Differential Interleukin-8 and Nitric Oxide Production in Epithelial Cells Induced by Mucosally Invasive and Noninvasive *Trypanosoma cruzi* Trypomastigotes

C. S. Eickhoff, L. Eckmann, and D. F. Hoft

Department of Internal Medicine, Saint Louis University Health Sciences Center, St. Louis, Missouri, and Department of Medicine, University of California at San Diego, La Jolla, California

Received 20 March 2003/Returned for modification 20 May 2003/Accepted 20 June 2003

An estimated 16 to 18 million people in South and Central America are infected with *Trypanosoma cruzi*, the protozoan parasite causing Chagas’ disease (7). Fifty thousand deaths per year result from the cardiac and gastrointestinal pathology due to chronic *T. cruzi* infection. *T. cruzi* is most commonly transmitted to humans through contact with the infected feces of the reduviid bug. *T. cruzi* metacyclic trypomastigotes (MT) in reduviid bug feces can initiate infection through cuts in the skin or through contact with mucosal surfaces, including the eyes and mouth. *T. cruzi* MT infect epithelial cells, develop, and replicate intracellularly as amastigotes and are released from lysed host cells as blood form trypomastigotes (BFT). BFT are then able to infect new epithelial cells and most other types of mammalian host cells and repeat the intracellular replication cycle. *T. cruzi* MT are able to initiate infection through the conjunctival and gastrointestinal mucosa. On the other hand, we have shown that BFT are unable to infect through mucosal surfaces (14). The mechanisms responsible for the mucosal invasiveness of MT, but not BFT, are not known.

Tissue infection with *T. cruzi* elicits significant inflammation responsible for the pathology associated with Chagas’ disease. BFT, but not MT, infection of macrophages induces proinflammatory cytokine and nitric oxide (NO) production (8). The effects of *T. cruzi* infection on proinflammatory responses in epithelial cells, however, have not been reported. We found that mice challenged orally with MT do not develop detectable mucosal inflammation until 4 to 7 days after infection, sufficient time for three to four cycles of replication and 100- to 1,000-fold amplification of BFT (16). We hypothesize that the inability of MT to induce inflammatory mediators could be important for *T. cruzi* mucosal infectivity. On the other hand, it may be advantageous for later stages of infection with BFT to induce inflammation, prolonging the life of the host by preventing uncontrolled parasite replication.

We studied the ability of *T. cruzi* strain Tulahuén MT and BFT to induce chemokine responses in mucosal epithelial cells. Culture-derived MT were prepared by subculture of axenically cultivated epimastigotes into modified Grace’s medium (Sigma, St. Louis, Mo.) for 7 to 10 days. These culture-derived MT are morphologically similar to insect-derived MT and are mucosally infective (20). Insect-derived MT were prepared by allowing *T. cruzi*-infected reduviid bugs (*Dipetalogaster maximus*) to feed on anesthetized mice. Engorged insects were then incubated in glass vials for 3 to 5 h, excreta were pooled, and parasite concentrations were determined by direct counting with a hemocytometer. BFT were collected by sacrificing highly parasitemic mice and copurifying parasites with mononuclear cells in Ficol-Paque density gradients (Amersham Pharmacia Biotech Inc., Piscataway, N.J.). Human cervical (HeLa) and gastric (AGS) epithelial cells obtained from the American Type Culture Collection (Manassas, Va.) were infected with MT and BFT in Dulbecco modified Eagle medium or McCoy’s 5A medium (Life Technologies, Gaithersburg, Md.) supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml) in either 24-well culture plates or 4-well tissue culture slide chambers (Nalge Nunc International, Naperville, Ill.).

We first measured the production of interleukin-8 (IL-8) in supernatants of cultures of human epithelial cells infected with BFT and culture-derived MT by enzyme-linked immunosorbent assay (ELISA) as previously described (11). We chose HeLa cells, which are commonly used for in vitro studies of *T. cruzi* infection, as an initial model for studies of chemokine responses produced by epithelial cells. Because we have shown that *T. cruzi* MT invade through the gastric epithelium after oral challenges (16), we chose AGS cells (derived from gastric adenocarcinoma tissue) as a more relevant model of *T. cruzi* mucosal infection. HeLa and AGS cells infected with BFT for 48 h produced marked increases in IL-8 in a dose-dependent manner (Fig. 1a and b, respectively). After the same period of infection, culture-derived MT induced only 20% as much IL-8 as did BFT in HeLa cells and failed to induce IL-8 in AGS cells, even at the highest infection level tested (>5:1 parasite-to-cell ratio). Insect-derived MT did not induce IL-8 in any of
the cell lines tested (data not shown). In all cases, IL-8 induced by BFT was maximal in cultures by 48 h (data not shown). Increased IL-8 protein production after BFT infection was paralleled by increased IL-8 mRNA expression detected by reverse transcription-(RT) PCR at 24 h after *T. cruzi* infection (Table 1). BFT infection of HeLa cells induced >20-fold more IL-8 mRNA than did infection with culture-derived MT.

The lack of IL-8 induction after MT infection could be related to a “silent” invasion of epithelial cells or active inhibition of the IL-8 responses in these cells. To test whether or not MT could actively inhibit IL-8 production, HeLa cells were infected with culture-derived MT and stimulated with IL-1β (10 ng/ml), a potent activator of IL-8 secretion. Infection with culture-derived MT induced minimal IL-8 levels (<100 pg/ml), whereas IL-1β stimulated >3,000 pg of IL-8 per ml, both in the presence and in the absence of culture-derived MT infection. Experiments were performed with insect-derived MT with similar results. Therefore, MT did not actively inhibit the induction of IL-8 by IL-1β stimulation.

To determine whether the differential effects of BFT and MT infections of human epithelial cells are unique to IL-8 induction or more global differential effects on chemokine production are stimulated, we measured the levels of GRO-α (another CXC chemokine with neutrophil chemotactic properties) and MCP-1 (a representative CC chemokine) in supernatants from MT- and BFT-infected cultures by ELISA as previously described (11). GRO-α and MCP-1 levels in epithelial cell supernatants were both markedly increased in wells infected with BFT (Table 2). Maximal BFT infections induced >200 pg of GRO-α per ml in the HeLa and AGS cell lines, while culture-derived MT failed to induce detectable levels of this chemokine. In addition, BFT infection of HeLa cells induced MCP-1 production that was not seen in culture-derived MT-infected HeLa cell supernatants. Thus, these experiments demonstrate that infection with BFT and MT induced global differential effects on chemokine production in epithelial cells.

To test whether these differences in IL-8, GRO-α, and MCP-1 production were the result of differential infectivity of BFT and culture-derived MT in vitro, infected HeLa cells were Giemsa stained and examined microscopically. HeLa cells infected with BFT and culture-derived MT were found to have similar percentages of infected cells; 48 h after infection, 7 to 8% of cells were infected by both BFT and culture-derived MT. Infected cells also contained similar numbers of intracellular parasites (three to four amastigotes per infected cell). In fact, by day 4 of infection, the percentage of cells infected by culture-derived MT was almost twice that of cells infected by BFT, suggesting that the lack of chemokine induction by MT was accompanied by enhanced intracellular replication of *T. cruzi*.

### Table 1. IL-8 RT-PCR

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of molecules/µg</th>
<th>Normalized IL-8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-8 mRNA</td>
<td>β-actin mRNA</td>
</tr>
<tr>
<td>Uninfected</td>
<td>$1.2 \times 10^4$</td>
<td>$1.3 \times 10^7$</td>
</tr>
<tr>
<td>BFT</td>
<td>$4.0 \times 10^5$</td>
<td>$2.5 \times 10^7$</td>
</tr>
<tr>
<td>CMT</td>
<td>$2.4 \times 10^5$</td>
<td>$3.2 \times 10^7$</td>
</tr>
</tbody>
</table>

*HeLa cells (2 \times 10^5) were infected with 2 \times 10^6 parasites. After 24 h, extracellular parasites were washed away and total RNA was extracted. RT-PCR was performed with IL-8- and β-actin-specific primers by using a positive control template to calculate the number of specific mRNA molecules per microgram of mRNA. Shown are numbers of IL-8 and β-actin mRNA molecules per microgram and levels of IL-8 normalized for β-actin expression (calculated by dividing the number of IL-8 mRNA molecules per microgram of total RNA by the number of β-actin mRNA molecules per microgram of total RNA [×10,000] and then dividing by this value obtained for uninfected cultures).*

### Table 2. GRO-α and MCP-1 induction by BFT†

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Cell line</th>
<th>Inoculum (no. of organisms)</th>
<th>Cytokine secretion (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRO-α</td>
<td>HeLa</td>
<td>1.25 \times 10^5</td>
<td>203 ± 28</td>
</tr>
<tr>
<td>GRO-α</td>
<td>HeLa</td>
<td>2.5 \times 10^5</td>
<td>117 ± 40</td>
</tr>
<tr>
<td>GRO-α</td>
<td>HeLa</td>
<td>5 \times 10^4</td>
<td>97 ± 28</td>
</tr>
<tr>
<td>GRO-α</td>
<td>AGS</td>
<td>1.25 \times 10^5</td>
<td>232 ± 6</td>
</tr>
<tr>
<td>GRO-α</td>
<td>AGS</td>
<td>2.5 \times 10^5</td>
<td>165 ± 8</td>
</tr>
<tr>
<td>GRO-α</td>
<td>AGS</td>
<td>5 \times 10^4</td>
<td>33 ± 8</td>
</tr>
<tr>
<td>MCP-1</td>
<td>HeLa</td>
<td>1.25 \times 10^5</td>
<td>276 ± 11</td>
</tr>
<tr>
<td>MCP-1</td>
<td>HeLa</td>
<td>2.5 \times 10^5</td>
<td>250 ± 21</td>
</tr>
<tr>
<td>MCP-1</td>
<td>HeLa</td>
<td>5 \times 10^4</td>
<td>157 ± 19</td>
</tr>
</tbody>
</table>

*HeLa and AGS cells were incubated overnight in 24-well plates at 2 \times 10^5 and 5 \times 10^5 cells per well, respectively, and then infected with BFT or MT. After 24 h, infected cell monolayers were washed to remove extracellular parasites. Supernatants were collected 48 h after infection and tested by ELISA for GRO-α and MCP-1 by ELISA. Results shown are in picograms per milliliter (average ± the standard error with the medium background subtracted). Results shown are representative of two experiments.*
MT. Multiple studies have demonstrated that *T. cruzi* parasites express stage-specific proteins and other molecules, including some that are generated by stage-specific posttranscriptional and posttranslational modifications (1, 5, 6, 10, 13, 18, 21). It is possible that the differential chemokine and NO responses induced in epithelial cells by BFT and MT are due to the distinct glycoconjugates present on their surface membranes shown previously to differentially induce cytokines and NO production in macrophages (3, 4, 8, 9). BFT and MT were equally infective in epithelial cells, indicating that the lack of chemokine and NO responses in epithelial cells infected with MT could not be explained by decreases in infection. In fact, higher levels of parasite replication were detected 4 days after infection with MT, suggesting that lower levels of microbial activity were induced in these cultures. These findings are consistent with our hypothesis that the inability of BFT to establish infection in mice after mucosal challenge may be due to potent trypanocidal activity induced in epithelial cells upon mucosal invasion.

It may benefit the parasite for BFT to induce inflammation and microbial mechanisms so that overwhelming infection does not kill the host, allowing the host-parasite relationship to continue. Studies have shown that NO levels in mice correlate with protection but not eradication of parasite infection (23). In addition, other groups have shown that removal of the inducible NO synthase gene in knockout mice results in extreme susceptibility to acute *T. cruzi* infection (19). Therefore, NO production induced by inflammatory signals may be important for the maintenance of a stable host-parasite relationship. However, inflammatory diseases of the gut, as well as the heart, are the major cause of morbidity and mortality in Chagas’ disease patients. Treatments decreasing the production of chemokines and other inflammatory mediators could be useful in limiting or slowing the progression of pathology associated with Chagas’ disease.

**REFERENCES**


Editor: J. M. Mansfield