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## Increased association of *Trypanosoma cruzi* with sialoadhesin positive mice macrophages

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**Abstract** *Trypanosoma cruzi* is a parasite with large amounts of sialic acid (SA) residues exposed at its surface that seems to be involved in macrophages infection. Some macrophages, present in *T. cruzi* infected tissues, expresses sialoadhesin (Sn), a receptor that recognizes SA. Thus, the involvement of Sn in the association of *T. cruzi* to macrophages was investigated. Sn was induced in mice peritoneal macrophages by homologous serum (HS) cultivation. Epimastigotes and trypomastigotes associated more to HS cultured macrophages than to fetal bovine serum (FBS). Blocking of Sn with antibodies reduced the association of trypomastigotes to similar level as for FBS cultured macrophages. Desialylation reduced the association of parasites to HS cultured macrophages indicating the Sn importance. Furthermore, the entrance mechanism of trypomastigotes to Sn positive macrophages has a phagocytic nature as demonstrated by scanning electron microscopy and cytochalasin D treatment. Sn positive macrophages may important in the initial trypomastigote infection, thus in the establishment of Chagas disease.

**Keywords** *Trypanosoma cruzi* · Sialic acid · Macrophages · Sialoadhesin · Homologous serum · Entrance mechanism · Chagas disease

### Introduction

*Trypanosoma cruzi* is the etiological agent of Chagas disease affecting about 18 million people in Latin America. This protozoan presents three developmental forms (Brener 1973). Epimastigote and amastigote are proliferative forms found in the invertebrate gut and mammalian host cells, respectively. Trypomastigotes is the infective form able to adhere and gain access to the interior of cells. The understanding of how trypomastigotes infects host cells is far from being established (Araujo-Jorge et al. 1992; Rosestolato et al. 2002); however, evidences point to a phagocytic entrance mechanism in professional phagocytes (Rosestolato et al. 2002). This infection plays a crucial role in the maintenance and amplification of this disease. Several surface molecules have been implicated in the adhesion and internalization of *T. cruzi* into host cells (De Souza 2002). There are a number of evidences showing that sugar residues at the parasite glycoalk are involved in the interaction with host cells (Araujo-Jorge 1998). Among the sugar residues, sialic acid (SA) has gained much attention in the invasion process mainly due to the high amount present at the parasite surface (Souto-Pradon and de Souza 1985, 1986; Vermelho and Meireles 1994) and the existence of a surface trans-sialidase capable of removing SA and transferring it to the parasite surface mucins (Belen Carrillo et al. 2000; Acosta-Serrano et al. 2001).

Of the mammalian infected host cells, the macrophage is probably the first cell to encounter *T. cruzi* (Kierszenbaum et al. 1974; Deutschlander et al. 1978). Macrophages are a highly heterogeneous population (Rutherford et al. 1993) of phagocytes present in

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virtually every tissue of the body (Gordon 1995). A subpopulation of macrophages presents sialoadhesin (Sn), a surface receptor that recognizes SA. Sn is the founding member of the Siglecs family of SA binding Ig-like lectins (Crocker 2002). Although Sn function has not been fully understood, it is probably related to adhesion and signaling (Munday et al. 1999; Crocker 2002). Macrophage subpopulations expressing high levels of Sn are present in the spleen and lymph nodes; other organs of the body, including the heart (Crocker et al. 1997) express Sn in low or medium levels (Crocker et al. 1988; Crocker and Gordon 1989; Crocker et al. 1997; Munday et al. 1999; Crocker et al. 1990). Although mice peritoneal macrophages express low amounts of Sn, the *in vitro* expression of this receptor can be induced by the cultivation of these macrophages in the presence of homologous serum (Crocker and Gordon 1986; Crocker et al. 1988b; McWilliam et al. 1992). Thus, the interaction of cells to Sn positive macrophages can be further explored *in vitro*.

Previous studies with enzyme treatment (Araujo Jorge and de Souza 1984) and lectins (Araujo Jorge and de Souza 1988) have shown the involvement of sialic acid residues on the interaction of *T. cruzi* with macrophages. In addition, several surface-exposed sialoglycoconjugates have been identified in this parasite (Souto-Padron and de Souza 1985; Vermelho and Meirelles 1994; Acosta-Serrano et al. 2001). Macrophages expressing Sn are found in tissues where *T. cruzi* normally develops, indicating that they may favor the infection at these sites (Araujo-Jorge 1989; Crocker et al. 1997). In order to analyze the involvement of Sn on the penetration of *T. cruzi* we decided to carry interaction experiments using macrophages that expresses this receptor. The results obtained clearly show the involvement of sialic acid residues on the interaction of *T. cruzi* with these macrophages.

## Materials and methods

### Peritoneal macrophages

Peritoneal washes were performed in male Swiss mice (25–30 g) with Hank's solution and resident macrophages seeded on glass coverslips in 24-well plates. After 1 h at 37°C in a 5% CO<sub>2</sub> atmosphere, cells were washed and cultured in 199 medium with 2% fetal bovine serum (FBS) or 2% homologous serum (HS) (Crocker et al. 1988). After 24 h, cells were washed twice and used or, as for most experiments, further cultured in the same conditions for another 24 h period before use. HS was obtained by harvesting mice blood by cardiac puncture with a 3 ml syringe and a 26 G needle. Blood was allowed to clot in the syringe for 6 h at 25°C, serum was gently collected with a Pasteur pipette, transferred to tubes and centrifuged at 600 g for 10 min. Serum was collected, inactivated (30 min, 56°C), aliquoted and frozen at –20°C.

### Erythrocyte binding assay

Expression of Sn was measured by the capacity of macrophages to bind washed erythrocytes (McWilliam et al. 1992). Human blood from healthy donors was harvested into heparinized syringes, transferred to tubes and centrifuged at 500 g for 10 min. Plasma and leukocytes were discarded; erythrocytes were washed three times with PBS and stored at 8°C. Before being used, erythrocytes were washed with PBS and a 0.5% vol/vol suspension in 199 medium was prepared. Interactions of positive control (HS) or negative control macrophages (FBS) with erythrocytes were performed for 1 h at 37°C. After that, cells were washed twice in PBS and coverslips containing the cells were mounted over 10 µl of 1% glutaraldehyde in PBS on a glass slide. Quantification of the interaction was assayed by randomly counting at least 200 macrophages for each coverslip, in triplicates, under a Zeiss Axioplan microscope equipped with differential interference contrast using a 100× oil immersion objective. Percentage of macrophages with adhered erythrocytes and the mean number of erythrocytes per macrophage was calculated. An association index was obtained by multiplying the mean number of associated erythrocytes per macrophage by the percentage of macrophages with erythrocytes. Photographs were taken from these preparations.

### Parasites

Epimastigote (Y strain and Dm28 clone) and trypomastigotes (Y and CL strains), forms of *T. cruzi* were used. Epimastigotes were maintained at 28°C by weekly transfers in liver infusion tryptose (LIT) medium (Camargo 1964) supplemented with 10% fetal bovine serum (FBS). After 6 days in cultures parasites were collected, washed and resuspended in 199 medium. Trypomastigotes were obtained from the supernatant of infected Vero cells (Rosestolato et al. 2002). After 5–7 days of infection, the supernatant containing trypomastigotes were collected, centrifuged at 1000 g for 10 min and resuspended in 199 medium. Parasites were quantified and used for macrophage interaction experiments.

### Sialoadhesin blocking assay

Sn was blocked by the previous incubation of macrophages with SER-4 and 3D6 monoclonal antibodies (Crocker and Gordon 1989). Macrophages were washed and incubated with 1 µg/ml of both antibodies for 30 min. After that, macrophages were washed with 199 medium and incubated with trypomastigotes, Y strain.

### Desialylation of *T. cruzi*

Epimastigotes and trypomastigotes of *T. cruzi* were desialylated by incubation for 60 min in the presence of

0.006 U/ml of neuraminidase from *Vibrium cholerae* (Sigma) in Hank's solution, pH 5.5 (Monteiro et al. 1998). After that, parasites were washed with Hank's solution and allowed to interact with macrophages. After the interaction, cells were washed, fixed, stained and scored as described below.

### Macrophage interactions and quantification

Interactions were performed for 2 h at 37°C using a 10:1 parasite:macrophage ratio. After that, cells were washed twice with medium, fixed with absolute methanol for 5 min, stained with Giemsa, dehydrated in acetone-xylol, and mounted in Entellan. Quantification of the interaction was assayed by randomly counting at least 200 macrophages for each coverslip in quadruplicates under a Zeiss Axioplan Microscope using a 100× oil immersion objective. An association index was calculated by multiplying the mean number of associated (internalized and adhered) *T. cruzi* per host cell by the percentage of infected macrophages (Seabra et al. 2004). Photographs were also taken from these preparations.

### Cytochalasin D treatment

To verify the influence of Sn in the entrance mechanism of *T. cruzi* trypomastigotes, Y strain, macrophages

cultured with HS were preincubated for 30 min with 5 µg/ml of cytochalasin D. After that, trypomastigotes were added to macrophages for 1 h at 37°C (Rosestolato et al. 2002). Cells were washed, fixed, stained and internalized or adhered parasites were observed.

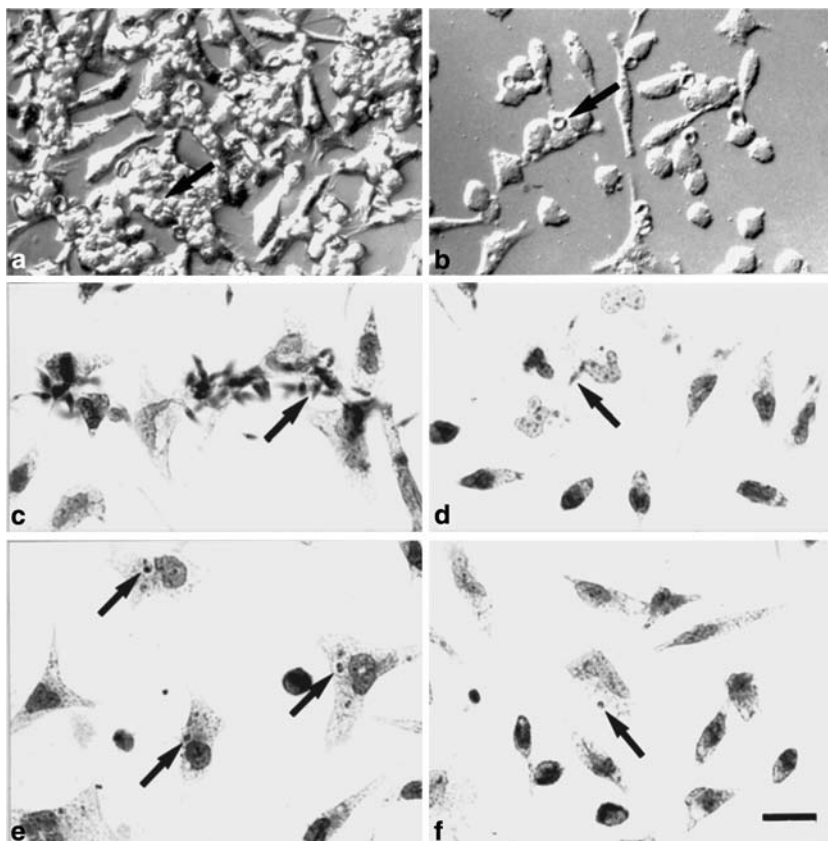
### Scanning electron microscopy

Interaction of trypomastigotes with macrophages was performed for 1 h. After that, cells were gently washed, fixed in 2.5% glutaraldehyde and 4% freshly prepared formaldehyde in 0.1 M cacodylate buffer, pH 7.2 for 1 h at room temperature. Cells were washed twice, post-fixed with 1% OsO<sub>4</sub>, dehydrated in ethanol, critical point dried in CO<sub>2</sub>, covered with a layer of gold and observed in a Zeiss 962 Digital scanning electron microscope.

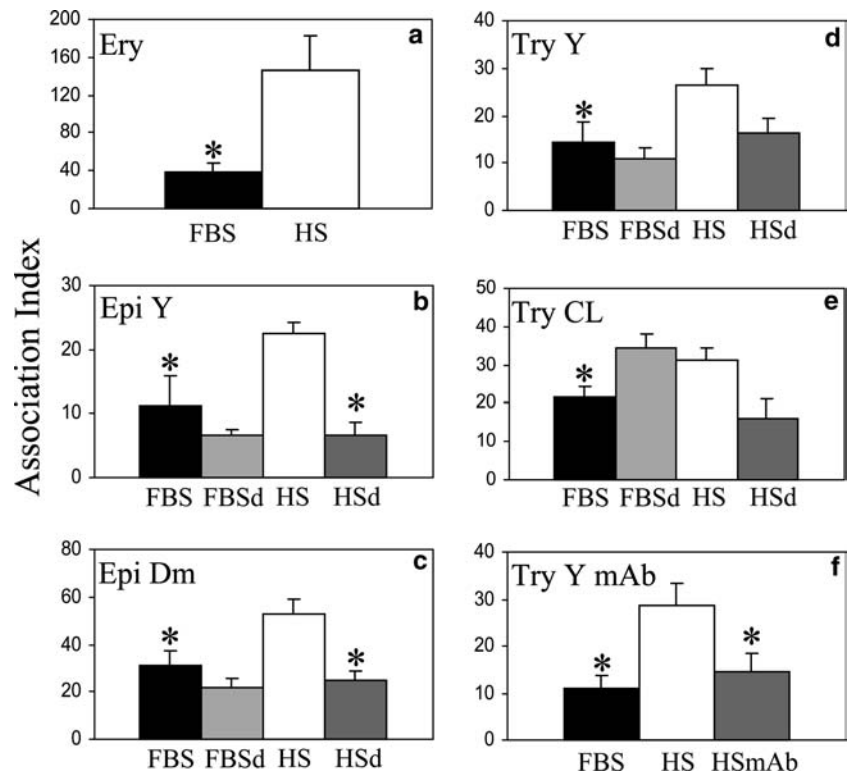
## Results

High amounts of Sn were detected on macrophages cultured for 48 h with HS when compared to FBS as determined by the amount of erythrocytes bound to their surface (Fig. 1a, b). Quantification of the interaction confirmed that macrophages cultured with HS had a higher erythrocyte association index (Fig. 2a). Macrophages cultured for 24 h with HS had fewer

**Fig. 1** Light micrographs of macrophages cultured with homologous serum (HS: left) or fetal bovine serum (FBS: right) after interaction with erythrocytes (a, b), epimastigotes (c, d) or trypomastigotes (e, f) of *Trypanosoma cruzi*. a, b Note the enormous amount of erythrocytes (arrow) bound to macrophages cultured with HS. c, d Epimastigotes (arrow) bound more to macrophages cultured with HS than to FBS. e, f Macrophages cultured with HS were more infected by trypomastigotes (arrow). a and b, differential interference contrast microscopy; c to f, Giemsa stained cells seen by bright field microscopy. Bar, 10 µm



**Fig. 2** Association index of erythrocytes (a), epimastigotes of the Y strain (b) and the Dm28 clone (c), trypomastigotes of *Trypanosoma cruzi* of the Y (d) and CL strain (e) after desialylation or after blockage of the sialoadhesin receptor with the monoclonal antibodies with macrophages cultured with fetal bovine serum (FBS) or homologous serum (HS). Macrophages were cultured for 48 h with the indicated serum and allowed to interact with cells. Desialylation (FBSd, HSd) and antibodies blocking (HSmAb) were performed as described in materials and methods. Values are means  $\pm$  standard deviation of three independent experiments. \* Significantly different ( $P < 0.05$ ) from values for macrophages cultured with HS as calculated by the Student *t* test



erythrocytes bound to their surface when compared to cells cultured for 48 h. However, these macrophages had significantly more associated erythrocytes than cells cultured with FBS (not shown).

Epimastigotes and trypomastigotes, independently of the strain, associated more to macrophages cultured with HS than FBS (Fig. 1c, d, e, f). The higher association was further established by the quantification of these interactions (Fig. 2b, c, d, e, f). Blockage of the Sn receptor with the monoclonal antibodies decreased the association index of trypomastigotes to macrophages cultured with HS to the same level as parallel FBS cultured cells (Fig. 2f). Desialylation of epimastigotes, independent of the strain, caused a significant reduction in the association index to macrophages cultured with HS (Fig. 2b, c). Desialylation of trypomastigotes, Y and CL strain, also lowered the association index to macrophages cultured with HS (Fig. 2d, e). Desialylation of epimastigotes (both strains) and trypomastigotes (Y strain) also lower the association indexes to macrophages cultured with FBS (Fig. 2b, c, d). However, desialylation of trypomastigotes from the CL strain caused an increase in the association index to FBS cultured macrophages (Fig. 2e).

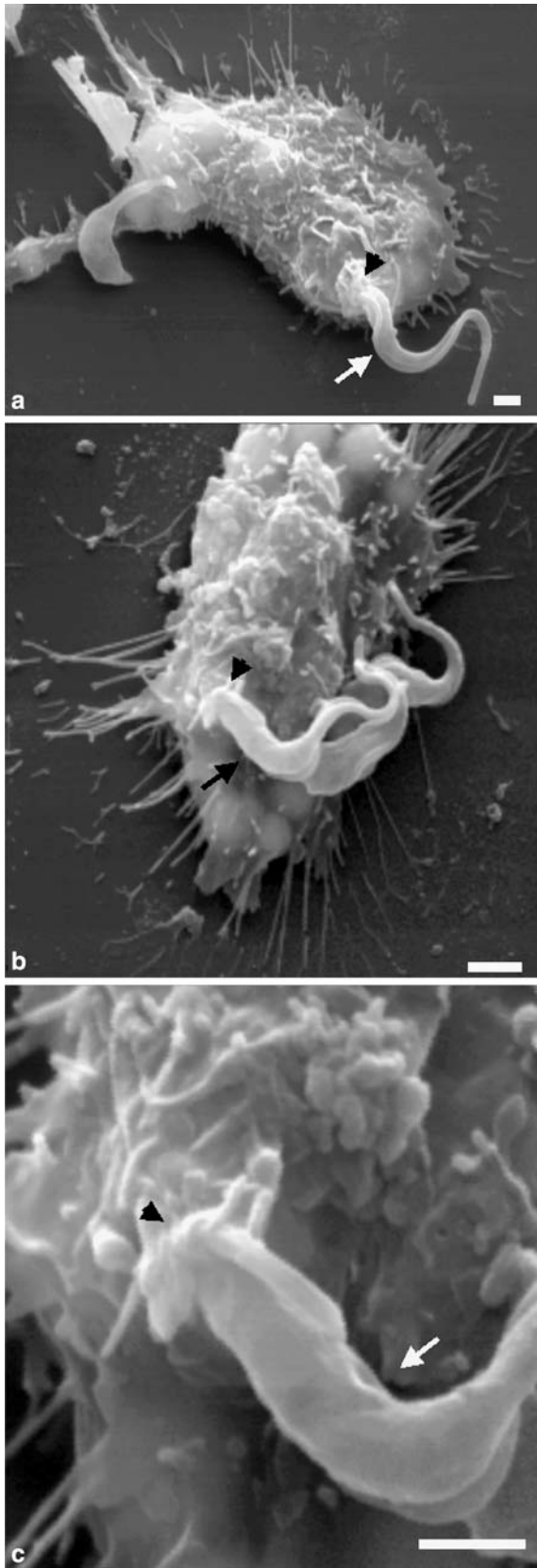
Higher association of trypomastigotes to macrophages cultured with HS was also observed by scanning electron microscopy (Fig. 3). Plasma membrane projections from the macrophage were easily visualized (Fig. 3b, c). Cytochalasin D treatment caused rounding of macrophages as described before (Rosestolato et al. 2002) and abolished trypomastigotes internalization; only adhered parasite were observed (not shown).

## Discussion

Infection of macrophages by trypomastigotes of *T. cruzi* is a crucial phase in the development of Chagas disease, which is far from being understood. A better comprehension of this interaction may lead to new understanding of this disease and probably new treatment strategies. In this context, many researchers have tried to determine the molecules involved in adherence and internalization of *T. cruzi* into macrophages. The presence of Sn positive macrophage in organs where *T. cruzi* preferentially infects, the high amounts of SA at the parasite surface and the control of this sugar residue exposure by trans-sialidase, led us to investigate how Sn influences the interaction of this parasite to macrophages.

Peritoneal macrophages cultured with HS express Sn (Crocker et al. 1988). This result was confirmed in our experiments by the increased binding of erythrocytes to macrophages (McWilliam et al. 1992). We also observed by the same binding technique that the amount of Sn increases with time in culture. Thus, our experimental conditions reproduce previous experiments.

Epimastigotes and trypomastigotes of *T. cruzi* are highly sialylated forms (Souto-Padron and de Souza 1985; Vermelho and Meirelles 1994; Acosta-Serrano et al. 2001). Thus, as expected, both forms did associate more to macrophages expressing Sn. This was confirmed by blocking this receptor with antibodies. In that condition, the same level of association obtained with control macrophages (cultured with FBS) was found for macrophages cultured with HS. Further confirmation of



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**Fig. 3** Internalization of trypanomastigotes into macrophages cultured with homologous serum as seen by scanning electron microscopy. (a) Trypanomastigotes (arrow) entering macrophages. Note the membrane projection (arrowhead) from the macrophage around the parasite. (b) Three trypanomastigotes (arrow) can be observed on the macrophage. (c) Higher magnification of the interaction site of b. Note membrane projection (arrowhead) from the macrophage around the parasite. Bar, 2  $\mu$ m.

by the action of neuraminidase, a reduction in the association index to macrophages cultured with HS was detected. Although this reduction was only significant for epimastigotes, desialylated trypanomastigotes also associated less to macrophages cultured with HS, further confirming the influence of Sn to this interaction. The percentage reduction in association index after desialylation was different dependent on the *T. cruzi* strains. This heterogeneous result could be explained by the difference found in the *T. cruzi* strains (Andrade 1999). The lower association index seen after desialylation of epimastigotes and trypanomastigotes (Y strain only) to FBS cultured macrophages does not agree with previous reports (Araujo Jorge and de Souza 1984, 1988). Many factors may explain this discrepancy as follows. Macrophages used in this work were cultured for 48 h. Although this may not seem to change much, these macrophages produce less nitric oxide after interferon gamma activation than the ones cultured for 24 h (DaMatta RA unpublished observations). The increase in the cultivation period may also change the receptor repertoire of macrophages. Finally, variation in the experimental conditions (Araujo-Jorge et al. 1989) and reagents may also explain these differences.

The expression of Sn increased the association of *T. cruzi* to macrophages. Thus, we used this model to probe the entry mechanism of this parasite into macrophages. Scanning electron microscopy confirmed the higher association of trypanomastigotes to these macrophages. More interesting, plasma membrane projections were observed on the parasite macrophage contact site. Taken together, these results corroborates with the hypothesis where *T. cruzi* enters macrophages preferentially by a phagocytic mechanism and that Sn may help in the adherence process. Following the same experimental procedure of Rosestolato et al. (2002), we could also determine that trypanomastigotes do need integral filamentous actin to enter macrophages. Thus, our results corroborate with the phagocytic nature entrance mechanism of trypanomastigotes into professional phagocytes.

Recently, it has been suggested that Sn may enhance macrophage clearance of sialylated bacteria, such as *Neisseria meningitidis*, in tissue and at sites of inflammation (Jones et al. 2003). It is clear from our work that Sn can increase the association of *T. cruzi* to macrophages. How could the expression of Sn influence the outcome of Chagas disease? One of the preferential sites of acute infection of *T. cruzi* is the spleen (Lima et al. 2001) and lymph nodes (Nunes et al. 1992). The heart is also an organ that has Sn positive macrophages (Croc-

the involvement of Sn in the interaction with *T. cruzi* was obtained with desialylation of parasites. When SA was removed from epimastigotes and trypanomastigotes

ker et al. 1997). Thus, it is reasonable to suggest that *T. cruzi* may use Sn positive macrophages to initiate infection in these tissues. Further studies using double labeling of parasites and Sn positive macrophages as well as Sn-deficient macrophages are required to determine the in vivo involvement of Sn in the interaction of *T. cruzi* and macrophages.

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