Metabolic, Endocrine and Genitourinary Pathobiology

Infertility in Murine Acute *Trypanosoma cruzi* Infection Is Associated with Inhibition of Pre-Implantation Embryo Development

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We previously showed that *Trypanosoma cruzi* acute infection induced infertility in a great proportion of female mice, which resulted from a defect taking place before implantation. In this study, we have analyzed every step of reproduction from mating to implantation to identify the most sensitive event. Our results show that mating, ovulation, fertilization, and first division of the zygote of infected mice take place normally compared with uninfected mice, indicating that the defect occurred after the two-cell stage. *In vivo* development of two-cell embryos to the blastocyst stage was indeed dramatically delayed; some embryos even arrested their development before having reached the eight-cell stage while others degenerated. The effect was less pronounced when embryos were allowed to develop *in vitro*, indicating that the infectious context of the mother plays a role in maintaining growth retardation. The delay of embryonic development was associated with insufficient divisions of the blastomeres and led to abnormal blastocyst outgrowth that may explain implantation failure. Inhibition of cell division was correlated with the maternal parasitemia. This work clearly shows that *T. cruzi* infection dramatically impedes embryonic development, offering a model for further *in vivo* studies of embryotrophic factors produced by the oviduct of infected females. (Am J Pathol 2006, 169:1730–1738; DOI: 10.2353/ajpath.2006.060309)

Infertility, concerning women and men equally, affects about 10% of couples throughout the world. Its prevalence, however, varies dramatically between countries, depending on the incidence of causes. It is generally

higher in developing countries where preventable causes are not well controlled. Causes of infertility include anatomical, genetic, endocrinological, and immunological disorders as well as, in 20 to 30% of cases, pelvic inflammatory diseases and infections of the upper genital tract mainly due to sexually transmitted diseases.^{1,2} Pathogens may also prejudice the female reproductive ability even when the reproductive tract is not itself infected, by inducing systemic disturbances that affect the hormonal and cytokine equilibrium necessary for successful reproduction, the embryo itself, or its environment.^{3–5}

Along this line, we recently showed that the acute infection with the protozoa *Trypanosoma cruzi*, the agent of Chagas disease in Latin America, totally impaired reproduction of mice by drastically reducing their fertility and inducing massive fetal death.⁶ Indeed, about 80% of infected mice were infertile, whereas in those that developed a gestation, all embryos died following parasite invasion of the placenta, causing ischemic necrosis and maternal production of high levels of tumor necrosis factor- α (TNF- α).⁷ Because our previous work suggested that infertility resulted from a defect occurring before implantation, we presently studied all of the steps of reproduction from mating to implantation to specify the impact of acute *T. cruzi* infection. Our results indicate that the infertility phenotype does not result from any abnormality in ovulation, fertilization, and cleavage of the zygote into a two-cell stage embryo but from a dramatic deleterious effect of infection on further cell cycles, compaction, and cavitation.

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Materials and Methods

Mice, *T. cruzi* Infection, and Mating

BALB/c mice were purchased from B&K Universal (Hull, UK) and maintained in a conventional animal house. Two-month-old females were infected subcutaneously (in the footpad) by inoculation of 100 blood trypomastigotes in 50 μ l of sterile, endotoxin-free phosphate-buffered saline (PBS) (of the Tehuantepec strain of *T. cruzi* maintained in our laboratory). The control group of uninfected age-matched mice received 50 μ l of PBS. To obtain pregnancies during the ascending phase of parasitemia, female mice were put with uninfected males at the end of the 6th day postinfection (p.i.) as previously described.⁶ The presence of a vaginal plug, indicating that mating had occurred, was checked every morning for 4 days. Females showing a vaginal plug were immediately separated from the males. The morning of sighting a vaginal plug was denoted day 0.5 of gestation (G0.5). In that way, the preimplantation period of the gestation (ie, until day of gestation 4.5) occurred before days 11 to 15 p.i.

Induction of Superovulation

Superovulation in female mice at day 5 p.i. and age-matched mice was induced by intraperitoneal injections of 10 IU of pregnant mare's serum gonadotropin (Folligon; Intervet, Boxmeer, The Netherlands) in 0.25 ml of PBS, followed 48 hours later by 10 IU of human chorionic gonadotrophin (Pregnyl; Organon Belgium, Brussels, Belgium) in 0.25 ml of PBS. These mice were then caged with males overnight and inspected for vaginal plugs the following morning.

Collection of Oocytes and Embryos

Fully grown primary oocytes [germinal vesicle (GV) stage] were collected by puncture of ovaries from non-mated mice. Mice were sacrificed by cervical dislocation, and the peritoneal cavity was immediately opened. The ovaries were collected and individually transferred into a 35-mm Petri dish containing 1 drop of Krebs-Ringer-bicarbonate culture medium⁸ supplemented with 4 mg/ml bovine serum albumin (BSA; Sigma, St. Louis, MO) (KRB4-BSA) and containing 45 μ g/ml 3-isobutyl-1-methylxanthine (Sigma) to prevent oocytes from undergoing their spontaneous *in vitro* maturation. Oocytes were released from the antral follicles using two hypodermic needles under a stereomicroscope. They were freed from the remaining follicular cells by aspiration with a Pasteur pipette of 100- μ m internal diameter.

Ovulated metaphase II oocytes were collected from the oviducts of mated mice at G0.5. After animal sacrifice and opening of the peritoneal cavity, the oviducts were taken and placed in KRB4-BSA medium plus hyaluronidase (300 μ g/ml). Under a stereomicroscope, the wall of the oviducts was cut at the level of the enlarged ampulla, which releases the cumulus oophorus. The oocytes were

then collected with a Pasteur pipette and rinsed twice in KRB4 medium.

Two-cell stage embryos were collected by flushing the oviducts at G1.5 and collected in KRB4-BSA medium while blastocysts were flushed from the uterine horns at G3.5 in Dulbecco's modified Eagle's medium/25 mmol/L glucose (Cambrex, Verviers, Belgium) supplemented with 10% fetal calf serum. Flushing was carried out with a Pasteur pipette of 200 μ m in diameter.

Culture of Oocytes and Embryos for the Study of Maturation and Development *in Vitro*

To allow the GV-stage oocytes (primary oocytes) to undergo their meiotic maturation *in vitro*, they were rinsed 3 times in KRB4-BSA to eliminate 3-isobutyl-1-methylxanthine and incubated in four-well multidishes (Nalge Nunc, Naperville, IL) for 16 hours at 37°C in a humidified atmosphere of 5% CO₂. Oocytes from a single female were pooled in each well containing 0.8 ml of KRB4-BSA under 0.3 ml of paraffin oil (Sigma). Oocytes were checked for either germinal vesicle breakdown alone (supposed to be arrested at the metaphase I stage) or both germinal vesicle breakdown and extrusion of the first polar body (matured, metaphase II stage).

Two-cell stage embryos were cultured for 4 days at 37°C in 0.8 ml of KRB4 medium supplemented with 4 mg/ml BSA under 0.3 ml of paraffin oil in a humidified atmosphere of 5% CO₂.

The fully grown blastocysts collected at G3.5 were cultured in 0.8 ml of Dulbecco's modified Eagle's medium/25 mmol/L glucose/10% fetal calf serum under 0.3 ml of paraffin oil. They were gently placed one by one on a sterile glass coverslip put on the bottom of each of the wells of the four-well dishes.

In some experiments, embryos obtained from different mice were incubated separately to correlate the results with the maternal parasitemia.

Fluorescent Labeling of Outgrown Blastocysts

To analyze the *in vitro* differentiation of blastocysts, the glass coverslips on which they adhered were removed from the culture medium after 6 days, rinsed three times in PBS, fixed in PBS containing 1.7% paraformaldehyde for 20 minutes, and permeabilized in PBS containing 2% Triton X-100 for 30 minutes. F-actin (microfilaments) was stained for 2 hours 30 minutes in PBS containing 1% fluorescein isothiocyanate-conjugated phalloidin (Sigma) and 1% Tween 20. Coverslips were then rinsed in PBS-Tween 20 and mounted in Vectashield medium (Vector Laboratories, Burlingame, UK). Chromatin was stained with TOTO-3 (thiazole orange homodimer; Molecular Probes, Eugene, OR) diluted at 1:200 in the mounting medium (Vectashield). F-actin and chromatin were visualized by confocal fluorescence microscopy using a TCS 4D confocal laser-scanning microscope (Leica, Wetzlar, Germany) equipped with an Ar/Kr laser source.

Table 1. Reproductive Capacity of Mice Acutely Infected with *T. cruzi* or Noninfected, From Ovulation to Two Cell Embryos

Parameter studied	Mouse group	
	Noninfected	Infected
Number of oocytes in ovaries	22.1 ± 1.3 (9)*	24.0 ± 2.5 (10)
Number of ovulated oocytes [†]	26.4 ± 2.0 (10)	29.1 ± 1.8 (10)
Proportion of fertilized oocytes [‡]	59.9 ± 10.1 (4)	46.8 ± 5.2 (5)
Number of embryos/mouse at G1.5	7.2 ± 0.8 (6)	8.4 ± 0.8 (9)

*Number of female mice.

[†]Total number (fertilized or not) per mouse that had ovulated, whether they presented a vaginal plug or not.

[‡]In mice that presented a vaginal plug.

Estimation of the Cell Number in Embryos

The embryos were prepared for cell counting by the air-drying method of Tarkowski⁹ slightly modified by Tomkins et al.¹⁰ In brief, under a stereomicroscope, embryos were rapidly rinsed one by one in 3 drops of PBS and 2 drops of anhydrous sodium citrate 1% in distilled water successively and incubated for 10 minutes in a third drop of sodium citrate to induce osmotic swelling. They were then immediately placed on a glass slide and fixed with acetic ethanol [ethanol/acetic acid 3:1 (v/v)] to fix the nuclei, which were subsequently stained with Giemsa for 10 minutes.

Determination of the Number of Implantations Sites

Mated mice that presented a vaginal plug were sacrificed at G5, ie, a few hours after implantation normally occurs.¹¹ The uterine horns were collected and implantation sites were macroscopically counted.

Results

The Infertility of Mice Acutely Infected with *T. cruzi* Results from a Defect Occurring after Mating and before Implantation

As previously published, the infertility of mice acutely infected with *T. cruzi* did not result from a lower rate of mating.⁶ Results from the experiments performed in this study for evaluating fertilization and embryonic development confirmed these observations. Indeed, the rate of mating, assessed by the observation of vaginal plugs, was $71.3 \pm 8.7\%$ ($n = 86$) and $78.7 \pm 7.72\%$ ($n = 86$), $P > 0.05$, for uninfected and infected mice, respectively. Our previous results also suggested that the infertility of acutely infected mice resulted from a failure that occurred before implantation, since no implantation scars were visible in their uterus. However, this observation was done 9 days after mice had mated, ie, 4.5 days after implantation. To confirm this result, we have sacrificed infected mice 5 days after successful mating, ie, just after the time of implantation, and looked for implantations macroscopically visible on dissected uterine horns. Although 3/4 (75%) uninfected females presented implan-

tations (8.0 ± 1.2 /mouse), only 2/9 (22%) infected females did (7.5 ± 0.5), in agreement with a previous observation.⁶ These results clearly show that infertility of acutely infected mice results from a defect occurring after mating and before implantation, justifying a careful analysis of every step of reproduction before implantation.

T. cruzi Infection Does Not Affect the Yield of Primary Oocytes, Oocyte Maturation, Ovulation, Fertilization, and First Cleavage of the Zygote

Acutely infected females were sacrificed before mating (ie, at day 7 p.i.) to evaluate the number of oocytes harvested from antral follicles. As shown in Table 1, puncture of ovaries of both uninfected and infected females supplied us with similar numbers of oocytes, which all displayed a normal morphology (primary oocytes: GV stage) within their *zona pellicula*.

We then looked for their capacity to undergo maturation, a cytological process that makes the oocytes able to become fertilized. This normally takes place in the ovaries as a result of the preovulatory surge of luteinizing hormone.¹² It can also be stimulated *in vitro* by releasing the oocyte from the antral follicle in a suitable medium in which it undergoes germinal vesicle breakdown and first polar body extrusion (1pb) within 12 hours (see Materials and Methods). As shown in Figure 1, after 16 hours of culture, most of the primary oocytes (80%) from uninfected mice had indeed reached the metaphase II stage (1pb+). As usual, few of them, being meiotically incompetent, were still arrested at the primary state (GV+). Some others were in an intermediate state of maturation (metaphase to anaphase I of meiosis, characterized by the absence of both GV and 1pb), or were degenerated. Maturation of primary oocytes took place similarly when they were obtained from *T. cruzi*-infected females.

We next studied the capacity of mice to ovulate and the ability of the ovulated secondary oocytes to be fertilized. To obtain a large number of oocytes, superovulation was induced by treating the females with gonadotrophic hormones before mating. They were sacrificed at day 0.5 of gestation (G), and both secondary oocytes and zygotes (presence of pronuclei and the second polar body, pb2) were counted after disruption of their oviducts. Despite the hormonal treatment, the same propor-

Figure 1. In vitro maturation of primary oocytes from *T. cruzi* uninfected and acutely infected mice. Primary oocytes were collected from the ovaries of female mice at day 7 p.i. and of age-matched uninfected mice and were allowed to mature *in vitro* for 16 hours. Maturation stages after this incubation were morphologically determined as follows: primary, presence of a germinal vesicle; intermediate, neither GV nor first polar body (metaphase to anaphase I); secondary, presence of the first polar body (metaphase II stage); and degenerated, fragmented. Results from two experiments performed with 88 and 118 oocytes from uninfected mice (gray bars) and 117 and 133 from infected mice (black bars) were pooled. Data are expressed as the mean $\pm$ SEM of the proportion of each stage.

Figure 2. Distribution of embryonic stages at day 3.5 of gestation in *T. cruzi* acutely infected mice. Female mice infected at day 0 with 100 parasites were sacrificed at G3.5, corresponding to days of infection 11 to 14. Embryos were collected from the uterine horns, and their stage of development was determined by microscopic examination. Three experiments giving similar results were pooled. Results are expressed as mean $\pm$ SEM of the proportion of embryonic stages/gravid mouse from 34 infected female mice (black bars) and 27 age-matched uninfected female mice (white bars) that gave a total of 192 and 165 embryos, respectively. Asterisks indicate significant differences between mouse groups (* $P < 0.05$, ** $P < 0.005$, Mann-Whitney-Wilcoxon *U* test).

tion of mice failed to ovulate in both infected and uninfected groups. Seventy percent (10/14) and 77% (10/13) of the mice did ovulate in response to the hormonal stimulation, respectively. Table 1 shows that the number of ovulated oocytes and the proportion of them that had been fertilized were also similar in both groups of mice, as was the morphology of oocytes and zygotes (not shown).

To assess whether zygotes normally underwent their first cleavage, the presence of two-cell embryos was checked in nonstimulated mated females at G1.5. After flushing of their oviducts, we found a similar number of two-cell stage embryos in both groups of mice (Table 1). This clearly shows that *T. cruzi* infection does not impair the very first step of embryonic development. Because no unfertilized oocytes were found, it indicates that ovulation and fertilization also took place normally in infected mice that had not been hormonally stimulated.

It may thus be concluded from these first results that acute *T. cruzi* infection does not affect reproduction steps from ovulation to the two-cell stage.

Acute T. cruzi Infection Induces Delay in Embryonic Development and Inhibits Cell Division in Embryos in Relation to Maternal Parasitic Load

We then studied the embryonic development later in gestation. Acutely infected mice displaying a vaginal plug were sacrificed at day 3.5 of gestation (G3.5). Embryos were flushed from the uterine horns, and the different embryonic stages were identified and counted. The results from three experiments giving similar results were

pooled. Embryos were not found in all mice, but *T. cruzi* infection did not significantly decrease either the proportion of mice containing embryos [62.8 and 77.3% in uninfected ($n = 27$) and infected ($n = 34$) mice, respectively] or the mean total number of embryos/mouse (6.1 ± 0.7 and 5.7 ± 0.4 , respectively, $P > 0.05$, Mann-Whitney-Wilcoxon *U* test). As expected, most of the embryos (71.8%) collected at G3.5 from control uninfected mice had reached the blastocyst stage (Figure 2).¹¹ On the contrary, in acutely infected females, only 38.3% of the embryos were identified as blastocysts ($P < 0.05$), with a quite normal morphology. As a consequence, the proportion of infected females harboring blastocysts was also lower compared with uninfected females (70.6 versus 92.6% of females, respectively, Fisher test: $P = 0.031$). Such significantly reduced proportion of blastocysts was associated with a higher proportion of degenerated embryos (containing blastomeres of variable size and displaying cell fragmentation) and a higher proportion of earlier embryonic stages. It is worth noting that a very few embryos of infected females were still at the two- and four-cell stage, a situation not observed in uninfected mice. These results clearly show that acute *T. cruzi* infection delays the embryonic development between days 1.5 and 3.5 of gestation and partly induces embryonic degeneration, leading to a lower number of blastocysts.

To correlate this developmental delay with lengthening of the cell cycles, nuclei of the morulae and the blastocysts were counted. The morulae from infected and uninfected mice contained similar number of nuclei (27.6 ± 1.0 , $n = 16$, versus 29.0 ± 1.6 , $n = 30$, respectively). By contrast, the blastocysts from infected females were made of a significantly reduced number of cells when compared with the control situation (47.4 ± 1.8 , $n = 34$,

versus 55.9 ± 2.0 , $n = 30$, $P = 0.001$). This strongly suggests that cell cycles were lengthened if not arrested.

Because at G3.5 infected mice are at the beginning of the parasitemic phase, we looked for a relation between the abnormalities in embryo development and the maternal parasitic load. The average cell number of the morulae was significantly and inversely correlated with the maternal parasitemia ($r = -0.448$, $P = 0.007$, $n = 30$), a similar correlation being obvious for blastocysts. This strongly suggests that the inhibition of cell division in embryos of infected mice was related to the maternal parasite burden.

In Vitro Incubation Partially Rescues Embryos from Developmental Delay

To gain insight into the relation between *T. cruzi* infection and the slowing of embryonic development, embryos collected at days 1.5 and 3.5 of gestation were incubated *in vitro*, to allow their development out of the infectious *in vivo* context.

Two-cell stage embryos collected at G1.5 were cultured for 3 days *in vitro* and scored 24, 48, and 72 hours later for the stage they reached. Figure 3 shows that most of the embryos from uninfected females reached the four-cell stage after 24 hours, the morula stage after 48 hours, and the blastocyst stage after 72 hours. When harvested from infected females, embryos displayed a normal morphology but apparently progressed at a slightly lower pace. Fewer embryos from these females were indeed over the four-cell stage after 24 hours of culture, no embryos reached the blastocyst stage after 48 hours of culture, and finally, a higher proportion of them were degenerated at 72 hours. Moreover, some embryos from infected females persistently remained at early stages, an event that did not occur in the control group.

To determine whether embryos from infected females are able to undergo the peri-implantation steps of their development (hatching from the zona pellucida and trophoderm outgrowth and differentiation into giant trophoblast cells), embryos were collected at G3.5. Both morulae and blastocysts were cultured *in vitro* for 6 days and scored every 24 hours for hatching, attachment, and spreading. After 24 hours, all morulae had developed into blastocysts, at the same rate in both groups (Figure 4). Afterward, however, the blastocysts from uninfected females hatched and spread more rapidly than those from infected mice, leading to significant differences in the respective proportions of stages scored at days 2 and 3 (Figure 4). Nevertheless, after 5 to 6 days of culture, embryos from infected mice had reached the spread blastocyst stage. It is worth mentioning that mortality of embryos from infected females incubated *in vitro* even for 6 days was not higher than the one of control embryos. This is in sharp contrast to what we observed *in vivo* at G3.5 (Figure 2), and after 3 days of *in vitro* culture of two-cell embryos collected at G1.5 (Figure 3).

Some blastocysts collected at G3.5 from uninfected ($n = 8$) or infected mice ($n = 7$) were allowed to

Figure 3. *In vitro* development of two-cell embryos collected at day 1.5 of gestation in *T. cruzi* acutely infected mice. Female mice infected at day 0 with 100 parasites were sacrificed at G1.5, corresponding to days of infection 9 to 12. Two-cell embryos were collected from the oviducts and incubated *in vitro* to follow their development. The different embryonic stages, identified by microscopic examination, were counted after 24, 48, and 72 hours. Results are expressed as mean $\pm$ SEM of the proportion of embryonic stages/gravid mouse from nine infected females (black bars) and six age-matched uninfected mice (gray bars) that gave a total of 76 and 43 embryos respectively. The insert in the third panel shows, for both groups, the cumulative proportion of embryos that did not even reach stage IV of development after 72 hours. Asterisks indicate significant differences between mouse groups (* $P < 0.05$, Mann-Whitney-Wilcoxon *U* test).

outgrow on glass coverslips for 5 days to examine more in detail their morphology by confocal fluorescent microscopy after labeling of both F-actin and chromatin. All spread blastocysts from uninfected mice had a size of $128 \pm 8 \mu\text{m}$ in diameter and displayed a clearly visible and central ovocylinder (Figure 5A). On the contrary, five of seven (71%) blastocysts from infected females displayed a different morphology. They indeed spread normally, but their inner cell mass (ICM) failed to differentiate into an ovocylinder (Figure 5B) or underwent quite limited spreading while differentiating an ovocylinder (Figure 5C).

Figure 4. In vitro development of morulae and blastocysts collected at day 3.5 of gestation in *T. cruzi* acutely infected mice. Female mice infected at day 0 with 100 parasites were sacrificed at G3.5, corresponding to days 11 to 14 of infection. Embryos were collected from the uterine horns, and morulae and blastocysts were incubated *in vitro* to follow their development. The different embryonic stages, identified by microscopic examination, were counted every 24 hours during 6 days. Two experiments giving similar results were pooled. Results are expressed as the mean $\pm$ SEM of the proportion of embryonic stages/gravid mouse from 14 infected females (black bars) and 17 age-matched uninfected mice (gray bars) that gave a total of 49 and 60 embryos (morulae and blastocysts), respectively. **Asterisks** indicate significant differences between mouse groups (* $P < 0.05$, Mann-Whitney-Wilcoxon *U* test).

Altogether, these results show that, when incubated out of the infectious context provided by the maternal genital tract, most embryos collected early in gestation

from infected females apparently recover from the delay induced *in vivo*. However, some blastocysts collected later in gestation outgrow abnormally.

Figure 5. Morphology of blastocyst outgrowths from *T. cruzi* acutely infected mice. Blastocysts collected at day 3.5 of gestation in acutely infected or age-matched uninfected mice were cultured *in vitro* for 5 days. To visualize by fluorescence confocal microscopy, the TE and the ovocylinder (corresponding to the ICM)-spread blastocysts were stained with phalloidin (red) and TOTO-3 (blue), which stain actin F and DNA, respectively. **A:** Normal blastocyst from an uninfected mouse; **B and C:** blastocysts from an infected mouse; **B:** TE is correctly spread but the ICM is not well defined; and **C:** TE is poorly spread but the ICM appears normal.

Discussion

We previously showed that *T. cruzi* acute infection induced infertility in a great proportion of female mice.⁶ Our present results show that, in infected mice, the defect takes place after the embryos have reached the two-cell stage. Indeed, in infected mice, 1) ovulation and fertilization occurred normally, 2) the first division of the zygote was neither affected nor delayed, 3) development from the two-cell stage to the blastocyst stage was slowed, 4) some embryos did not reach the eight-cell stage, while others degenerated, 5) the slowing of embryonic development resulted in an insufficient number of blastomere division, leading to abnormal blastocyst hatching and failure of implantation, and 6) the inhibition of cell division correlated with the maternal parasitemia.

In this model in which gestation occurred during the ascending phase of the parasitemia, acute *T. cruzi* infection did not affect the ovarian function because infected mice responded as control mice to gonadotrophic hormones. Thirty-six hours after mating (G1.5), they also harbored a similar number of two-cell embryos as controls, indicating that follicular growth, maturation, ovulation, and fertilization, together with the first cleavage of zygotes, all took place normally in infected females. Of course, this also indicates that sperm transit and interaction between male and female gametes were not hampered. Schuster and Schaub, however, have shown that acute *T. cruzi* infection is associated with anestrus.¹³ Such apparent contradiction with our results might be explained by the difference in the parasite burden at the time of study of the ovarian function. Indeed, these authors showed that anestrus in infected mice was related to high parasitemia, whereas in our experiments, estrus and mating occurred before the parasitemic phase, ie, before the triggering of the systemic inflammatory response.^{14,15}

In contrast, the preimplantation development from the two-cell stage to the blastocyst stage was drastically impeded in *T. cruzi*-infected females, either by an inhibition of cell divisions or by degeneration. However, at

G3.5, the proportion of infected females harboring blastocysts (38%) was still higher than that developing gestation (approximately 20%), indicating that embryonic developmental delay did not entirely account for infertility. Blastocyst formation marks the segregation of the first two cell lineages in the mammalian pre-implantation embryo: the inner cell mass that will form the embryo proper and the trophoblast (TE) that gives rise to the trophoblast lineage.^{16,17} Some of the blastocysts collected at G3.5 from the uterus of infected mice did outgrow abnormally when they were further incubated *in vitro*, displaying either a small TE or no well-differentiated ICM (ovocylinder). These anomalies are probably a direct consequence of the delay in cell cycle progression,^{18,19} and might explain implantation failure.²⁰ Some blastocysts probably progress correctly. However, as implantation depends on the synchronized development of the blastocyst and the endometrium,²¹ their maturation might be too slow to implant during the receptive time frame of the uterus.²⁰ Altogether, these qualitative and quantitative defects of blastocyst development likely explain the infertility of acutely infected mice, observed in 80% of them.⁶

The delay of embryo differentiation was associated with the maternal parasite burden, since the number of blastomeres nuclei in embryos was inversely correlated with the parasitemia. This is in line with our previous results showing that only the mice with the lowest parasitemia were able to develop a gestation.⁶ This undoubtedly highlights the deleterious effect of *T. cruzi* infection on pre-implantation embryo development.

The mechanism by which *T. cruzi* infection inhibits embryo development is currently not known. Infections of the reproductive tract are important causes of infertility.² In our model, pre-implantation embryo development occurs during the ascending phase of the parasitemia, ie, a period during which extracellular trypomastigotes disseminate in the fluids of the host, and various tissues become intracellularly infected. This raises the possibility of a direct effect of parasites or of molecules secreted by

them on embryos. In line with this, *T. cruzi* molecules have been shown to inhibit cell cycle progression of lymphocytes by down-regulating mRNA levels of cyclin D2 and cdk4, molecules that control progression through the G₁ phase of the cell cycle.²² The presence of rare amastigotes has been reported in the genital tract of infected female mice.²³ In our model, we did not look for intracellular parasites in the reproductive system during the pre-implantation period. However, their presence is unlikely since we showed previously that no parasite could be seen in the uterus later on (G17–19), when the parasitemia is higher.⁶ The presence of extracellular trypomastigotes in hydrosalpingeal fluid is also unlikely since we never observed trypomastigotes in the medium after having flushed the embryos out of the oviducts or the uterine horns.

Interestingly, when embryo development was allowed to occur *in vitro*, ie, out of the maternal infectious context, the developmental delay was reduced. Indeed, more than 90% of the two-cell embryos collected at G1.5 from infected mice progressed similarly as controls, and the morulae obtained at G3.5 became blastocysts within 24 hours, as did those from uninfected mice. This indicates the developmental delay induced by *T. cruzi* infection requires a permanent *in vivo* interaction with the reproductive tract. The development of the embryo before implantation is regulated by many embryotrophic molecules, growth factors, and cytokines produced in the lumen of the oviduct by maternal cells and the embryo itself.^{24,25} Systemic infection or inflammation may disturb this network and induce infertility, even if the reproductive tract is not itself infected. *T. cruzi* is known to possess strong proinflammatory activities,²⁶ and the acute infection is associated with a strong inflammatory response, with systemic production of TNF- α ¹⁵ and interferon- γ (IFN- γ).²⁷ Blastocysts express receptors for TNF- α , which induces apoptosis of blastomeres *in vitro*.²⁸ IFN- γ has also been shown to induce alterations of the inner cell mass and trophoblast development.²⁹ Overproduction of these cytokines in the oviducts and/or uterine horns of infected mice might thus be responsible for the deleterious effects observed. Alternatively, a systemic inflammatory response may lead to the inability of the endometrium to support implantation.³⁰ Thus, the inflammatory response in *T. cruzi* infection might be the cause of infertility, although the precise mechanism of its action remains, for now, elusive. In addition, the known correlation between parasite burden and inflammation¹⁵ might account for the relation we observed between poor pre-implantation embryonic development and maternal parasitemia. Besides the parasite burden, the strain of *T. cruzi* might also influence the effect of the infection on fertility. Indeed, Gonzalez Cappa et al have shown that one strain induced an inflammatory reaction in the genital tract whereas another did not.³¹ Therefore, our findings may likely not be generalized to all *T. cruzi* infections.

In humans, the majority of infected adult individuals are in the chronic phase of the infection, because such infection is generally acquired during infancy or early in adulthood.³² Therefore, it is less probable to put in evidence of a relation between *T. cruzi* acute infection and

female infertility, and no data, from us or others, currently support this possibility.

This work clearly shows that the infertility of mice acutely infected with *T. cruzi* results from a defective embryonic development between fertilization and implantation, offering a model for further *in vivo* studies of embryotrophic factors produced in the oviduct.

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References

- Daar A, Merali Z: Infertility and assisted reproductive technologies in the developing world. Current practices and controversies in assisted reproduction. Report of a meeting on Medical, Ethical and Social Aspects of Assisted Reproduction. WHO Headquarters, Geneva, Switzerland, 2001 September 17–21. Edited by E Vayena, PJ Rowe, D Griffin. Geneva, Switzerland, World Health Organization, 2002, pp 15–21
- The ESHRE Capri Workshop Group: Physiopathological determinants of human infertility. *Hum Reprod Update* 2002, 8:435–447
- Vanroose G, de Kruif A, Van Soom A: Embryonic mortality and embryo-pathogen interactions. *Anim Reprod Sci* 2000, 60–61:131–143
- Krishnan L, Guilbert LJ, Wegmann TG, Belosevic M, Mosmann TR: T helper 1 response against *Leishmania major* in pregnant C57BL/6 mice increases implantation failure and fetal resorptions. Correlation with increased IFN- γ and TNF and reduced IL-10 production by placental cells. *J Immunol* 1996, 156:653–662
- Brabin L, Brabin BJ: Parasitic infections in women and their consequences. *Adv Parasitol* 1992, 31:1–81
- Mjihdi A, Lambot MA, Stewart LJ, Detournay O, Noel JC, Carlier Y, Truysens C: Acute *Trypanosoma cruzi* infection in mouse induces infertility or placental parasite invasion and ischemic necrosis associated with massive fetal loss. *Am J Pathol* 2002, 161:673–680
- Mjihdi A, Truysens C, Detournay O, Carlier Y: Systemic and placental productions of tumor necrosis factor contribute to induce fetal mortality in mice acutely infected with *Trypanosoma cruzi*. *Exp Parasitol* 2004, 107:58–64
- Mulnard J, Puissant F: Development of mouse embryos after ultracentrifugation at the pronuclei stage. *Arch Biol* 1984, 95:461–474
- Tarkowski AK: An air-drying method for chromosome preparations from mouse embryos. *Cytogenetics* 1966, 5:394–400
- Tomkins PT, Carroll C, Houghton JA: The cytogenetic preparation of oocytes on preimplantation embryos; a modification of the method of Tarkowsky. *Lab Pract* 1986, 35:81–83
- Hogan B, Beddington R, Costantini F, Lacey E: *Manipulating the Mouse Embryo: A Laboratory Manual*. New York, Cold Spring Harbor Laboratory Press, 1994
- Channing CP, Hillensjo T, Schaerf FW: Hormonal control of oocyte meiosis, ovulation and luteinization in mammals. *Clin Endocrinol Metab* 1978, 7:601–624
- Schuster JP, Schaub GA: *Trypanosoma cruzi*: the development of estrus cycle and parasitemia in female mice maintained with or without male pheromones. *Parasitol Res* 2001, 87:985–993
- Truysens C, Torrico F, Angelo-Barrios A, Lucas R, Heremans H, De Baetselier P, Carlier Y: The cachexia associated with *Trypanosoma cruzi* acute infection in mice is attenuated by anti-TNF- α , but not by anti-IL-6 or anti-IFN- γ antibodies. *Parasite Immunol* 1995, 17:561–568
- Truysens C, Torrico F, Lucas R, De Baetselier P, Buurman WA, Carlier Y: The endogenous balance of soluble tumor necrosis factor receptors and tumor necrosis factor modulates cachexia and mortality in mice acutely infected with *Trypanosoma cruzi*. *Infect Immun* 1999, 67:5579–5586
- Ralston A, Rossant J: Genetic regulation of stem cell origins in the mouse embryo. *Clin Genet* 2005, 68:106–112

17. Johnson MH, McConnell JM: Lineage allocation and cell polarity during mouse embryogenesis. *Semin Cell Dev Biol* 2004, 15:583–597
18. Alexandre H: Involvement of DNA-polymerase activities in mouse-blastocyst differentiation in vitro. *Differentiation* 1985, 29:152–159
19. Aziz M, Alexandre H: The origin of the nascent blastocoele in preimplantation mouse embryos: ultrastructural cytochemistry and effect of chloroquine. *Roux's Arch Dev Biol* 1991, 200:77–85
20. Paria BC, Reese J, Das SK, Dey SK: Deciphering the cross-talk of implantation: advances and challenges. *Science* 2002, 296:2185–2188
21. Cai L, Zhang J, Duan E: Dynamic distribution of epidermal growth factor during mouse embryo peri-implantation. *Cytokine* 2003, 23:170–178
22. Kierszenbaum F, Majumder S, Paredes P, Tanner MK, Sztejn MB: The *Trypanosoma cruzi* immunosuppressive factor (TIF) targets a lymphocyte activation event subsequent to increased intracellular calcium ion concentration and translocation of protein kinase C but previous to cyclin D2 and cdk4 mRNA accumulation. *Mol Biochem Parasitol* 1998, 92:133–145
23. Lenzi HL, Castelo-Branco MT, Pelajo-Machado M, Oliveira DN, Gattass CR: *Trypanosoma cruzi*: compromise of reproductive system in acute murine infection. *Acta Trop* 1998, 71:117–129
24. Hardy K, Spanos S: Growth factor expression and function in the human and mouse preimplantation embryo. *J Endocrinol* 2002, 172:221–236
25. Strandell A, Thorburn J, Wallin A: The presence of cytokines and growth factors in hydrosalpingeal fluid. *J Assist Reprod Genet* 2004, 21:241–247
26. Ropert C, Ferreira LR, Campos MA, Procopio DO, Travassos LR, Ferguson MA, Reis LF, Teixeira MM, Almeida IC, Gazzinelli RT: Macrophage signaling by glycosylphosphatidylinositol-anchored mucin-like glycoproteins derived from *Trypanosoma cruzi* trypomastigotes. *Microbes Infect* 2002, 4:1015–1025
27. Torrico F, Heremans H, Rivera MT, Van Marck E, Billiau A, Carlier Y: Endogenous IFN-gamma is required for resistance to acute *Trypanosoma cruzi* infection in mice. *J Immunol* 1991, 146:3626–3632
28. Pampfer S, Wu YD, Vanderheyden I, De Hertogh R: Expression of tumor necrosis factor-alpha (TNF alpha) receptors and selective effect of TNF alpha on the inner cell mass in mouse blastocysts. *Endocrinology* 1994, 134:206–212
29. Fontana V, Choren V, Vauthay L, Calvo JC, Calvo L, Cameo M: Exogenous interferon-gamma alters murine inner cell mass and trophoblast development. Effect on the expression of ErbB1, ErbB4 and heparan sulfate proteoglycan (perlecan). *Reproduction* 2004, 128:717–725
30. Mayorga M, Iborra A, Estany S, Martinez P: Protective effect of vitamin E in an animal model of LPS-induced inflammation. *Am J Reprod Immunol* 2004, 52:356–361
31. Gonzalez Cappa SM, Mirkin GA, Solana ME, Tekiel VS: *Trypanosoma cruzi* pathology. Strain dependent? *Medicina (B Aires)* 1999, 59(Suppl 2):69–74
32. Carlier Y, Pinto Dias JC, Ostermayer Luquetti AO, Hontebeyrie M, Torrico F, Truyens C: *Trypanosomiasis américaine ou maladie de Chagas*. *Encyclopédie Médico-Chirurgicale, Maladies Infectieuses*. Editions scientifiques et médicales. Paris, Elsevier SAS, 2002, 21 p