocytes and monocytes (2, 43, 47-49). Since T-suppressor cells are less sensitive to corticoid (54), a relative increase of suppressor T-cells during pregnancy (29) has been taken to explain pregnancy-associated immunodepression (7, 14). Furthermore, humoral immunity is either unaffected or enhanced during pregnancy (22, 27, 33), suggesting that cell-mediated reactions are suppressed (4).

Biological activity of corticoids is generally related to free corticoid, unbound to corticoid-binding globulin (2), and further studies are directed to the role of free corticoid in the regulation of immunity.

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