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Pregnancy impairs resistance of C57BL/6 mice to *Leishmania major* infection

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Summary

To determine if gestational factors affect the severity of *L. major* infection, this study assessed the levels of IL-4 mRNA and IFN-gamma mRNA in popliteal lymph node cells of pregnant C57BL/6 mice mated at 5hours, 16hours and 15days post *L. major* infection using PCR. Infected pregnant C57BL/6 mice developed larger cutaneous footpad lesions compared with non-pregnant infected C57BL/6 mice. The resolution of footpad lesions commenced after 8th week in C57BL/6 mice mated at 16hrs post *L. major* infection but 12 weeks in C57BL/6 mice mated at 5hrs and 15 days post *L. major* infection. C57BL/6 mice that were infected 20 days post partum resolved *L. major* infection effectively. But, the lesions in infected pregnant C57BL/6 mice and infected non-pregnant C57BL/6 mice were not as large as in susceptible BALB/c mice. The mean litter weights were similar in pregnant infected C57BL/6 mice mated at different stages of *L. major* infection but were slightly lower than weights of litters from pregnant uninfected C57BL/6 mice. In 5 days infected pregnant C57BL/6 mice, the levels of IFN-gamma were raised compared with the levels of IL-4 but those mated at 15 days post *L. major* infection had highest level of IFN-gamma mRNA. In 10 days pregnant infected C57BL/6 mice, levels of IL-4 were raised compared with IFN-gamma but mice mated at 16hrs post *L. major* infection had highest level of IL-4. In 15 days pregnant infected mice, the levels of IL-4 were higher than IFN-gamma irrespective of the stage of *L. major* infection when the mice were mated. Mice infected with *L. major* 20 days post-partum produced more IFN-gamma than IL-4 from 16hrs post *L. major* infection onwards. It may be concluded that increased IL-4 in pregnant infected C57BL/6 mice impairs the resistance of C57BL/6 mice to *L. major* infection especially in mice that were pregnant before effective immunity (5 hours post *L. major* infection) is mounted against *L. major* infection.

Keywords: *Mice, L. major, cytokines, gestation, pregnancy outcomes.*

Résumé

Pour déterminer si les facteurs de gestation affectaient la sévérité de l'infection L. majeure, cette étude a evalue les niveaux d'IL-4 mRNA et mRNA de l'IFN-gamma dans les cellules du noeud de la lymphe du poplitee de gestation C57BL/6 de souris accouplee à 5h, 16h et a 15jours post infection de L. majeure utilisant le PCR. Les souris en gestation C57BL/6 non en gestation infectées ont développé les plus grandes lésions cutanées de la semelle en comparaison avec des souris non en gestationinfecté C57BL/6. La résolution de lésions de la semelle a commencé après la 8eme semaine dans les souris C57BL/6 accouplees à 16hrs Les souris qui ont ete infectees 20 jours post partum one resolue effectivement l'infection L. majeure. Mais les lesions dans les souris souris C57BL/6 infectées en gestation et les souris C57BL/6 infectees non en gestation n'etaient pas aussi grandes que dans les souris BALB/c susceptibles. Les poids moyens des petits souris étaient semblable dans les souris en gestation C57BL/6 infectees qui ont ete accouplees a des etapes differentes de l'infection L. majeure mais était légèrement plus bas que les poids des petits des souris C57BL/6 en gestation non infectees. En 5 jours les niveaux d''IFN-gamma des souris en gestation infectees ont ete eleves en comparaison avec les niveaux d'IL-4 mais celles accouplees a à 15 jours post infection de L. majeure avait le plus haut niveaux de mRNA et de l'IFN-gamma. En 10 jours les les niveaux d'IL-4 des souris ont été élevés comparé avec cela d' IFN-gamma mais les souris accouplees a 16hrs post infection L. majeure avaient le niveau le plus eleve d'IL-4. Dans les souris infectees en gestation en 15 jours les niveaux d''IL-4 étaient plus elevees qu'IFN-gamma outre les etapes de l'infection L majeure quand les souris ont ete accoupleees. Les souris infecte avec L. majeure a 20 jours post partum ont produit plus d'IFN gamma que d'IL-4 a partir de 16 heures post infectionL.majeure auparavant. Il peut etre conclu que l'augmentation d'Il-4 dans les souris C57BL/6 infectees en gestation affaiblit la resistance des souris C57BL/6 a l'infection L. majeure particulierement les souris qui etaient en gestation avant que l'immunite ne soit effective (5 heures post L. infection majeure) elle se dresse contre l'infection L. majeure.

Introduction

Leishmania cause a spectrum of infectious diseases in mammalian hosts ranging from self-healing ulceration to progressive visceral dissemination [1]. Strains of inbred mice experimentally infected with *L. major* reproduce this spectrum of disease in association with distinct lymphokine responses. Cells from lymph nodes draining the

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cutaneuos lesions of resistant C57BL/6 mice produce interferon-gamma (IFN-gamma), whereas cells from susceptible BALB/c mice generate interleukin 4 (IL-4) [2]. BALB/c mice develop a progressive infection with ultimate death of the animal whereas, C57BL/6 mice develop only a minimal self-healing lesion at the site of parasite inoculation and successfully resolve the infection [1,2]. Resistance or susceptibility to *L. major* is associated with the type of CD4+ T cell-mediated immune response mounted by the mouse. Two distinct types of CD4+ T cell-mediated immune response are identified viz: - Th 1 secreting IL-2, IFN-gamma and TNF beta; and Th 2 secreting IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 [3,4]. Immune responses against *Leishmania* show overall cytokine response reminiscence of the Th 1 and Th 2 subsets. Susceptibility in BALB/c mice is associated with the induction of a Th 2 response and resistance in C57BL/6 mice is attributable to the mounting of a protective Th 1 response [5].

Maternal immune responses in pregnancy are biased towards humoral and away from cell-mediated immune response [6]. As Th 1 and Th 2 cells are involved in cell-mediated inflammation and antibody responses respectively [7], therefore Th 2 biased immune responses may predominate during pregnancy. Expression of Th 1 and Th 2 related cytokines varies in the blood circulation of pregnant women belonging to different trimesters. Th 1 cytokines predominates in 1st trimester (early stage of pregnancy) while Th 2 cyokines are more detectable in 3rd trimester (late stage of pregnancy) [7]. There is evidence that Th 1 cytokines can be harmful to pregnancy, whereas Th 2 cytokines are protective. IFN-gamma and IL-2 induce foetal loss [8]. Positive correlations have been reported between resorptions and the expression of IFN-gamma, IL-2 and TNF [9]. In contrast to these deleterious effects of Th 1 cytokines, the Th 2 cytokine IL-10 protects against foetal death in murine model of spontaneous resorption [10], and there is a negative correlation between resorptions and IL-10 expression [10].

Malaria or HIV/AIDs have been shown to have adverse effects on pregnancy outcomes [10, 11]. Moreover, clinical evidence suggests that toxoplasmosis, tuberculosis and leprosy tend to be aggravated during pregnancy [12]. Similarly, murine gestation lowers resistance to *L. monocytogenes* and *T. gondii* [6]. But immunological reasons were not forwarded to explain these observations. This present study determines the cytokine interactions between anti-leishmanial immunity and pregnancy using mice model. The hypothesis is that Th 2 response that protects fetoplacental unit may adversely affect the course of infection in normally resistant species.

Methodology

Infection of mice

Mice were injected in the footpad with 3 x 10^6 stationary phase promastigotes suspended in 0.05ml of incomplete Dulbecco's Minimum Essential Medium (DMEM) before mating of the mice. Age-matched groups of control (non-pregnant female C57BL/6 and BALB/c) mice were also infected with the same number of parasites

Mating of mice

The ratio of one male to two female of 6-8 weeks old mice were housed in the same cage for mating and the presence of vaginal plugs in the females was checked every 5hrs to confirm conception. Pregnant females were removed from the mating cages and housed in separate cages.

Experimental design

A total of 45 C57BL/6 mice were used for this experiment. The mice were grouped according to stage of *L. major* infection as earlier described [4] as follows: - pre-IFN-gamma production stage (5 hrs of *L. major* infection) (Group A), excessive IFN-gamma production stage (16 hours post *L. major* infection)(Group B) and reduced IFN-gamma stage (day 15 post *L. major* infection)(Group C). In each of the group of A-C, 12 mice were used for the study from which three were sacrificed at 5 days post plug, 10 days post plug and 15 days post plug. According to Robertson et al [21], 5 days post plug, 10 days post plug and 15 days post plug are classified as early- (1st trimester), middle- (2nd trimester) and late- (3rd trimester) stages of pregnancy in mice. Three mice in each of the groups were reserved for assessment of the course of long term *L. major* infection. Each of the above mentioned groups of mice were matched by a group of same age controls (pregnant non-infected C57BL/6 mice, non-pregnant non-infected C57BL/6 and BALB/c mice, infected non-pregnant C57BL/6 mice and BALB/c mice).

Another group of mice (9 in number) were infected with L. major at 20 days post partum, three of which were sacrificed at 5 hours post *L. major* infection, 16 hours post *L. major* infection and 15 days post *L. major* infection.

Assessment of infection

Measuring the thickness of the infected footpad weekly with a metric caliper assessed the progression of cutaneous infection. The lesion size was calculated by subtracting the value of the uninfected contra-lateral footpad from that of the infected ones.

Pregnancy outcome

The number and weight of the litters were documented at birth. The litters were weighed individually using a sensitive weighing scale and the mean weight was recorded. Mortality rate of the litter was determined by dividing the number of litters that died within 1-week post birth with the total number of delivered pups.

Quantitation of cytokines mRNA by competitive PCR

Total RNA was extracted from the cells using chloroform-isopropanol method and suspended in RNAase free water. cDNA was synthesised from diluted RNA by incuba-

tion and treatment with 1st strand mix, dTT and primer working buffer. PCR was performed on this cDNA in 5µl volume containing dNTP, sterile water, PCR buffer, 5 primer, 3 primer, Taq polymerase, and competitor. Thereafter, 15µl of the reaction product was analysed on a 1% agarose gel in TBE X1 buffer containing 0.1mg/ml ethidium bromide. Quantitation of cDNA was done by calculating how much of the competitor fragment will be required to achieve equal amount of products [13].

Statistical analysis

The data generated in Figures 1b and 1c were analysed using Student t-test after calculating mean and standard deviation.

Results

Course of L. major infection in pregnant C57BL/6 mice

Figure 1a demonstrates the progression of footpad lesions (thickness of the infected footpad minus that of the uninfected footpad) as measured by metric caliper. The footpad lesion in pregnant infected BALB/c mice was significantly higher than that of non-pregnant infected BALB/c, pregnant infected C57BL/6 mice and non-pregnant infected C57BL/6 mice from 2nd week of infection onward. The lesion resolved in non-pregnant infected C57BL/6 after 6th week as opposed to the unresolved lesions in pregnant infected and non-pregnant infected BALB/c. However, in the pregnant infected C57BL/6 mice, lesion sizes showed significant increase when compared with non-pregnant infected C57BL/6 from 4th week of infection onward, particularly in mice mated at 5hrs and 15 days post *L. major* infection. Though the lesions finally resolved in the pregnant infected C57BL/6 mice, the resolution of footpad lesions commence after 8th week in C57BL/6 mice mated at 16hrs post *L. major* infection and 12 weeks in C57BL/6 mice mated at 5hrs and 15 days post *L. major* infection. C57BL/6 mice that were infected 20 days post partum were able to resolve *L. major* infection effectively (Figure 1a).

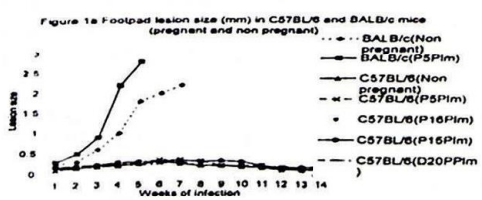

Fig. 1a:

L. major infection decreased litter weight and increased litter mortality

Litter weight was taken at birth with a weighing scale. Litters from pregnant infected BALB/c mice had least mean litter weight but the litter weight of all groups of pregnant C57BL/6 mice were similar (Figure 1b). The numbers of litters (8.2 ± 1.8) produced by infected C57BL/6 mice were similar to those of controls (8.5 ± 1.6) but highest mortality rates were observed in the litters from pregnant infected BALB/c mice, C57BL/6 mice mated at 5 hrs- and 15 days post *L. major* infection (Figure 1c).

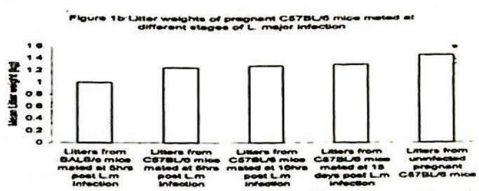

Fig. 1b:

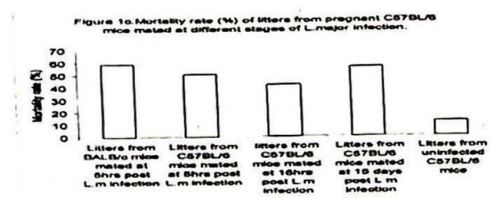

Fig. 1c:

Expression of IFN-gamma mRNA and IL-4 mRNA in lymph nodes of pregnant C57BL/6 mice

Figures 2a – 2d show the expression and production of IL-4 and IFN-gamma mRNA by lymph node cells of mice in infected and non-infected mice. In all 5 days pregnant infected C57BL/6 mice, the levels of IFN-gamma were raised compared with the levels of IL-4. In this group of mice, those mated at 15 days post *L. major* infection had highest level of IFN-gamma mRNA (Figure 2a). In 10 days preg-

nant infected C57BL/6 mice, IL-4 were raised compared with IFN-gamma but mice mated at 16hrs post *L. major* infection had highest level of IL-4 (Figure 2b). In pregnant C57BL/6 mice that were sacrificed at 15 days of pregnancy, the levels of IL-4 were higher compared with non-pregnant mice irrespective of the stage of *L. major* infection at which the mice were mated. When Figures 2a-2c were compared, it is obvious that the level of IL-4 consistently increases with gestation while IFN gamma consistently reduces with gestation. Figure 2d shows that mice infected with *L. major* 20 days post-partum produced more IFN-gamma than IL-4 from 16hrs post *L. major* infection.

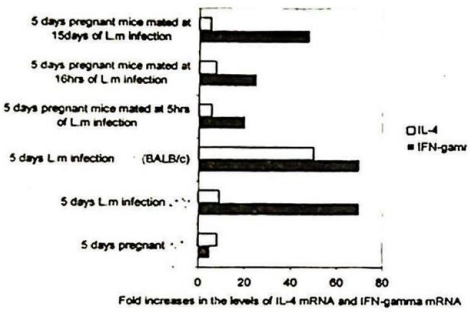

Fig. 2a:

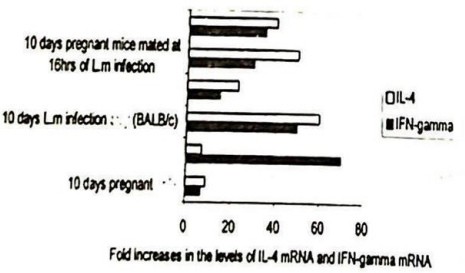

Fig. 2b:

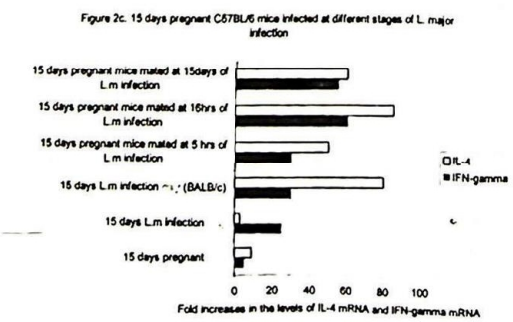

Fig. 2c:

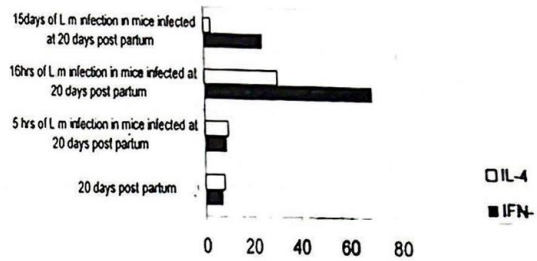

Fig. 2d:

Discussion

The footpad lesion sizes of pregnant susceptible BALB/c mice were larger than those of non-pregnant BALB/c mice. To this end, most (80%) of the pregnant infected BALB/c mice died 2 weeks earlier (Week 5) than non-pregnant infected BALB/c mice (Week 7). Moreover, pregnant resistant C57BL/6 mice were not able to resolve *L. major* infection as early as non-pregnant infected C57BL/6 mice. Till termination of experiment at week 14, the footpad lesion sizes of pregnant infected C57BL/6 mice remained slightly larger than those of non-pregnant infected C57BL/6 mice. These observations show that pregnancy impairs the ability to resolve intracellular infections.

Effective immunosuppressive mechanisms to dampen potential antifoetal immune reactions are required during pregnancy. Several such mechanisms have been described, including production of TGF- beta by decidual cells [14,15], IL-10 that suppresses cell-mediated immunity [4] and several unidentified serum factors [16]. *L. major* is a well-characterized macrophage parasite that requires strong Th1/cell-mediated response to resolve [5-8]. The present study evaluated whether pregnancy exerts an influence that impairs a successful immune response against *L. major* infection. The results showed that pregnant C57BL/6 mice failed to effectively control concurrent *L. major* infection, and that this was associated with a diminished IFN-gamma (Th 1 cytokine) and increase IL-4 (Th 2 cytokine) production by lymph node cells especially at days 10 and 15 of pregnancy in infected pregnant mice. Thus pregnancy inhibits a successful cell-mediated response against *L. major* as pregnancy advances. The cytokine interactions between *L. major* infection and pregnancy may act in both directions, as strong Th 1 immune responses against *L. major* may impair successful pregnancy.

Th 2 cytokine, which is represented in this study by IL-4, is associated with safe pregnancy till term [6]. This offers a plausible explanation for the down-regulation of the Th 1 anti-*L.major* response, but other gestational factors may be involved. Immunosuppressive properties are displayed by a number of gestational hormones

and proteins, including human gonadotropin, progesterone, TGF-beta, alpha-fetoprotein and early pregnancy factors [16]. Progesterone influences immune cytokine production, and cause an overall systemic shift towards a Th 2 response [17]. Th 2 cytokines selectively down regulates Th 1 reactivity by inhibiting the production of inflammation cytokines such as IFN-gamma and TNF-alpha that activate macrophages and are required for the parasite clearance during *L. major* infection [18]. IL-10 (Th 2 cytokine) aids gestation and inhibit macrophage activation while IL-4 exacerbates *L. major* infection [19]. Previous studies showed that natural killer cells (NK cells) are activated and inhibited by IFN-gamma and IL-10 respectively [4]. Pregnant mice exhibit diminished NK cell responses [19] whereas NK cells aid the differentiation of T cells toward a curative Th 1 pattern during *L. major* infection [18,20].

The lesions of resistant C57BL/6 mice stabilize at a moderate size for weeks and none of the mice developed the progressive infection characteristic of highly susceptible BALB/c strain. This is an indication that pregnancy did not totally abrogate the curative Th 1 anti-*L.major* response in non-pregnant C57BL/6 mice. This observation could be connected with diminished levels of IFN-gamma in pregnant C57BL/6 mice. The negative effects of pregnancy on resistance to *Leishmania* infection could further be demonstrated in rapid progression of footpad lesion in pregnant susceptible BALB/c mice.

As shown in this study, the impairment of protective immunity to *L. major* varies with stage of *L. major* infection. The regression of footpad lesion did not commence in C57BL/6 mice mated at 5hrs and 15 days post *L. major* infection until 12th week post infection whereas footpad lesion from those mated at 16hrs post infection healed from 8th week post infection. At 5hrs post *L. major* infection, the host might have developed no form of adaptive immune response. This will allow for the running of normal course of cytokine production during pregnancy. Thus, the Th 1 cytokine (especially IFN-gamma) of early stage of pregnancy will provide initial protection against *L. major* infection. Switching to Th 2 cytokine (especially IL-4) at late stage of pregnancy down regulates the IFN-gamma that provides initial protection against *L. major*, thus the increase in lesion sizes. There is massive production of IFN-gamma in 16hrs post *L. major* infection in resistant C57BL/6 mice. In contrast, IL-4 is excessively produced in susceptible BALB/c mice [3]. The IFN-gamma being produced in C57BL/6 mice mated at 16hrs post *L. major* infection might supplement IFN-gamma secreted as a result of early pregnancy to reduce the footpad lesion sizes despite Th 2 cytokines of late pregnancy.

Our study demonstrated that pregnancy affects the equilibrum of cytokines towards Th 2 and away from the Th 1 during the anti-*L.major* response in pregnant mice. These changes in cytokine patterns explain the pregnancy impairment of host resistance to *L. major* infection especially if time of conception is close to the time of infection.

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