Pregnancy and Humoral Immune Response in Mice Chronically Infected by Trypanosoma cruzi

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The effect of pregnancy on the humoral immune response induced by Trypanosoma cruzi was studied in groups of chronically infected and pregnant mice (IP) or chronically infected and nonpregnant mice (INP) of strain BALB/c. Groups of noninfected and nonpregnant mice (NIP) or noninfected and pregnant mice (NIP) served as controls. The pregnant mice were killed on day 17 of pregnancy. Anti-T. cruzi immunoglobulin G (IgG) and IgM antibodies, detected by immunofluorescence or enzyme-linked immunosorbent assay or both, underwent a pregnancy-associated decrease of 20 to 40%, whereas complement-mediated lytic antibodies were unaffected by pregnancy. Immunoblotting analysis indicated identical specificities of the anti-T. cruzi antibodies in IP and INP groups. The levels of all the immunoglobulin isotypes (particularly IgG2a and IgG3), circulating immune complexes, rheumatoid-like factor, and anti-DNA antibodies were considerably increased during chronic infection (NIP versus INP), which could be related to the high degree of polyclonal B-cell activation occurring in T. cruzi infection. However, pregnancy significantly decreased (by 20 to 60%) such parameters. IgG levels were particularly affected (by 40 to 60%), and the decreases could be ordered as follows: IgG3 > IgG2a > IgG1 > IgG2b for IP versus INP. Comparisons between the noninfected groups indicated differences only in IgG levels. These results indicate the following. (i) The specific humoral anti-T. cruzi immune response is weakly affected by pregnancy, which is not sufficient to modify the course of the mother’s infection. (ii) Pregnancy does not modify the expression of the anti-T. cruzi antibody repertory. (iii) Pregnancy reduces the polyclonal B-cell activation, particularly the levels of the IgG isotypes undergoing the greatest activation.

Trypanosoma cruzi infection is a major public health problem, estimated to affect over 24 million people in Latin America. Although many works have studied the dramatic congenital Chagas’ disease (1), very little information is available about other interactions between pregnancy and T. cruzi infection. In another study (Y. Carlier, M. T. Rivera, C. Truyens, F. Puissant, and J. Millaire, Am. J. Trop. Med. Hyg., in press), we studied fetal growth and reproductive capacity of mice chronically infected by T. cruzi. In the present study, we investigate the effect of pregnancy on the humoral immune response to T. cruzi. Indeed, the specific response seems to be very important in limiting the levels of circulating parasites (3). The chronic phase of T. cruzi infection is characterized by extremely low levels of these parasites associated with high levels of specific antibodies, some of which induce a complement-mediated lysis of blood trypomastigotes (17, 18). Another feature of the humoral immune response induced by T. cruzi infection is the high degree of nonspecific polyclonal B-cell activation, beginning soon after infection (8, 10, 22, 23) and persisting throughout the chronic phase of infection (9).

Pregnancy, characterized by a tolerance to the fetal allograft, is expected to be associated with a nonspecific immunodepression (for a review, see reference 13) which could influence the course of infectious and parasitic diseases (for a review, see references 19 and 20). Indeed, an increased susceptibility to newly acquired infections and reactivation of chronic ones have been observed during pregnancy, leading to more severe infections and higher mortality rates (2, 6, 21, 28, 29). However, in another study (Carlier et al., in press), we showed that pregnancy has no influence on chronic T. cruzi infection, since reactivation was not observed and basic parasitemia was similar in pregnant and nonpregnant mice. Consequently, it was interesting to study the influence of pregnancy on the humoral immune response of T. cruzi-infected mice.

The aim of the present work is to answer three questions. (i) Does pregnancy influence the levels of anti-T. cruzi antibodies (detected by serologic or complement-mediated lysis tests)? (ii) Is pregnancy able to modify the expression of the anti-T. cruzi antibody repertory (detected by immunoblotting analysis of the antigens recognized by antibodies from chronically infected and pregnant mice [IP] and chronically infected and nonpregnant mice [INP])? (iii) Does pregnancy modify the polyclonal B-cell activation associated with T. cruzi infection (comparing levels of immunoglobulins, circulating immune complexes [CIC], rheumatoid factor, and anti-DNA antibodies of IP and INP groups)?

MATERIALS AND METHODS

Experimental protocol. Nulliparous BALB/c mice, 8 weeks old and weighing 19.4 ± 1.9 g at the beginning of the experiment, were divided into four groups: (i) 15 IP, (ii) 27 INP (control group for infection), (iii) 21 pregnant and noninfected mice (NIP) (control group for pregnancy), and (iv) 14 nonpregnant and noninfected mice (NINP) (a general control group). They were kept in plastic cages in a controlled animal house.

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**T. cruzi infection.** To obtain a chronic infection, mice were infected intraperitoneally with 100 blood parasites (Tehuantepec strain) as described elsewhere (Carlier et al., in press).

**Mating and gestation.** Mice were mated when 120 days old, i.e., on postinfection day 60 (during the chronic phase) for the infected mice. On 4 successive days, females were checked for vaginal plugs. Mice found positive for this were considered day 1 of their gestation period.

**Killing and spleen and blood collection.** Mice were killed by cervical dislocation between day 137 and 140 of their life, i.e., on day 77 of infection for the infected groups and on day 17 of gestation for the pregnant groups. This date was specifically chosen in the last week of pregnancy (week 3) for mice, because this is the period in which pregnancy-associated immunodepression is known to be maximal (21). Pregnancy was checked by laparotomy. All offspring data have been reported elsewhere (Carlier et al., in press). Spleens were recovered after dissection and weighed to the nearest milligram. Blood samples were collected by cardiac puncture in a heparinized syringe. After centrifugation, plasma was recovered, aliquoted, and frozen until used. Individual samples were used for all serologic investigations, except for lytic anti-"T. cruzi" antibody studies, for which a plasma pool of each mouse group was prepared.

**Immunoglobulin levels.** Immunoglobulin G (IgG), IgA, IgM, IgG1, IgG2a, IgG2b, and IgG3 levels were evaluated by a solid-phase enzyme-linked immunosorbent assay (ELISA) with the appropriate antisera (Sigma Chemical Co., St. Louis, Mo.). The specificity of the antisera was controlled. Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 µl of the appropriate antisera diluted between 1/10,000 and 1/1,000,000 in 0.05 M carbonate buffer, pH 9.4 (overnight, at room temperature) and then saturated with 1% bovine serum albumin. For the assay, according to the studied subclasses, plasma samples were diluted between 1/1,000 and 1/40,000, and 100-µl samples were incubated in plates for 2 h at room temperature. Then, the plates were washed and incubated with 100 µl of the correspondingly diluted (1/1,000) anti-IgG, -IgA, -IgM antisera conjugated with alkaline phosphatase (Sigma). Nitrophenyl phosphate was used as the substrate. A492 was read. Results were expressed as the percentage of the value of a normal serum pool.

**Anti-ssDNA antibody levels.** Anti-single-stranded (ss) DNA activities were detected by a solid-phase ELISA with heat-denatured calf thymus DNA as the antigen (Sigma). Briefly, microtiter wells were incubated with 40 µg of protamine sulfate (2 h at room temperature) and then coated with 5 µg of ssDNA in 50 µl (5 h at room temperature). For the assay, plasma samples were diluted 1/100 and 50-µl samples were incubated overnight at 4°C. After the washes, antibody binding was revealed by a goat anti-mouse IgG antisem conjugated with alkaline phosphatase, diluted 1/1,000, and incubated 5 h at 4°C. Results are expressed as A492.

**Anti-rabbit IgG antibody levels.** Rheumatoid-like factor antibodies were detected by using purified rabbit IgG as the antigen (0.6 µg/50-µl well, incubated overnight at room temperature), in a solid-phase ELISA similar to that used for anti-ssDNA antibodies.

**CIC.** CIC were detected in serum with polystyrene plates coated with 1 µg of C1q per well (16). A 30-µl quantity of serum was mixed with 30 µl of phosphate-buffered saline and precipitated by 60 µl of polyethylene glycol. The precipitate was suspended in 0.2 M EDTA-0.8% Tween buffer, pH 7.5, and distributed into duplicate wells. The bound CIC were revealed by a goat anti-mouse IgG antisem coupled to alkaline phosphatase. The level of CIC in serum was obtained by referring to a standard curve constructed with mouse aggregated gamma globulins.

**Anti-"T. cruzi" antibodies.** Antibody tests were performed as previously described (4), with slight modifications. Briefly, ELISA was performed by using 0.5 µg (protein) of "T. cruzi" epimastigote soluble antigenic extract per well to coat microtiter wells (100 µl). All incubations were done for 1 h at 37°C. Mouse plasma samples (100 µl) diluted 1/500 were then incubated. After the washes, 100 µl of peroxidase-conjugated anti-mouse IgG (Institut Pasteur Production, Paris, France) or IgM (Cappel Laboratories, Cochranville, Pa.) was added and diluted 1/1,000 and 1/8,000, respectively. After the addition of H2O2 as the substrate and ortho-phenylene diamine as the chromogen, A405 was read. Mean absorbance of the NINP sera, considered nonspecific background, was subtracted from all sample results.

An immunofluorescence test was performed on "T. cruzi" epimastigotes fixed in 1% glutaraldehyde. Plasma samples were serially (twofold) diluted, beginning with the 1/4 dilution (for which all mouse sera from noninfected groups were negative). Fluorescein isothiocyanate-conjugated anti-mouse IgG or IgM (Institut Pasteur Production) was used at a dilution of 1/200. Results were expressed as the last titer giving a positive reaction.

**Lytic anti-"T. cruzi" antibodies.** Complement-mediated lysis of "T. cruzi" trypomastigotes was assessed according to Krettli et al. (18), with slight modifications. Briefly, trypomastigotes were obtained according to Gutteridge et al. (15), from rats previously irradiated (700 rad) and inoculated with 1.5 x 105 parasites. Rats were killed on postinfection day 24. Heparinized blood was collected by cardiac puncture and put through a DEAE-cellulose column to separate blood cells bound to the column from trypomastigotes which left the column, free to be collected. For the lysis test, parasites were incubated at 37°C for 30 min in 60 µl of a mouse serum sample diluted in Dulbecco modified Eagle medium. After centrifugation, parasites were resuspended in 30 µl of medium. Triplicate 5-µl samples of this suspension, containing 2 x 105 to 3 x 105 parasites, were distributed in wells of Terrasaki plates with an equal volume of fresh human serum as the complement source. After incubation for 60 min at 37°C, the living parasites in each well were counted in a Thoma's chamber, and the percentage of lysis was calculated.

**Immunoblotting.** To compare the antibody specificities of INP and IP groups, the transfer technique described by Towbin et al. (27) was used. Epimastigotes of "T. cruzi" (105) were lysed in a solution of 0.5% Nonidet P-40, 0.5 mM TLCK (Nα-p-tosyl-L-lysine chloromethyl ketone; Sigma), and Tris hydrochloride buffer, pH 6.8, and submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (gradient of 10 to 20% polyacrylamide) in 0.025 M Tris-glycine buffer, pH 8.9. A low-molecular-weight standard (Pharmacia, Inc., Piscataway, N.J.) was included in each slab, and electrophoresis was performed under constant voltage (50 V) at room temperature for 15 h. The slab gels were then soaked for 1 h in cooled transfer buffer (192 mM glycine, 25 mM Tris with 20% methanol, pH 8.3) to avoid further shrinkage during transfer. The separated proteins in gels were then transferred to nitrocellulose paper (Sartorius; porosity, 0.2 µm) with the Bio-Rad Trans-blot cell for 5 h at 4°C, at a constant intensity of 250 mA and with stirring. At the end of transfer, standards were stained with 1% amido black and slabs were stained with Coomassie blue solution to assess the efficiency of the transfer. To detect the transferred...
antigenic proteins, nitrocellulose membranes were saturated in 4% bovine serum albumin solution in phosphate-buffered saline for 30 min, then cut into strips, and incubated overnight at room temperature with mouse serum samples diluted 1/100, with constant stirring. After being washed in phosphate-buffered saline–0.3% Tween, the strips were incubated for 60 min with peroxidase-labeled anti-mouse immunoglobulin conjugate at a dilution of 1/750 (Institut Pasteur Production). They were then washed and revealed by chloronaphthol peroxide diluted in Tris-saline buffer for 30 min and rapidly washed in phosphate-buffered saline before drying. Scanning studies were performed with a Shimadzu CS930 densitometer.

Serum albumin levels. It has been suggested that hemodilution, due to the increased vascular compartment related to the presence of placenta and fetuses, could be a factor contributing to decreased levels of circulating immunological parameters (12, 20). Therefore, serum albumin levels, which are not influenced by the immune response, were quantified in the different mouse groups as indices of hemodilution (12). The Technicon automatic colorimetric procedure with bromocresol green was used according to Doumas et al. (11). Results are expressed in milligrams per milliliter.

Analysis of results. The Mann-Whitney Wilcoxon nonparametric test was used to compare the data groups and to estimate the $P$ values (24). To express the magnitude of such differences between the groups, a relative variation percentage was calculated by using the ratio of mean levels of the parameter being compared. Increases were indicated as positive values, and decreases were indicated as negative values.

RESULTS

Immunoglobulin levels. Comparisons between INP and IP groups (Fig. 1; Table 1) showed an important pregnancy-associated decrease during infection for IgG ($-39\%; P < 0.001$), whereas IgM levels were less affected ($-21\%; P < 0.05$) and IgA was unaffected. The magnitudes of the decreases were different among the IgG subclass levels and were ordered as follows: IgG3 > IgG2a > IgG1 > IgG2b ($-59$ to $-44\%; P < 0.001$). By comparing NINP and IP groups, it could be concluded that pregnancy without infection led to a significant decrease only of IgG levels ($-46\%; P < 0.001$). Decreases in the IgG subclass levels were ordered IgG2b > IgG3 > IgG1 > IgG2a ($-53$ to $-19\%; 0.001 < P < 0.01$). Comparisons between NINP and IP groups showed that infection by T. cruzi induced a slightly significant increase of IgA ($+48\%; P < 0.01$) and IgM levels ($+81\%; P < 0.001$) and a particularly important increase of IgG ($+364\%; P < 0.001$). IgG subclasses increased in the order IgG2a > IgG3 > IgG1 > IgG2b ($+1,389$ to $+72\%; P < 0.001$), with a remarkable increase for IgG2a ($+1,389\%$) and IgG3 ($+548\%$).

CIC, CRF, and anti-DNA antibody levels. Comparisons between INP and IP groups (Fig. 2; Table 1) showed that CIC were slightly decreased by pregnancy ($-19\%; P < 0.05$), whereas circulating rheumatoid-like factor antibody (CRF) and anti-DNA antibodies were more affected (more than $-42\%; 0.001 < P < 0.01$). Pregnancy alone (NINP versus [vs] NIP) had no influence. T. cruzi infection (NINP vs INP) considerably increased the level of the three evaluated parameters ($+351$ to $+593\%; P < 0.001$).

Anti-T. cruzi antibody levels. Pregnancy (INP vs IP) induced a significant decrease of IgG antibodies, detected by both an immunofluorescence test and ELISA ($-22$ to $-37\%; P < 0.001$), whereas only IgM antibodies detected by an immunofluorescence test were modified ($-29\%; P < 0.01$) (Fig. 3; Table 1).

Lytic anti-T. cruzi antibody levels. The percentage of lysis decreased regularly from serum dilution 1/2 to 1/128 (Fig. 4). For this last dilution, lysis was no longer detected. Statistical analysis showed no significant difference between INP and IP data.

Specificity of anti-T. cruzi antibodies. Examples of immunoblots are shown in Fig. 5. NINP and NIP blots were all

| Table 1. Comparisons of the different mouse groups for the parameters studied |
|-----------------------------|-----------------------------|-----------------------------|
| Parameter (test) | Comparison of mean group values | Comparison of mean group values |
|                | NINP/NIP | NINP/NINP | INP/IP |
|                | % | % | P | P |
| IgA             | NS | +48 | <0.01 | NS |
| IgM             | NS | +81 | <0.01 | -21 | <0.05 |
| IgG             | -46 | <0.001 | +364 | <0.001 | -39 | <0.001 |
| IgG1            | -44 | <0.001 | +151 | <0.001 | -49 | <0.001 |
| IgG2a           | -19 | <0.01 | +1,389 | <0.001 | -53 | <0.001 |
| IgG2b           | -53 | <0.001 | +72 | <0.001 | -44 | <0.001 |
| IgM             | NS | +548 | <0.001 | -59 | <0.001 |
| CIC             | NS | +593 | <0.001 | -19 | <0.05 |
| CRF             | NS | +351 | <0.001 | -43 | <0.01 |
| aDNA Ab         | NS | +588 | <0.001 | -42 | <0.001 |
| aTc IgG Ab (IF) | NS | +22 | <0.001 | -22 | <0.001 |
| aTc IgM Ab (IF) | NS | -29 | <0.001 | -29 | <0.001 |
| aTc IgG Ab (ELISA) | NS | -37 | <0.001 | -37 | <0.001 |
| aTc IgM Ab (ELISA) | NS | -37 | <0.001 | -37 | <0.001 |
| Spleen weights  | NS | +71 | <0.001 | +13 | <0.05 |
| Serum albumin   | NS | +58 | <0.01 | -20 | <0.01 |

* Abbreviations: Ab, antibody; IF, immunofluorescence test; aTc, anti-T. cruzi.

* Estimated from the nonparametric Mann-Whitney Wilcoxon test.
negative. INP and IP blots showed 16 antigenic bands of variable intensity. Eight main bands were selected for comparison studies. No significant difference could be observed between frequencies of the main antigenic molecules recognized by antibodies from INP and IP plasma (Table 2). The mean relative area peaks obtained by densitometric analysis were similar for six of the eight bands studied. Two bands showed a lower density in IP than in INP blots (Table 2).

**Spleen weights.** Spleen weights were increased during T. cruzi infection (for NINP vs INP, +71%; \( P < 0.001 \)). Association of pregnancy and T. cruzi infection led to higher spleen weight values (+13%; \( P < 0.05 \)), whereas pregnancy alone was without effect (NINP vs NIP).

**DISCUSSION**

Comparing the infected mouse groups INP and IP, specific anti-T. cruzi antibodies underwent a pregnancy-associated
TABLE 2. Main antigens in immunoblots from infected mouse groups

<table>
<thead>
<tr>
<th>Band</th>
<th>No. of plasma samples with band (%)a</th>
<th>Relative peak area of bandb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>INP</td>
<td>IP</td>
</tr>
<tr>
<td>C</td>
<td>11 (91.7)</td>
<td>12 (92.3)</td>
</tr>
<tr>
<td>D</td>
<td>8 (66.7)</td>
<td>8 (61.5)</td>
</tr>
<tr>
<td>G</td>
<td>5 (41.7)</td>
<td>6 (46.1)</td>
</tr>
<tr>
<td>K</td>
<td>7 (55.3)</td>
<td>6 (46.1)</td>
</tr>
<tr>
<td>M</td>
<td>4 (33.3)</td>
<td>7 (53.8)</td>
</tr>
<tr>
<td>O</td>
<td>7 (58.3)</td>
<td>6 (46.1)</td>
</tr>
<tr>
<td>Q</td>
<td>12 (100.0)</td>
<td>13 (100.0)</td>
</tr>
<tr>
<td>U</td>
<td>7 (58.3)</td>
<td>5 (38.5)</td>
</tr>
</tbody>
</table>

a Antigens recognized by anti-T. cruzi antibodies, as detected by scanning densitometry.
b Differences between INP and IP were nonsignificant for all bands, as determined by the nonparametric Mann-Whitney Wilcoxon test.
c Differences between INP and IP were nonsignificant for all bands, except for bands D and U, for which P < 0.05, as determined by the nonparametric Mann-Whitney Wilcoxon test.

decrease (20 to 40%), whereas lytic antibodies, considered good indices of protective immunity (17), were unaffected by pregnancy. Protective antibodies have been shown to belong mainly to the IgG2b subclass (25), which was decreased less by pregnancy than the other immunoglobulin subclasses were. These observations can be related to the absence of mortality and the similar parasitemia previously observed in the pregnant (IP) and nonpregnant mice (INP) (Carlier et al., in press). Consequently, in answering the first question, whether the specific humoral anti-T. cruzi immune response is affected by pregnancy, the decrease is weak and not sufficient to modify the course of the mother's infection. As far as we know, such results have never been previously reported in chronic T. cruzi infection. In other chronic parasitic diseases, pregnancy has been shown either to be without influence, as in Toxoplasma infection (21), or to lead to relapses with high rates of lethality, as in Plasmodium infection (2, 6, 28, 29), although such a dramatic effect is more frequently obtained when pregnancy is associated with acute infections (21).

Since the different immunoglobulin and antibody isotypes did not undergo a uniform decrease of their levels, it is possible that pregnancy also exerts different influences on the expression of the anti-T. cruzi antibodies. However, only quantitative modifications, without interference with the specificities recognized by the anti-T. cruzi antibodies, were observed in the immunoblotting analysis of IP and INP sera. Consequently, in answering the second question, it can be assumed that pregnancy is not associated with a restriction in the expression of the anti-T. cruzi antibody repertoire.

The effect of chronic T. cruzi infection on the immunological status of nonpregnant mice (NINP vs INP) is characterized by a major increase in serum of the levels of all the immunoglobulin isotypes, with the occurrence of CIC, CRF, and anti-DNA antibodies. Sera from chronically infected mice have been demonstrated to contain 6 to 8 times more T. cruzi unrelated antihapten or antigen antibodies (anti-arsonate, -trinitrophenyl, -fluorescein isothiocyanate, and -tobacco mosaic virus) than the controls did (unpublished results). Therefore, it can be postulated that the high levels in serum of immunoglobulins, CIC, CRF, and anti-DNA antibodies are probably related to a nonspecific activation. This could confirm the high polyclonal B-cell activation previously claimed to occur during T. cruzi infection (8-10, 22, 23). However, it must be noted that, in our model of chronic infection, the polyclonal B-cell activation is higher for isotypes IgG2a and IgG3, whereas in a previous study, the restriction mainly concerned IgG2a and IgG2b (9). Since the latter experiment (9) showed no variation due to the mouse strain, such a difference could be related either to the methodology used (serum immunoglobulin levels in our own study instead of plaque-forming cells in the D'Imperio Lima ones) or to the parasite strain (Tehuantpec vs CL). The splenomegaly observed in the INP group is a classical feature of T. cruzi infection, reflecting the mouse's response to invasive organisms (26).

The effect of pregnancy on T. cruzi-associated polyclonal B-cell activation is shown in the comparison of INP and IP groups, with a significant decrease (20 to 60%) of all the parameters studied (except for IgA). It must be stressed that IgG levels were particularly affected (by 40 to 60%), principally the IgG2a (~53%) and IgG3 (~59%) isotypes. Thus, the third question can be answered, that pregnancy decreases all the products of the polyclonal B-cell activation, particularly the isotypes undergoing the highest activation.

Pregnancy was found to affect the products of polyclonal B-cell activation more than the anti-parasitic antibodies. The polyclonal B-cell activation occurring in T. cruzi infection would be dependent on both a parasite-derived mitogenic activity (7, 10, 14) and T cells, which are probably different from T cells involved in the specific immune response (5, 22a). So, it could be postulated that pregnancy would affect T-cell subsets involved in the nonspecific response more than subsets involved in the specific response, thus explaining the lower effect of pregnancy on anti-T. cruzi response.

Interestingly, pregnancy in noninfected mice (comparing NINP and NIP groups) was associated with a significant decrease only in IgG isotypes. This effect of pregnancy cannot be due to hemodilution, since albumin serum levels were unaffected by pregnancy without infection.

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LITERATURE CITED