

IMMUNOHISTOCHEMICAL CHARACTERIZATION OF THE INFLAMMATORY INFILTRATE IN PLACENTAL CHAGAS' DISEASE: A QUALITATIVE AND QUANTITATIVE ANALYSIS

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Abstract. Chagas' disease, a systemic illness endemic to some regions of South America, is caused by the protozoan *Trypanosoma cruzi*. Transplacental infection may occur during any phase and cause fetal death. This study is the first to characterize the inflammatory cells in chagasic villitis by immunohistochemistry. Paraffin sections of 8 placentas with villitis by *T. cruzi* (4 live births and 4 stillbirths), as well as 8 control placentas without inflammation, were stained with hematoxylin and eosin, monoclonal antibodies for CD45RO, CD20, CD45RO/OPD4, CD8, HNK1, CD15, MAC387, and CD68 proteins, and a polyclonal antibody for S-100 protein. Quantification of positive cells was performed in 3 different high-power fields. In all cases of chagasic villitis, the inflammatory infiltrate was composed mainly of CD68+ macrophages, T lymphocytes, and a few natural killer cells. Among T cells, CD8+ cells outnumbered CD4+ cells in all placentas (CD4+:CD8+ ratios ranged from 0.04 to 0.38). B cells were absent or rare. In stillbirths, villitis was diffuse and severe with numerous *T. cruzi*, while in live births it was focal with few parasites. Other features that characterized villitis in stillbirths were 1) frequent trophoblastic necrosis, 2) presence of MAC387+ macrophages and CD15+ granulocytes attached to the sites of trophoblastic necrosis, 3) low CD4+:CD8+ ratios in most cases, 4) increased numbers of S-100 positive cells in the villous stroma. In conclusion, CD68+ macrophages and CD8+ T lymphocytes were the major cell population in villitis caused by *T. cruzi*. However, the pattern of inflammatory reaction differed between stillbirths and live births and was probably related to the number of parasites in the placental villi.

Chagas' disease, a systemic illness endemic to some regions of South America, is caused by the protozoan *Trypanosoma cruzi*. This disease begins with a short acute phase characterized by high parasitemia, followed by a life-long chronic phase with few parasites. *Trypanosoma cruzi* is spread by an arthropod vector but may also be transmitted through blood transfusion or congenitally. The severity of congenital chagasic infection is variable and may cause intrauterine growth retardation, prematurity, deformations, abortion, stillbirth, or may be asymptomatic.^{1,2}

Transplacental infection may occur at any phase of the maternal disease, although the risk is higher in the acute phase because of the great number of circulating parasites.³ The trypomastigote (flagellated) forms of the parasite penetrate actively through the trophoblast, become amastigotes, and multiply mainly within the Hofbauer cells. Rupture of these nests or pseudocysts releases the parasites and cellular contents in the interstitium where they provoke an inflammatory reaction and necrosis of the placental villi.¹ Intact trypomastigotes, which may also be liberated, penetrate other cells and/or may reach the fetal circulation causing congenital infection.¹

We have for the first time examined the inflammatory cells in chagasic villitis by immunohistochemistry, using monoclonal antibodies to stain paraffin-embedded tissue sections.

MATERIALS AND METHODS

This study was approved by the University of Campinas Medical Sciences School Research Ethical Committee. Informed consent was not necessary because the study was retrospective and no personal identifiers were used. The study was performed in 8 placentas (4 live births and 4 stillbirths) with chagasic villitis and 8 (all live births) control

placentas without inflammation. In the placentas with villitis, the parasites were identified in paraffin-embedded sections stained with hematoxylin and eosin and with antibodies to *T. cruzi*, using an immunohistochemical method.⁴ The placentas were fixed in 10% buffered formalin, the blocks embedded in paraffin, and histologic sections were stained with hematoxylin and eosin. One paraffin block from each case was chosen for the immunohistochemical studies. Immunohistochemical staining was performed according to the streptavidin biotin peroxidase method.⁵ The monoclonal antibodies used in this study were CD45RO (UCHL1), CD20 (126), CD45RO/OPD4, CD8, HNK1 (anti-human natural killer [NK] cell-like), CD15 (M1), MAC387, and CD68 (KP1). The polyclonal antibody used was to S-100 protein. All antibodies were obtained from Dakopatts (Copenhagen, Denmark).

Analysis of positive cells. Serial tissue sections were used and each of 9 different antibodies was applied on a different section of the same block. Mononuclear cells labeled with these antibodies were counted in 3 different high-power fields with villitis for each section. The positive cells were then expressed as a percentage of all inflammatory cells in each section (at least 100), except for CD45RO/OPD4+ cells and CD8+ cells, the percentage of which was calculated in relation only to cells with the appearance of lymphocytes. The analysis of S-100 protein-positive cells was semiquantitative as follows: 0 = no reactive cells; + = 1 cell/ high power field (hpf), ++ = 2–5 cells/hpf, and +++ = >5 cells/hpf.

RESULTS

The live births with chagasic villitis did not show clinical manifestations of congenital infection at birth, except for

TABLE 1

Histologic findings in placentas with villitis caused by *Trypanosoma cruzi*

No.	Birth*	Intensity of inflammation	Granulomatous reaction	Trophoblastic rupture†	Quantity of parasite nests†
1	LB	Moderate	Absent	+	++
2	LB	Slight	Absent	0	+
3	LB	Slight	Absent	0	+
4	LB	Slight	Absent	0	+
5	SB	Severe	Present	+++	+++
6	SB	Severe	Present	+++	+++
7	SB	Severe	Present	+++	++
8	SB	Severe	Present	+++	+++

* LB = live birth; SB = stillbirth.

† 0 = absent; + = 1/high-power field (hpf); ++ = 2–3/hpf; +++ = >3/hpf.

case 1 (Table 1), which had hepatosplenomegaly and low birth weight. All stillborns were macerated, so that histopathologic analysis of the organs was not possible.

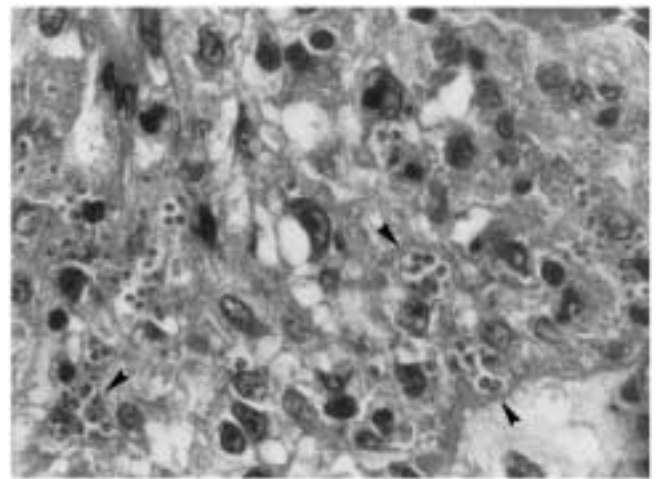
Histologic findings in placentas with chagasic villitis are shown in Table 1. The pattern of inflammatory reaction was different between stillbirths and live births. In stillbirths, villitis was diffuse and severe, with frequent trophoblastic necrosis and numerous *T. cruzi* (Figure 1A). Collections of mononuclear cells adhered to the sites of trophoblastic necrosis and a granulomatous reaction was occasionally observed in the intervillous space around these points. In live births, villitis was focal with few parasites, except in 1 case that had several intact pseudocysts in the villous stroma not surrounded by inflammatory cells (Figure 1B). Trophoblastic necrosis was rare or absent. These histologic findings in chagasic villitis are similar to those described in the literature and will not be discussed further.¹ The distribution of the cases with villitis caused by *T. cruzi* according to the percentage of reactive mononuclear cells with the antibodies CD45RO, CD20, CD45RO/OPD4, CD8, CD57, MAC387, CD68, and CD15 is listed in Table 2. The distribution of control cases is shown in Table 3.

Chagasic placentitis. *T lymphocytes, CD4+ T cells, and CD8+ T cells.* In chagasic villitis, CD45RO-positive T lymphocytes accounted for 20–40% of the inflammatory cells in most (62.5% of the cases) of the placentas; there was no significant difference between stillbirths and live births. T lymphocytes were present within the villous stroma, but in stillbirths they also adhered to the sites of trophoblastic necrosis and in intervillous space around these points. The CD8+ T lymphocytes outnumbered CD4+ T cells in all chagasic placentas (Figure 2). The CD4+:CD8+ ratios ranged from 0.04 to 0.38, and the lowest ratios were found in stillbirths (75% of the cases). Both CD4+ and CD8+ T cells were observed within the villous stroma and attached to the sites of trophoblastic necrosis.

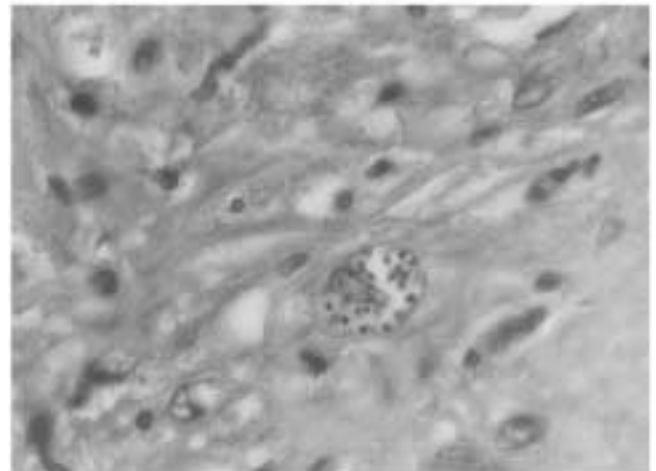
B lymphocytes. B lymphocytes identified with L26 antibody were present only in small amounts (0.1–20% of the inflammatory cells) within the villous stroma in 37.5% of the cases of chagasic villitis (2 stillbirths and 1 live birth).

Natural killer cells. NK1+ cells were found in the inflamed villous stroma and around the sites of trophoblastic necrosis in all chagasic placentas. There were only a few (< 1%) cells in 75% of the cases (Table 2).

Macrophages and monocytes. Macrophages of the villous



A



B

FIGURE 1. Chagasic villitis. A, stillbirth placenta with several parasite nests (arrowheads) in the placental villus. In contrast, in the live birth placenta (B), only 1 nest is found. (Hematoxylin and eosin stained, original magnification $\times 1,250$.)

stroma were identified by antibody CD68 (KP1), and fetal and maternal monocytes were identified by antibody MAC387. The latter did not react with macrophages of the villous stroma (Hofbauer cells). CD68+ macrophages were numerous in the stroma of the inflamed villi (Figure 3). However, in sites of trophoblastic necrosis there were adherent mononuclear cells that were also stained by antibody CD68. In stillbirths, CD68+ macrophages constituted 60–100% of the inflammatory cells in 75% of the cases. In the live births, these cells constituted 40–80% of the inflammatory cells in 75% of the cases. Ninety percent of the parasite nests (pseudocysts) were found in the cytoplasm of CD68+ macrophages. Monocytes labeled by antibody MAC387 were present in areas of villitis of all stillbirths with *T. cruzi* infection, but in only 1 of the live births (Table 2). They were observed predominantly attached to the sites of trophoblastic necrosis (Figure 4) and/or as a collection of cells below the intact trophoblast. They were apparently ma-

TABLE 2

Distribution of cases with villitis caused by *Trypanosoma cruzi* according to the percentage of mononuclear cells reactive to antibodies CD45RO, CD45RO/OPD4, CD8, NK1, CD20, CD68, MAC387, CD15, and S-100 protein

No.	CD45RO	CD45RO/OPD4	CD8	CD4/CD8 ratios	NK1	CD20	CD68	MAC387	CD15	S-100*
1	71.8%	37.3%	72.9%	0.33	<1%	0	62.8%	31.8%	0	+
2	24.3%	7.6%	23.8%	0.38	<1%	1.3%	63.3%	0	0	+
3	23.4%	33.3%	62.3%	0.36	<1%	0	35.5%	0	0	+
4	35.1%	8.4%	90.3%	0.04	<1%	0	47.1%	0	0	+
5	45.5%	19.4%	82.8%	0.20	2.6%	1.7%	81.6%	23.2%	33.6%	++
6	17.3%	10.7%	24%	0.36	<1%	0	82.1%	25%	6%	++
7	NA†	8.4%	71.9%	0.18	13.5%	0	77.9%	30.3%	25%	++
8	38.6%	13.2%	81.6%	0.13	<1%	1%	50.7%	16.9%	20.8%	++

* + = 1 cell/high-power field (hpf); ++ = 2-5 cells/hpf.
 † NA = not available.

ternal monocytes that migrated to the points of trophoblastic disruption. The granulomatous reaction observed in the intervillous space in placentas from stillbirths was composed mainly of CD68+ macrophages and MAC387+ monocytes.

Granulocytes. Granulocytes were identified by antibody CD15 (M1) only in placentas from stillbirths. They were found in sites of trophoblastic rupture (Figure 5) as small groups below the intact trophoblast or in areas of villous necrosis and constituted 0.1-40% of the inflammatory cells.

S-100 protein. In stillbirths, S-100+ cells were frequently observed in the stroma of some inflamed villi. These cells rarely contained parasite nests. In live births, S-100+ cells were found only occasionally in the villous stroma and there was no apparent relationship between them and areas of villitis.

Normal (control) placentas. In the control placentas, mononuclear cells positive for CD45RO, CD45RO/OPD4, CD8, CD20, MAC387, and CD15 were found in the fetal and/or maternal circulation but not in the villous stroma. Only a small number of CD68+ macrophages and occasional mononuclear S-100+ cells were present in the villous stroma (Table 3).

DISCUSSION

In endemic areas, the prevalence of South American trypanosomiasis (Chagas' disease) among pregnant women is between 23% and 81%. The risk of transmission is higher before the 34th week of gestation with a peak between the 22th and 26th weeks.³ Although congenital infection may occur at any phase of the maternal disease (acute or chronic, and regardless of clinical symptoms), placental inflammation

always represents a primary immune response of the fetus to *T. cruzi* because the placenta is the first fetal organ to encounter the parasite in the maternal circulation. The acute phase of *T. cruzi* infection is considered to last 30-90 days.⁶ This allowed us to immunohistochemically characterize the inflammatory cells of acute infection in a human tissue (placenta); studies of the acute phase have only been done in experimental models⁷⁻¹⁰ and none of them in the placenta.

This immunohistochemical study showed that in all cases of chagasic villitis the inflammatory infiltrate was composed mainly of CD68+ macrophages, T lymphocytes, and a few NK cells. Among T cells, CD8+ cells outnumbered CD4+ cells in all placentas (CD4+:CD8+ ratios ranged from 0.04 to 0.38). B cells were absent or rare. Some investigators have reported that CD8+ cells are the predominant subset of lymphocytes in tissues in acute experimental *T. cruzi* infection,⁹⁻¹¹ while others suggest the predominant subset to be CD4+ cells.^{7,8} Our findings in human placentas also show the predominance of CD8+ cells. We found a higher amount of CD8+ cells (23.8-90.3%) than that reported by Sun and Tarleton in mice, but the number of CD4+ lymphocytes (7.6-18.4%) in 62.5% of the placentas was similar to their results.¹⁰ The small number of CD4+ cells in the lesions probably does not reflect their true importance in the disease process.¹¹ The CD4+ Th1 lymphocytes appear to be the main cells responsible for induction of protective immunity in both acute and chronic infection with *T. cruzi*, and may be necessary for induction of the CD8+ cell infiltrate observed in the acute stage of the infection.^{6,11} The CD8+ lymphocytes, together with macrophages and interferon-γ, are considered important elements that control parasite replication during the acute phase and are also thought to be the

TABLE 3

Distribution of control cases according to percentage of mononuclear cells reactive to antibodies CD45RO, CD45RO/OPD4, CD8, NK1, CD20, CD68, MAC387, and CD15*

% of reactive cells	CD45RO 8 cases (%)	CD45RO/OPD4 8 cases (%)	CD8 8 cases (%)	NK1 8 cases (%)	CD20 8 cases (%)	CD68 7 cases (%)	MAC387 8 cases (%)	CD15 8 cases (%)
0	8 (100%)	8 (100%)	8 (100%)	8 (100%)	8 (100%)	0	8 (100%)	8 (100%)
0.1-20%	0	0	0	0	0	7 (100%)	0	0
20.1-40%	0	0	0	0	0	0	0	0
40.1-60%	0	0	0	0	0	0	0	0
60.1-80%	0	0	0	0	0	0	0	0
80.1-100%	0	0	0	0	0	0	0	0

* In the control cases, positive mononuclear cells for CD45RO, CD45RO/OPD4, CD8, NK1, CD20, MAC387, and CD15 were found within the fetal and maternal circulations but not within the villous stroma.

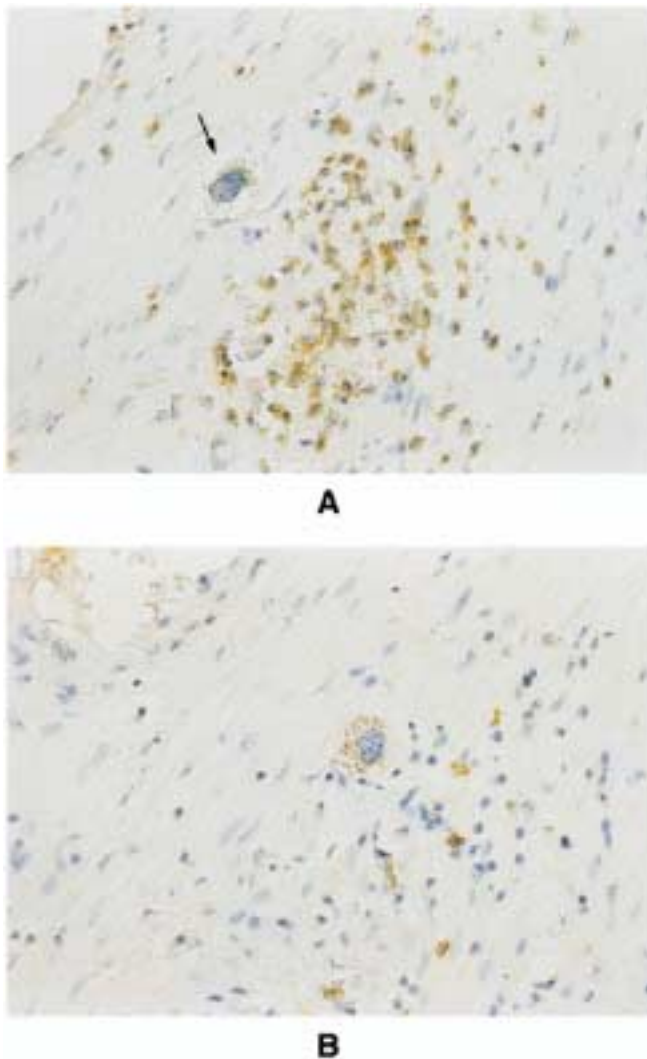


FIGURE 2. Chagasic villitis. **A**, CD8+ T lymphocytes around a parasite nest (pseudocyst) (arrow) **B**, same area as **A** showing CD4+ T cells. Note that the CD8+ T lymphocytes outnumber the CD4+ T cells. (Streptavidin biotin peroxidase method, original magnification $\times 330$.)

main effector cells responsible for tissue destruction.⁶ In chronic Chagas' disease, similar to what is observed in the acute phase, the inflammatory infiltrate is also composed mainly of CD8+ cells, with a low number of CD4+ cells.¹²⁻¹⁴ However, in contrast to the acute phase, parasites in the chronic phase of the disease are scarce or absent, leading to suggestions that autoimmunity may operate in the pathogenesis of this phase.

The CD68+ macrophages were the predominant cell type in chagasic villitis and usually outnumbered T lymphocytes, which is in contrast to what is found in *T. cruzi* infection of other tissues.^{10,12,14} In nonplacental tissues, T lymphocytes have been described as the most numerous cell in the inflammatory infiltrate.^{10,12,14} This macrophage reaction in the inflamed villi is not restricted to *T. cruzi* infection, but has also been observed in placentas with villitis of unknown etiology.¹⁵ The reason for this predominance of macrophages

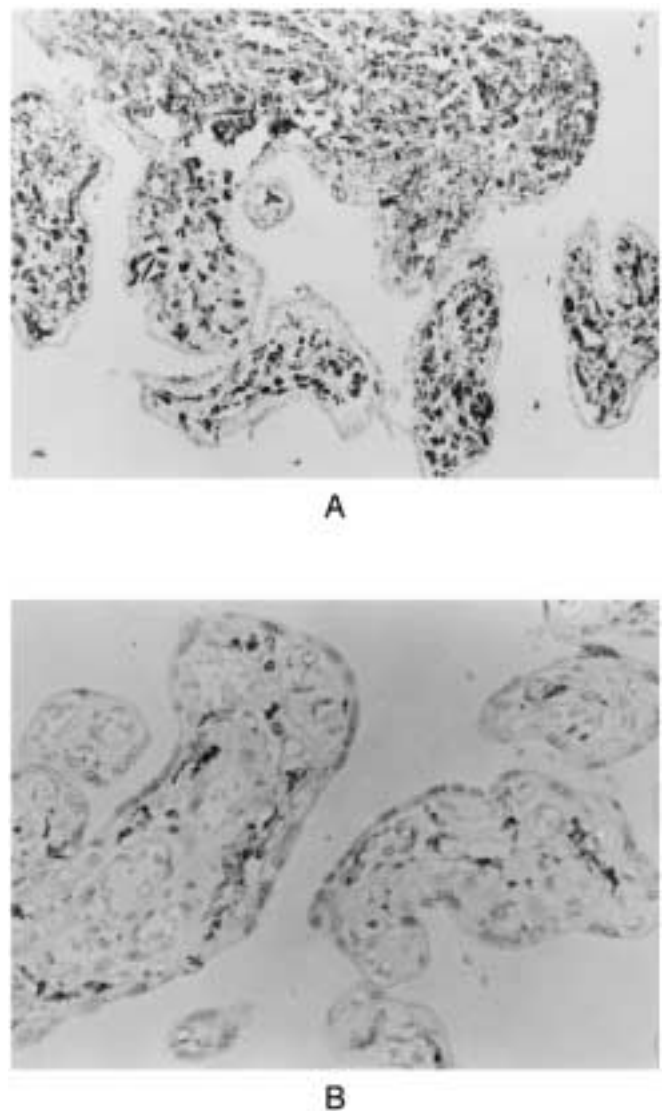


FIGURE 3. Chagasic villitis: **A**, inflamed villi show numerous CD68+ macrophages within the stroma. In contrast, in normal villi (**B**), only Hofbauer cells are reactive. (Streptavidin biotin peroxidase method, original magnification $\times 330$.)

in the placental inflammatory infiltrate, regardless of etiology, is unclear.

The CD68+ macrophages and some S-100+ cells, both found in the villous stroma, contained pseudocysts in our chagasic placentas. The intact pseudocysts were not surrounded by inflammatory cells. Only when the parasites were located outside the cells was there an inflammatory infiltrate in the villous stroma. This absence of inflammatory reaction to intracellular parasites is known since the first descriptions of Chagas' disease at the beginning of this century, but it has only been demonstrated only recently that *T. cruzi* infection interferes macrophage antigen presentation by macrophages.¹⁶

There were qualitative and quantitative differences in the pattern of inflammatory reaction to *T. cruzi* between the stillbirths and live births, which may be related to the number of parasites in the placental villi. Parasites were more abundant in stillbirths than in live births and their numbers di-

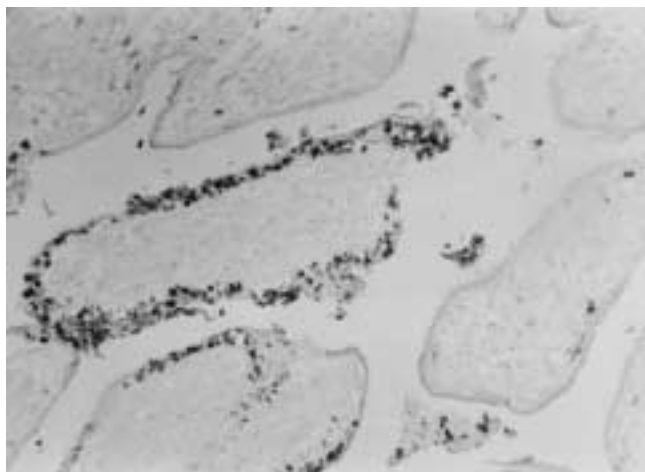


FIGURE 4. Chagasic villitis. Monocytes labeled by MAC387 adhered around the sites of trophoblastic necrosis. Note the absence of these cells in villi with intact trophoblasts (streptavidin biotin peroxidase method, original magnification $\times 130$).

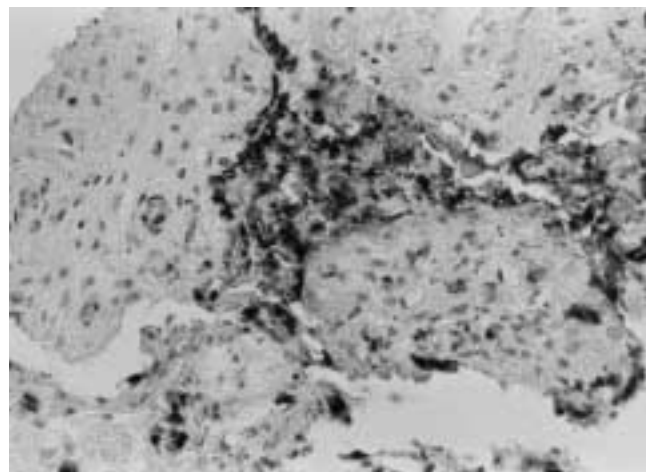


FIGURE 5. Chagasic villitis. Granulocytes labeled by CD15 adhered to areas of trophoblastic rupture. There are few cells within the villous stroma (streptavidin biotin peroxidase method, original magnification $\times 330$).

rectly correlated with the intensity and extension of the inflammatory reaction in the villi. These findings reinforce the hypothesis that the parasite provides the stimulus for the inflammatory response in the acute phase of the disease.¹¹ Other features of villitis in stillbirths were 1) frequent trophoblastic necrosis; 2) presence of MAC 387+ macrophages, CD15+ granulocytes, and T lymphocytes (CD8+ and CD4+) attached to the sites of trophoblastic necrosis; 3) the lowest CD4+:CD8+ ratios (≤ 0.20) in 75% of the cases; and 4) increased numbers of S100+ cells in the villous stroma. Villous stromal cells are known to express fetal class I antigens, but the trophoblast that covers the villi does not express classical major histocompatibility class (MHC) antigens (class I and class II).¹⁷ This has been related to protection of the tissue from both maternal immune recognition and cytotoxic cell attack.¹⁷ We believe that in chagasic placentitis the extensive destruction of trophoblast secondary to severe villous inflammation allows exposure of both parasitic and fetal MHC antigens of the villous stroma to maternal cells.¹ This may explain the extent of the reactions at the sites of trophoblastic necrosis, which are common in the severe villitis observed in the stillbirths. The cells attached to the sites of trophoblastic necrosis, which also participated in the inflammatory response in the villous tissue, appeared to be of maternal origin. It has been demonstrated in villitis of unknown etiology that most immunologic cells in inflamed villi are maternal.^{18,19} However, we should emphasize that a definitive differentiation between maternal or fetal origin of the inflammatory cells in our cases was not done.

The increased numbers of S-100+ cells observed in chagasic villitis of stillbirths are probably related to the extensive macrophage reaction that occurs in the chorionic villi of these cases. Dendritic cells, which are important antigen-presenting cells, are also S-100+. However, in our cases of chagasic villitis, in villitis of unknown etiology, and in normal placentas,¹⁵ S100+ cells did not show the morphology of dendritic cells. These S-100+ cells possibly represent a subtype of macrophages because they are CD68+¹⁵ and can contain parasite nests in the cytoplasm.

Why some placentas are more infected with *T. cruzi* than others is not well understood. The marked difference in placental tropism observed among strains of *T. cruzi* and the immunologic competence of the placenta in protecting the fetus against infection may play roles in the severity of congenital infection.^{3,20}

In conclusion, CD68+ macrophages and CD8+ T lymphocytes are the major cell populations in villitis caused by *T. cruzi*. However, the pattern of the inflammatory reaction is different between stillbirths and live births and probably related to the number of parasites in the placental villi. Furthermore, the placenta differs from other organs infected with *T. cruzi* in the predominance of macrophages over lymphocytes in the inflammatory infiltrate.

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