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ORIGINAL PAPER

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Calomys callosus (Rodentia: Cricetidae) trophoblast cells as host cells to *Toxoplasma gondii* in early pregnancy

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Abstract The potential of the RH strain of *Toxoplasma gondii* to invade trophoblast cells of the cricetid rodent *Calomys callosus* in a congenital infection in the initial third of pregnancy was investigated in this study using morphological and immunocytochemical approaches. The animals were intraperitoneally inoculated on the 1st day of pregnancy and the infection was observed on day 7. Various numbers of parasites could be observed inside the parasitophorous vacuoles in trophoblastic cells under light and electron microscopy. The trophoblast cells showed characteristics of healthy cells, and no alteration other than parasite vacuoles in their cytoplasm could be detected. Polyclonal or monoclonal anti-*T. gondii* antibodies (respectively, anti-*T. gondii* components and the major surface parasite antigen p30) labeled both the parasite surface and parasitophorous vacuole membranes, regardless of the number of parasites inside the compartment. In addition, p30-containing trails were detected in the extracellular matrix surrounding trophoblastic cells similar to those found with other parasites during locomotion and the invasion process. Our results show the ability of *T. gondii* to infect trophoblast cells during the early blastocyst-endometrial relationship and open new possibilities for more accurate study of the invasion process of this parasite and the role of the trophoblast as an embryo defense barrier.

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Introduction

Toxoplasma gondii, an obligate intracellular coccidian, is an important opportunistic pathogen for humans and animals in that it infects a wide range of hosts (Joiner and Dubremetz 1993; Hunter and Remington 1994). In human fetuses, toxoplasmosis is associated with severe congenital defects when the primary infection is acquired during the first trimester of pregnancy (Kasper and Boothroyd 1993). Experimental models for congenital toxoplasmosis have been described in the literature since the early 1950s (Cowen and Wolf 1950; Hutchison et al. 1982; Graham et al. 1984; Sims et al. 1988; Roberts and Alexander 1992). However, the role of the implanting trophoblast with regard to the initial events leading to the passage of *T. gondii* from the maternal to the embryonic cell population is not yet well known.

Trophoblast cells, belonging to a subset of the embryonic cell population, are directly involved in the process of embryo implantation, which in rodents is achieved by the penetration and lodging of the embryo in the endometrium and the establishment of an adequate nutritional supply for embryonic development (Mossman 1987). The means by which the trophoblast breaks the uterine barriers to reach the source of maternal blood show specific features according to the species. In rodents the epithelial cells in contact with the invading trophoblast cells exhibit severe disorganization, and they are removed by trophoblast phagocytosis. Once in contact with the basal lamina, the trophoblast rapidly spreads out both mesometrially and antimesometrially, phagocytosing the remaining epithelial cells, entering into contact with the endometrial stroma and reaching the maternal vasculature (Enders and

Schlafke 1969; Tachi et al. 1970; Billington 1971; Bevilacqua and Abrahamsohn 1989). Particularly in *Calomys callosus* this phase is a very early and rapid event, lasting for approximately 12 h after the onset of implantation (Ferro and Bevilacqua 1994).

Since *C. callosus* can inhabit grasslands and human dwellings and can be reservoirs for several pathogens, the infection of these animals with *T. gondii* may represent an important medical and social problem. Thus, the main purpose of this study was to observe the congenital infection of trophoblast cells by *T. gondii* in early pregnancy during embryo implantation by investigating the presence, characteristics, and replication capacity of the protozoan in this cellular population. The present study followed the fate of *T. gondii* in the uterus and trophoblast cells using morphology and immunocytochemistry techniques under light and electron microscopy.

Materials and methods

Parasites

The highly virulent type I RH strain of *Toxoplasma gondii* (Howe et al. 1996) was maintained by serial passage in Swiss mice by a standard procedure as previously described by Camargo et al. (1978) and Mineo et al. (1980).

Animals

The specimens of *Calomys callosus*, Canabrava strain, used in this study belong to a resident colony housed at the Institute of Tropical Medicine of São Paulo and were kindly provided by Dr. Judith Kloetzel. The animals were kept on a 12-h light: 12-h dark cycle in a temperature-controlled room (25° ± 2 °C) and were provided with food and water ad libitum. Virgin females aged between 3 and 4 months were caged with males overnight and checked for the presence of a vaginal plug. The day on which this sign was found was designated as the 1st day of pregnancy (dop).

Experimental infection and collection of implantation sites

On the 1st dop the animals were intraperitoneally inoculated with 0.2 ml of a suspension containing 10^2 *T. gondii* tachyzoites. Considering that the parasites probably gain access to the embryo through the maternal blood and that the interaction between trophoblasts and maternal blood in *C. callosus* is established after the 5th dop (Ferro and Bevilacqua 1994), in this investigation the animals were analyzed on the 7th dop. The animals were lethally anesthetized with ether and laparotomized and their uteri were removed and sectioned in thin slices containing the implantation sites. Samples were then fixed with different reagents according to the type of reaction to which these preparations were subsequently subjected as described below.

Morphology and immunocytochemistry studies

For conventional light microscopy, specimens were fixed by immersion in a solution of 95% ethanol, formol, and acetic acid in distilled water (3:1:1:5), dehydrated, and embedded in paraffin. Sections measuring 3 μm in thickness were either stained with hematoxylin-eosin or subjected to immunocytochemical reactions.

For ultrastructure studies the material was fixed by immersion for 24 h with 0.3% glutaraldehyde-4% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.2). Immunolocalization of the parasites was carried out using polyclonal antibody (p Ab) obtained from mice immunized with total *T. gondii* antigen and mouse monoclonal antibody (mAb) 6E9 directed to SAG-1 (p 30), the major surface antigen of *T. gondii* tachyzoites obtained as previously described (Mineo and Kasper 1994). This antigen is present on the parasite surface and on the surface-associated membrane of the parasitophorous vacuole during invasion (Grimwood and Smith 1995).

Immunohistochemical reactions

The paraffin sections on glass slides were (1) incubated for 10 min at room temperature with 3% H_2O_2 to block endogenous peroxidase, (2) treated with 2% normal goat serum diluted in phosphate-buffered saline (PBS) for 20 min at 37 °C to block nonspecific binding sites, (3) incubated for 12 h at 4 °C with pAb and anti-SAG-1 mAb, (4) rinsed in PBS, and (5) incubated with biotinylated goat anti-mouse IgG (Sigma Co., St. Louis, USA) for 30 min at 37 °C.

The reaction signal was amplified by the ABC system (Vector Inc., Burlingame, USA) and revealed with 3′3′tetradiaminobenzidine (DAB; Sigma Chemical Co., St. Louis, USA). The material was counterstained with Mayer's hematoxylin. Negative controls were carried out by substitution of the primary antibodies by PBS or by an IgG-class-matched irrelevant antibody. For immunofluorescence localization of the antigen the paraffin sections on glass slides were subjected to the same steps described above for immunoperoxidase reactions. They were treated with 2% normal goat serum in PBS, incubated with pAb, rinsed in PBS, and incubated with goat IgG fluorescein-conjugated anti-mouse IgG (Sigma Co., St. Louis, USA; diluted 1:50 in PBS) for 1 h at 37 °C. After three washes with PBS the slides were mounted in glycerol buffered with 0.1 *M* TRIS (pH 8.6) and observed on a Bio-Rad 1024-UV confocal system attached to a Zeiss Axiovert 100 microscope using a 63X NA 1.4 plan-Apochromatic (DIC) oil-immersion objective. All images were collected by Kalman, averaging at least 15 frames (512 × 512 pixels), using an aperture (pinhole) of maximally 2.0 mm. The collected DIC images were sharpened using Bio-Rad Laseshap 1024 software, version 2.1a, at a minimal setting.

For immunoperoxidase techniques carried out at the electron microscopy level, uterine fragments containing the implantation sites were cut in 1-mm-thick slices immediately after glutaraldehyde-paraformaldehyde fixation. The fragments were then incubated for 10 min at room temperature with 3% H_2O_2, and the immunocytochemical reaction was processed as described above for light microscopy. After DAB incubation the material was postfixed in 1% osmium tetroxide in 0.1 *M* phosphate buffer (pH 7.2), dehydrated in ethanol, and embedded in Epon-812. Ultrathin sections were either left unstained or stained with lead citrate and uranyl acetate and were then observed in a Zeiss EM 109 electron microscope.

For immunogold reactions the uterine fragments were dehydrated in ethanol and embedded in LR White resin (Sigma Co., St. Louis, USA). Ultrathin sections were then placed on nickel grids and hydrated with TRIS-buffered saline (TBS). The procedure followed the same steps carried out for paraffin sections. In brief, the sections were treated with 2% normal goat serum diluted in the same buffer, incubated with either pAb or mAb, rinsed in TBS, and again incubated with gold-conjugated goat anti-mouse IgG (gold-particle diameter 15 ηm; Sigma) for 30 min at 37 °C. The grids were finally stained with lead citrate and uranyl acetate and observed in the electron microscope. In the negative controls the primary antibody was substituted either by TBS or by an irrelevant antibody.

Alternatively, ultrathin sections, not subjected to any immunocytochemistry procedure, were stained with lead citrate and uranyl acetate and analyzed by electron microscopy for morphology studies.

Results

On the 7th dop, different populations of trophoblast cells can be found around the embryo (Fig. 1). At the embryonic pole the trophoblast cells form an excrescence named the ectoplacental cone, whereas at the lateral walls of the egg cylinder, flat, nonproliferative trophoblast cells are disposed as a loose network in which maternal blood is present. The peripheral cells from the lateral trophoblast and ectoplacental cone are exclusively giant cells. At this phase of pregnancy, these cells are in direct contact with stromal cells (mainly decidual and endothelial cells) as well as with the maternal blood, and they are the main cell population infected by *Toxoplasma gondii* in the embryonic organism.

In the maternal organism, *T. gondii* was profusely found within myometrial and serosa cells in contact with the intraperitoneal inoculation focus (Fig. 2a). On the contrary, in the endometrium, very few cells were found to be infected (Fig. 2b). Whenever *T. gondii* were present the cells were heavily stained by mAb and pAb immunoperoxidase. Positive reactions were also present inside leukocytes in vascular compartments (Fig. 2b). Cells involved in the formation of the embryo body itself did not show staining (Fig. 2c).

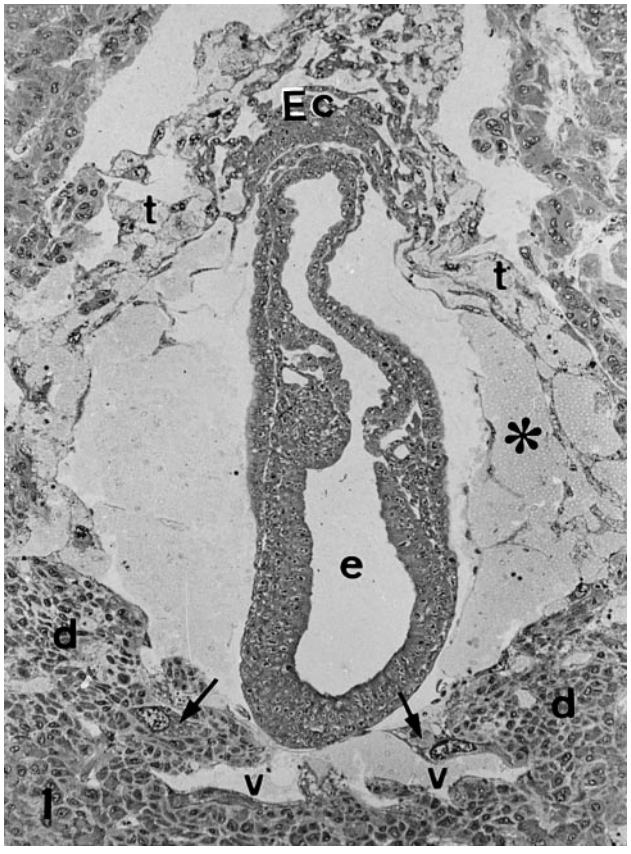

Fig. 1 Photomigrograph showing a *Calomys callosus* embryo (*e*) on the 7th dop in the implantation chamber. Note the network of trophoblast cells (*t*) around the embryo and the giant peripheral cells (*arrows*) invading the decidua (*d*) and maternal vessels (*v*). (*Ec* Ectoplacental cone, *asterisk* extravasated maternal blood cells) Toluidine blue, ×54

The cytoplasm of trophoblast giant cells frequently exhibited phagocytic vacuoles containing elements of maternal blood cells and parasitophorous vacuoles carrying various quantities of parasites (Fig. 3). Using mAb and pAb to *T. gondii*, we observed that staining was independent of the number of the parasites located within the parasitophorous vacuole and, thus, also independent of the size of this structure. Immunostaining was apparently present on the surface of the parasites and on the parasitophorous vacuole membrane (Fig. 3). Negative controls did not show any reactivity, assuring the specificity of the immunolabeling reactions (Fig. 3a).

Nomarski differential interference contrast associated with confocal fluorescence microscopy with pAb confirmed the presence of parasitophorous vacuoles in the cytoplasm of trophoblast giant cells on the 7th dop in *Calomys callosus* congenital infection (Fig. 4).

Ultrastructural analysis of the trophoblast revealed characteristics of healthy giant cells, even when more than one parasitophorous vacuole was found in each cell. The cytoplasm of infected and noninfected cells contained a high number of free polysomes, mitochondria with lamellar cristae and strands of endoplasmic reticulum, and Golgi complex cisternae. Parasites were also commonly seen in trophoblast projections invading the decidualized stroma or inside phagocytosed neutrophils (data not shown). Several parasites similar in size and structural characteristics seemed to share the same parasitophorous vacuole (Fig. 5a). Dense bodies similar to lysosomes were not found either surrounding or in close proximity to the parasitophorous vacuoles.

Immunogold labeling with pAb showed a positive reaction associated with the internalized parasites in the parasitophorous vacuoles (Fig. 5b). Surrounding infected trophoblast and decidual cells, trails of SAG-1 (p30)-immunopositive material were also ultrastructurally detected in the extracellular matrix. These trails were disposed as a loose network and were found in immunoperoxidase and immunogold reactions using either mAb or pAb to parasite components (Figs. 5c, d). Such trails were not seen in controls, in the absence of primary antibodies, or when irrelevant antibodies were used.

Discussion

Our results show that trophoblast cells from *Calomys callosus* may be infected by *Toxoplasma gondii* in early pregnancy during the implantation process. The observations were done at both the light- and the electron-microscopy level.

T. gondii are obligatory intracellular parasites. Therefore, at least two possible entry routes into the embryo from the intraperitoneal inoculation focus

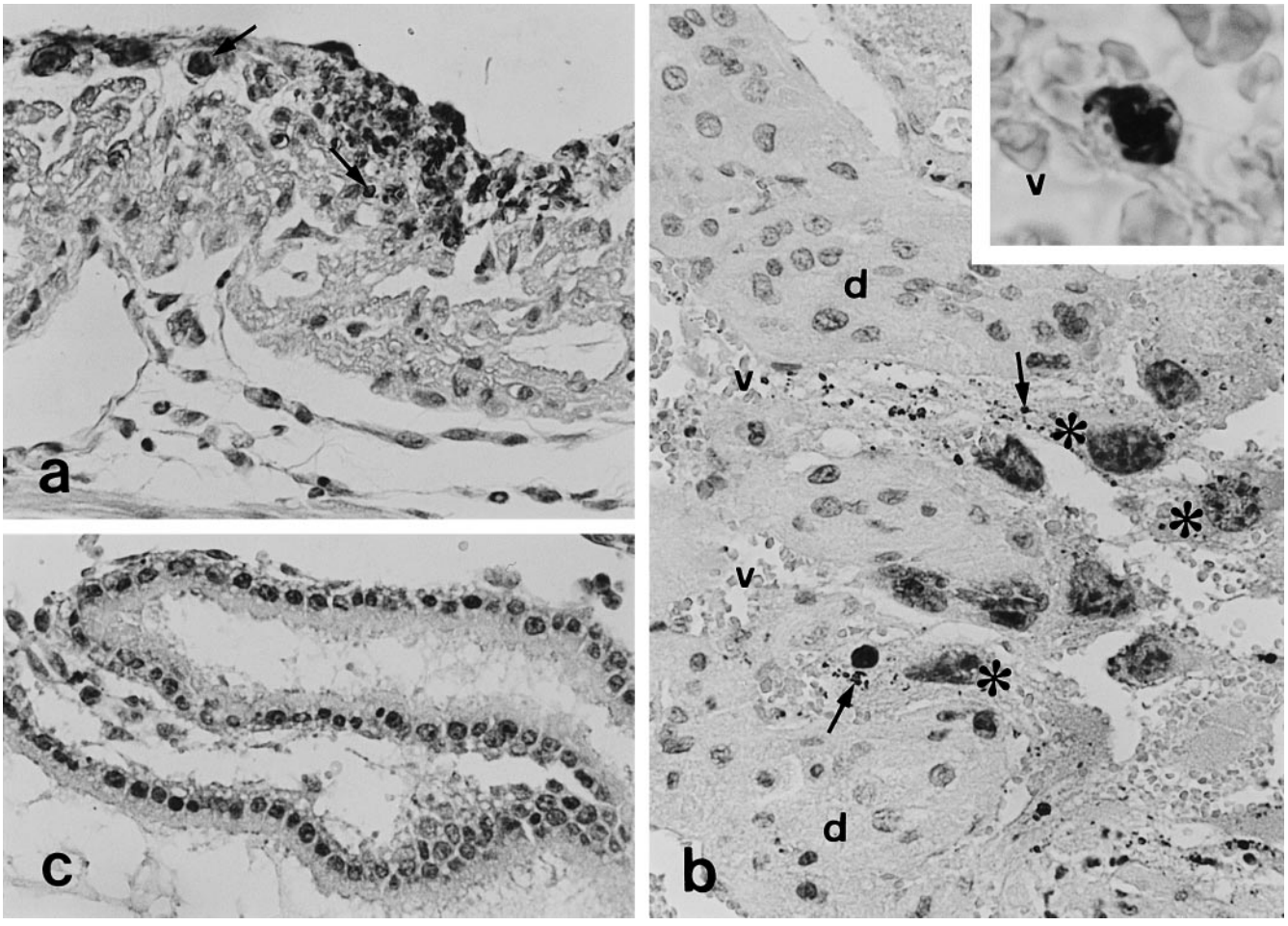

Fig. 2a–c At 7 days after inoculation the parasites (*arrows*) were abundant **a** in the outer wall of the uterus and **b** in trophoblast giant cells (*asterisks*). Leukocytes in vascular compartments (*v*) also presented parasitophorous vacuoles (*inset*). **b** Inside decidual cells (*d*) or **c** in other embryonic tissues, no parasite was found. Immunoperoxidase, pAb, Mayer's hematoxylin, ×80

might be speculated: direct invasion of local uterine cells and/or invasion of leukocytes by a vascular route. In either case, after lysis of the first infected cells the parasites could get into close contact with the embryonic organism. Considering these possibilities, however, the lack of significant parasite infection observed in the endometrium, in close proximity to the trophoblast favors the idea that *T. gondii* may have used the vascular route. The presence of leukocytes containing parasitophorous vacuoles as well as the close contact that trophoblast giant cells establish with the maternal blood during this phase also favor this possibility. In addition, among the different trophoblast cell populations that surround the embryo at the time of implantation, only trophoblast giant cells, known by their ability to interact directly with maternal blood components, were observed to be infected. Fadul et al. (1995) previously suggested the vascular route for the dissemination of *T. gondii* from the gut epithelium to other organs and systems of the infected host, implicating the monocytes as the carrier of the parasites within the vascular compartments.

In the present study the characteristics of embryo implantation and ultrastructural analysis of the trophoblast giant cells from infected animals were equivalent to those found in animals with normal pregnancy as reported by Ferro and Bevilacqua (1994). The presence of *T. gondii* inside these host cells apparently did not affect trophoblast morphology or invasiveness.

Although at the time of implantation the trophoblast cells exhibited intense phagocytic activity, the parasites were rarely found in phagosomes. The parasites presented healthy features and were confined almost exclusively to parasitophorous vacuoles. The presence of various synchronized parasites in the parasitophorous vacuole suggested a replication pattern similar to that observed for other infected eukaryotic cells described to date (Kasper and Boothroyd 1993). Together, these findings seem to indicate that *T. gondii* follows the same basic process of invasion, regardless of the type of host cell involved, infecting the trophoblast by active penetration (Fadul et al. 1995).

With our approach, a distinct immunostaining at the early stage of host cell infection was obtained. Independently of the immunostaining method or anti-

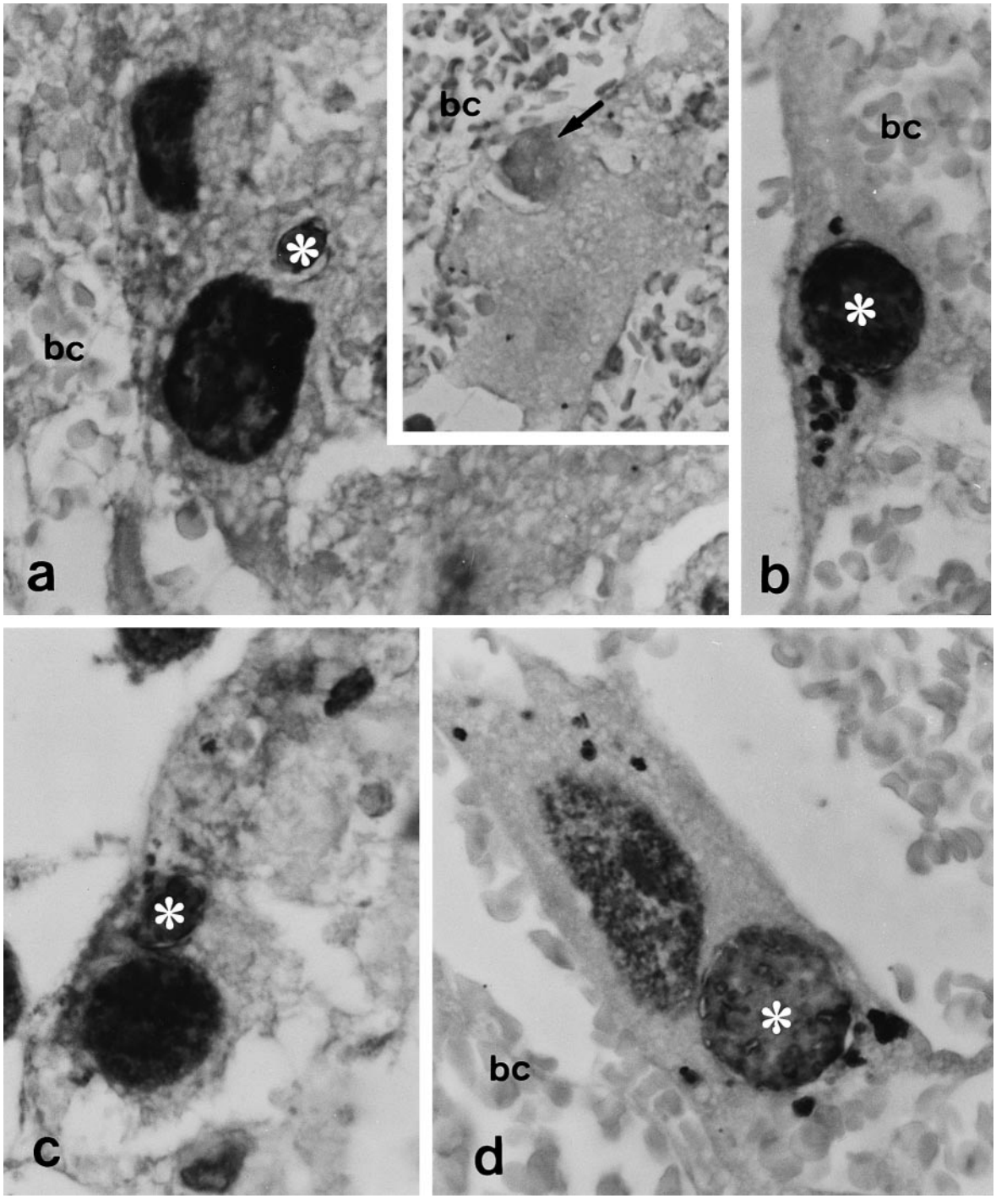

Fig. 3a–d Parasitophorous vacuoles containing parasites in *C. callosus* trophoblast giant cells. Parasites were strongly immunostained (*asterisks*) when either antibody was used: **a, b** mouse anti-*Toxoplasma gondii* pAb or **c, d** mAb to SAG-1 (p30). The *arrow* shows a parasitophorous vacuole in the control of the immune reaction where the primary antibody was omitted (*inset*). Maternal blood cells (*bc*) were observed among invading trophoblast cells. Immunoperoxidase, Mayer's hematoxylin, ×160

body used, the parasite and the parasitophorous vacuole of *T. gondii* were always positive to mAb SAG-1 (p30) and pAB against total *T. gondii* components. The presence of SAG-1 (p30), the major surface parasite antigen, on the plasma membrane and parasitophorous vacuole membrane of *T. gondii* has been described elsewhere (Dubremetz et al. 1985; Kimata and Tanabe 1987). According to Grimwood and Smith (1995), at the time of invasion, SAG-1 (p30) is removed from the parasite plasma membrane and becomes associated with the adjacent parasitophorous vacuole membrane. However, because it is continually synthesized, it can also be detected in these parasites when they are within the host cell. An effective detection of SAG-1 (p30) on

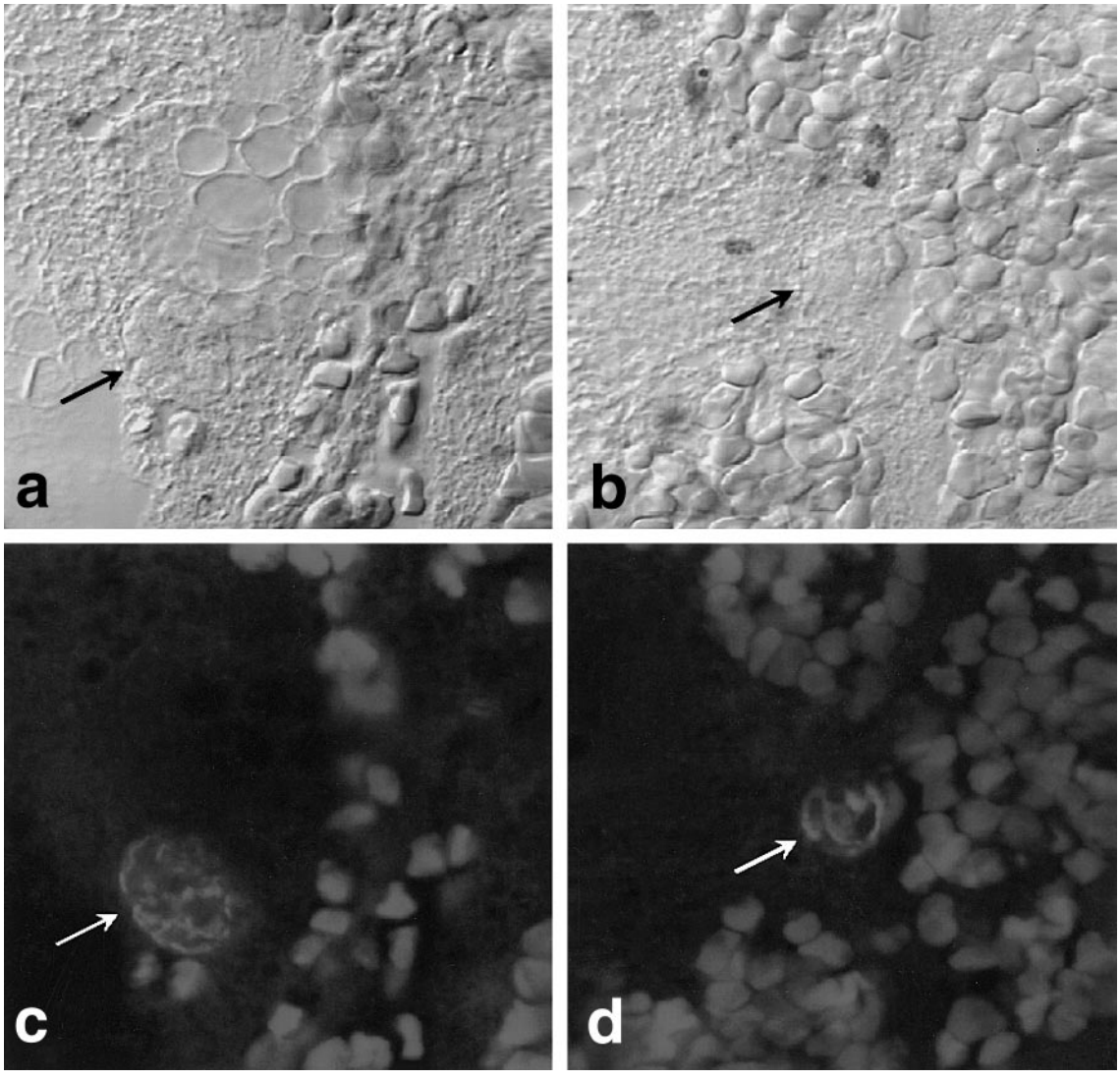

Fig. 4a–d Identification of *T. gondii*-positive cells in the trophoblast by **a, b** Nomarski differential interference contrast and **c, d** confocal fluorescence microscopy. *Arrows* indicate parasitophorous vacuoles containing *T. gondii* located near erythrocytes showing autofluorescence at the same wavelength as that of fluorescein. pAb, ×160

the parasitophorous vacuole membrane at this stage strengthens the hypothesis for a role of this parasite component in the initial events that lead to successful invasion and replication of *T. gondii* in host cells. In this regard, a significant inhibition of the parasite invasion has been demonstrated when this protein is blocked by specific antibodies (Mineo and Kasper 1994).

Specific immunostaining of the exoantigen SAG-1 (p30) was observed in the extracellular matrix surrounding infected trophoblast cells. Similarly, Sibley has also observed trail formation of SAG-1 from *T. gondii* tachyzoites (personal communication). This finding may indicate secretion of SAG-1 antigen from *T. gondii* while this protozoan is seeking host cells to invade and/or after cell infection. Secretion of exoantigen as an important mechanism for the achievement of host cell invasion has previously been described for tachyzoites of *T. gondii*, for gliding malaria sporozoites, and for other invasive apicomplexan parasites (Dubremetz et al. 1985; Stewart and Vanderberg 1992; Kasper and Boothroyd 1993). On the other hand, particularly with regard to SAG-1, export from infected cells has also been suggested, as this antigen can be found in vesicles close to the host cell surface, not related to the parasite membrane (Grimwood and Smith 1995).

The present investigation using an experimental in vivo model of congenital toxoplasmosis shows that *T. gondii* can infect trophoblast cells of *C. callosus* in the early stages of pregnancy. Although previous studies had confirmed the presence of tachyzoites within the trophoblast cells in the late stage of transplacentally induced toxoplasmosis in fetal pig and sheep (Buxton and Finlayson 1986; Dubey et al. 1990), no previous study had shown the trophoblast as a route of access for infection of *T. gondii* from maternal to embryonic tissues in the early stage of pregnancy. These findings open new opportunities for further studies of the kinetics of embryo infection by *T. gondii* and the mechanisms used by

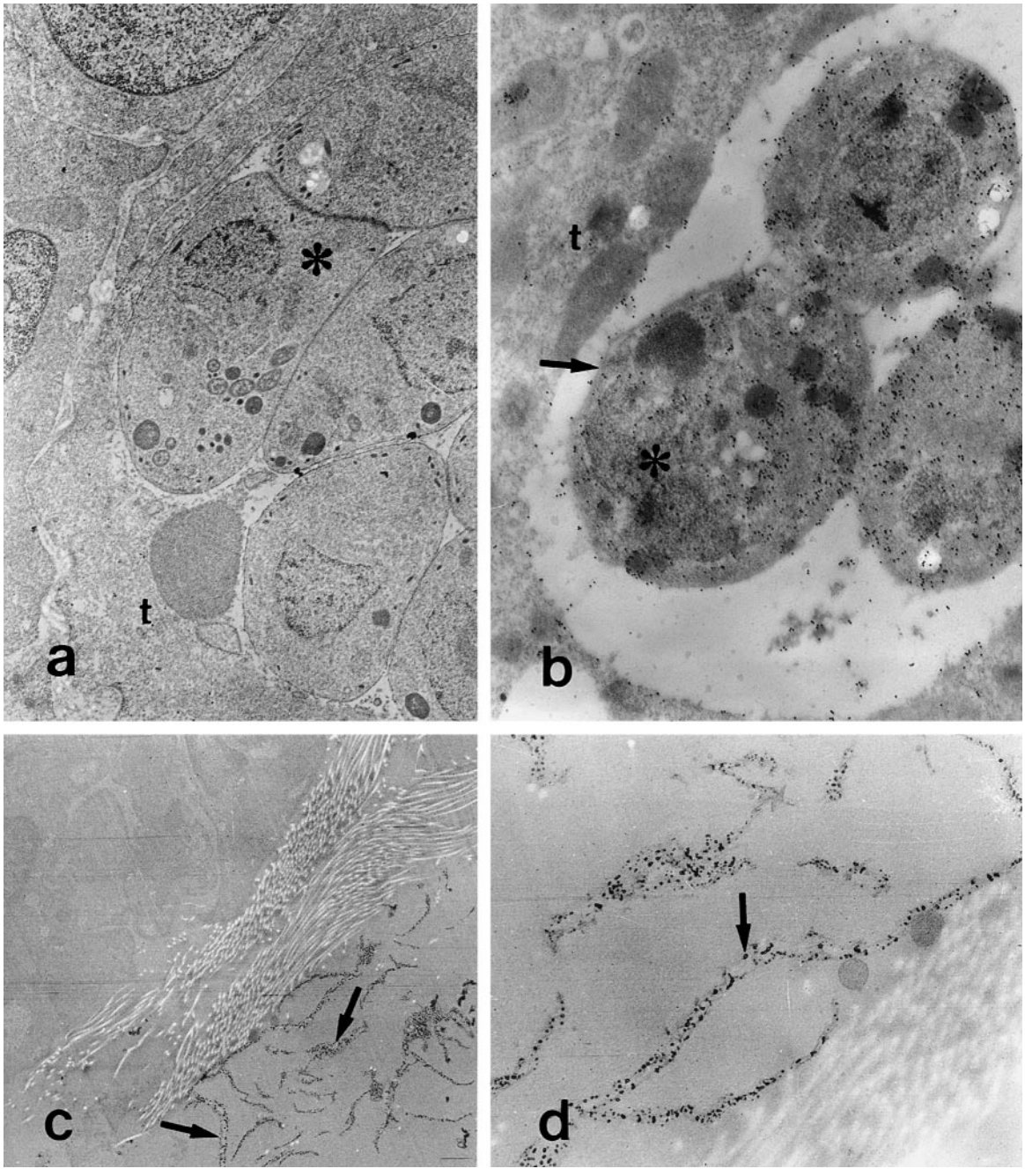

Fig. 5 **a** Dividing forms of *T. gondii* (*asterisk*) in *C. callosus* trophoblast cells (*t*) ×8,000. **b** Note the immunogold-positive staining (*arrow*) on the parasites (*asterisk*). pAb, ×14,000. **c** Specific immunostaining (*arrows*) was also observed on the extracellular matrix around infected cells. mAb (anti-SAG-1), ×4,000. **d** Under high magnification this immunoreactivity (*arrow*) bears some resemblance to trails. mAb (anti-SAG -1), ×12,000

the parasite during early pregnancy to ensure that it successfully surpasses the protective barrier represented by maternal tissues and trophoblasts.

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