Preferential Brain Homing following Intranasal Administration of *Trypanosoma cruzi*

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The Chagas’ disease parasite *Trypanosoma cruzi* commonly infects humans through skin abrasions or mucosa from reduviid bug excreta. Yet most studies on animal models start with subcutaneous or intraperitoneal injections, a distant approximation of the skin abrasion route. We show here that atraumatic placement of *T. cruzi* in the mouse nasal cavity produced low parasitemia, high survival rates, and preferential brain invasion compared to the case with subcutaneously injected parasites. Brain invasion was particularly prominent in the basal ganglia, peaked at a time when parasitemia was no longer detectable, and elicited a relatively large number of inflammatory foci. Yet, based on motor behavioral parameters and staining with Fluoro-Jade C, a dye that specifically recognizes apoptotic and necrotic neurons, brain invasion did not cause neurodegenerative events, in contrast to the neurodegeneration in the enteric nervous system. The results indicate that placement of *T. cruzi* on the mucosa in the mouse nasal cavity establishes a systemic infection with a robust yet harmless infection of the brain, seemingly analogous to disease progression in humans. The model may facilitate studies designed to understand mechanisms underlying *T. cruzi* infection of the central nervous system.

The protozoan parasite *Trypanosoma cruzi* causes Chagas’ disease, a debilitating condition that afflicts millions of people in the Americas. Patients with chronic symptomatic disease typically present abnormalities of the gastrointestinal tract (megaeosophagus and megacolon) and/or heart (cardiomegaly). Such megasymphdromes are caused, in part, by damage of the peripheral nervous system, particularly the myenteric (Auerbach’s) plexus, submucosal (Meissner’s) plexus, and nerve fibers, which may be largely destroyed (1, 23, 30).

Prior to overt megasymdrome, patients exhibit normal electrocardiogram readings and digestive processes, despite parasitological and/or serological evidence of continued *T. cruzi* infection. This indeterminate phase of the disease may persist for decades while presenting minor peripheral neuropathy (sensory impairment and diminished tendon jerks) in a relatively small (~10%) proportion of patients (17). Patients may show signs of neurorregeneration and/or neuroprotection, such as an age-dependent relative increase in the number of ganglion cells in the heart and enteric nervous system (23). Neurorregeneration in the enteric nervous system may occur even in chagasic megacolon (13).

Chronic indeterminate and symptomatic Chagas’ disease is preceded by the acute phase of the disease, which commonly starts when *T. cruzi* gains access to the body through skin abrasions or undamaged mucosa, usually in the face, from the contaminated semiliquid excrement of hematophagous reduviid insects. Entry through the conjunctival mucosa is readily diagnosed by the swelling of the eyelids (Romanha’s sign) (38). Another logical mucosal port of entry is the nasal cavity, which is directly accessible to moving trypanosomes deposited nearby within insect excreta. Notably, *Triatoma infestans*, a reduviid insect that frequently transmits Chagas’ disease, is attracted to the human face by the carbon dioxide exhaled during respiration (44, 45), a behavior likely favoring *T. cruzi* intranasal transmission.

Most studies with animal models of Chagas’ disease focus on the interaction of the parasite with the heart, gastrointestinal tract, and other organs. One notable exception is the central nervous system (CNS), even though *T. cruzi* infects the CNS in most patients with acute Chagas’ disease (20). Paradoxically, *T. cruzi* infection of the CNS is symptomatically and pathologically silent in immunocompetent individuals. Infection of the brain by most other pathogenic microbes carries severe and enduring detrimental effects (40), including brain infection by the *T. cruzi* counterpart in Africa, *Trypanosoma brucei*, which causes sleeping sickness and significant brain abnormalities in humans and cattle (22). Curiously, while cardiac problems are the main cause of morbidity in chronic Chagas’ disease, such problems are relatively benign in sleeping sickness, where conversely, CNS-dependent neurological problems dominate (5).

In the relatively few instances where *T. cruzi* invasion of the CNS was examined in animal models, parasites were inoculated into experimental sucking animals by the intraperitoneal route (12), or the brain infection was studied in the context of immunological responses, such as determining the prevailing inflammatory cell type (36), chemokine-dependent lymphocyte homing (37), or proinflammatory cytokine-dependent invasion (31).

As a first step toward understanding the molecular basis of *T. cruzi* interaction with cells in the CNS, we sought to develop an experimental mouse model that gives consistent invasion of the brain in immunocompetent adult animals. We found that atraumatic placement of *T. cruzi* in the nasal cavities of susceptible and resistant mice produced a systemic infection with
preferential invasion of the brain, as assessed by quantitative PCR, parasitemia, histology, and immunohistochemistry. Intranasally inoculated susceptible animals survived acute infection which was otherwise lethal if the parasites were injected subcutaneously. Furthermore, brain invasion did not trigger detectable neurodegeneration.

MATERIALS AND METHODS

Parasites. Trypanosoma cruzi (Tulahuen strain clone C2) was maintained in Vero cells at 37°C in 5% CO2 with 90% relative humidity in Dulbecco’s minimal essential medium supplemented with 2.5% (vol/vol) fetal bovine serum, as previously described (33). Invasive and highly virulent T. cruzi trypomastigotes were washed serially with serum-free Dulbecco’s minimal essential medium and sterile phosphate-buffered saline (PBS), counted in a hemacytometer, and resuspended in the appropriate volume of sterile PBS immediately prior to infection.

Infection. Six- to 8-week-old female C57BL/6 and BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were anesthetized with tribromoethanol (Avertin) solution and infected subcutaneously in the hind-limb footpad (30 μl) or intranasally (2 μl in air) every 2 min for 15 min, for a 20-μl total volume equivalent to 0.5 × 10^3, 5 × 10^3, or 25 × 10^3 parasites/mouse.

To induce basal ganglion neurodegeneration, C57BL/6 mice (10 weeks of age) were injected intraperitonally with increasing doses of 3-nitropropionic acid (3-NP) over 6 days (Sigma-Aldrich, St. Louis, MO) (20 mg/kg body weight every 12 h for 48 h, then 40 mg/kg body weight every 12 h for 48 h, and finally, 60 mg/kg body weight every 12 h for 48 h) (16). Semiquantitative behavioral assessment of motor disorders related to brain degeneration (general locomotor activity, truncal dystonia, and hind-limb dystonia) (16) was performed 1 day after the last injection of the neurotoxin. The same motor disorder assessment was performed at the peak of brain parasitism (25 days postinfection). For histological analysis, animals were sacrificed (on the indicated days) by CO2 asphyxiation and perfused intracardially with sterile PBS, and organs were removed, flushed with PBS, if necessary, and snap-frozen in liquid nitrogen or fixed in formalin solution. To determine parasitemia, tail vein blood was collected at the indicated days in a 1/10 volume of heparin (Sigma-Aldrich, St. Louis, MO), and parasites were counted by light microscopy (8). All procedures conducted were in accordance with the regulations set by the NIH Office of Laboratory Animal Welfare and were approved by the Institutional Animal Care and Use Committee at Tufts University.

Quantitative PCR. Genomic DNAs were purified from uninfected and T. cruzi-infected brains by use of a DNeasy kit (Qiagen, Valencia, CA). T. cruzi was quantified from brain tissue by real-time PCR (11). A standard curve was generated from DNAs prepared from weighed, uninfected tissues spiked with known amounts of T. cruzi trypomastigotes to determine CT values; these, in turn, were used to compute approximate numbers of T. cruzi/gram brain.

Histology/immunohistochemistry. Formalin-fixed samples were embedded in paraffin wax, and sections (5 μm) were stained with hematoxylin and eosin (H&E) or Fluoro-Jade C, a fluorescent histological marker of degenerating neurons (35) (Invitrogen, Carlsbad, CA). Antibody specific for F4/80 antigen (Abcam) was used to immunochemically identify murine cells with macrophage-like properties in brain slices (presumably microglia) prepared with a Ventana 300 automatic immunohistochemical stainer (Ventana Medical Systems).

For quantification of inflammatory foci in T. cruzi-infected tissue, three noncontinuous H&E-stained brain sections were counted per mouse; for each section, 25 fields within the basal ganglia were counted at a magnification of ×200, and the average number of parasite nests per 25 fields was determined for each animal and used to determine the average number of foci for three animals.

Statistical analysis. All experiments were carried out multiple times with more than three animals per experiment. Statistical analyses were conducted using GraphPad Prism software (version 4.0), and results are reported as means ± standard errors from one representative experiment. For analyses involving two treatments and comparison of multiple treatments, t tests and analysis of variance with post hoc Dunnett’s multiple comparison tests were performed, respectively.

RESULTS

Intranasal inoculation of T. cruzi produced low parasitemia and high brain invasion levels. Subcutaneous inoculation of T. cruzi into the footpad of resistant C57BL/6 mice infects the heart, gastrointestinal tract, and other organs, typically producing nonlethal disease in a broad dose range. To determine if such systemic infection is accompanied by brain invasion, we counted parasites in the tail vein blood (parasitemia) and in the brain by real-time PCR (11) after subcutaneous injection of 5 × 10^3 parasites into the mouse footpad. The results showed that parasitemia was maximal at 12 days postinoculation (PI) and undetectable a week thereafter (Fig. 1A). Brain parasitism was not detectable at the height of parasitemia, but it became measurable at the end of parasitemia, peaked at 25 days PI, and declined sharply thereafter (Fig. 1A).

Intranasally inoculated T. cruzi produced parasitemia and brain invasion analogous to those by subcutaneously injected parasites, except for the reversal in the extent of parasite load (Fig. 1B). Thus, parasitemia in intranasally infected mice was about one-third of that after subcutaneous injection, in contrast to the brain load, which increased about 2.0-fold (Fig. 1C). A similar progression and extent of parasite load were obtained when C57BL/6 mice were subcutaneously and intranasally infected with a five times greater parasite inoculum (25 × 10^3 parasites/mouse) (Fig. 1D to F).

Susceptible BALB/c mice intranasally inoculated with T. cruzi survived infection that would otherwise be lethal if they were injected subcutaneously. Given that intranasally inoculated resistant C57BL/6 mice produced low parasitemia and gave that resistance and susceptibility to T. cruzi infection are defined on the basis of parasitemia and survival produced by subcutaneous, intraperitoneal, or intravenous inoculation (21, 25, 41), we checked for the possibility of a paradigmatically susceptible mouse strain becoming relatively resistant if infected by the intranasal route. Intranasal administration of 500 T. cruzi trypomastigotes into susceptible BALB/c mice produced parasitemia following the kinetics and parasite load of resistant C57BL/6 mice (Fig. 2A). However, the intranasal inoculum, lethal if administered subcutaneously, did not kill infected BALB/c mice (Fig. 2B). A comparison of brain invasion in C57BL/6 and BALB/c mice was performed at the peak of infection, i.e., day 25 (Fig. 2C). All BALB/c mice had succumbed by 3 weeks after subcutaneous inoculation. Appropriately, C57BL/6 mice inoculated with 500 parasites exhibited less parasitism than those inoculated with 5,000 parasites, and BALB/c parasitism was similar to that found using the highest inoculum (25,000 parasites) in C57BL/6 mice. This finding could reflect a dampened immune response to T. cruzi infection noted previously in BALB/c mice. Regardless, intranasal doses provided a twofold increase compared to subcutaneous administration in both C57BL/6 and BALB/c mice at day 25 PI.

T. cruzi targeted the basal ganglia whether the infection was initiated by subcutaneous or intranasal inoculation and triggered a strong inflammatory response. To determine whether T. cruzi invades the brain randomly or targets a specific region, we determined parasite loads by real-time PCR in the cortex, basal ganglia, and cerebellum of C57BL/6 mice sacrificed at the peak of brain invasion (25 days PI). We found that T. cruzi invaded the basal ganglia preferentially, regardless of whether the parasites were inoculated by the subcutaneous or intranasal route. Furthermore, the parasite load in the basal ganglia following intranasal infection was greater (~3.0-fold) than the basal ganglia parasitism resulting from subcutaneous inoculation (Fig. 3). Regardless of the inoculation route, T. cruzi...
invasion of the basal ganglia was more prominent than that of the brain cortex, which in turn was greater than that in the cerebellum (Fig. 3). Due to the nature of the assay to quantify parasite load (real-time PCR), it was not practical to determine if \textit{T. cruzi} targeted individual components of the basal ganglia, namely, the striatum (putamen and caudate nucleus), globus pallidus, subthalamic nucleus, and substantia nigra.

Histological and histochemical analysis revealed \textit{T. cruzi} nests in the cortex and basal ganglia (Fig. 4A and C) and \textit{T. cruzi} inside F4/80-positive cells (Fig. 4C, inset). Quantitation of these nests or foci in the basal ganglia of C57BL/6 mice sacrificed...
ficed 25 days after intranasal and subcutaneous inoculation with 25,000 parasites showed a preferential increase (about 9.0-fold) in the brains of mice infected by the intranasal route (Fig. 5). The strong inflammatory response to intranasal inoculation compared to that in uninfected tissue (Fig. 4B) reflects the preferential parasite load produced by this procedure (Fig. 1). Interestingly, abundance, location, and cellular constituents of foci in histological sections of brains from BALB/c mice inoculated intranasally with 500 parasites were virtually identical to those for mice infected with 25,000 parasites (data not shown).

*T. cruzi invasion of the brain did not trigger neurodegeneration.* Alterations in the structure and physiology of the basal ganglia give rise to motor disorders such as parkinsonism and Huntington’s disease. One widely used model of basal ganglion neurodegeneration results from systemic intoxication with the fungal toxin 3-NP, which irreversibly inhibits mitochondrial respiration and selectively damages the striatum in most species, including humans, after accidental ingestion of contaminated mildewed sugar cane (4, 6, 29). Motor and behavioral

**FIG. 3.** Location and abundance of parasites in the brain following intranasal and subcutaneous inoculation. C57BL/6 mice were infected intranasally and subcutaneously with 25 × 10³ parasites, sacrificed at day 25 (peak brain parasitism), and assayed for *T. cruzi* by quantitative PCR in the brain cortex (CX), basal ganglia (BG), and cerebellum (CB). The experiment was repeated four times, using two or three mice per region tested. *, *P* < 0.001.

**FIG. 4.** Histology of the frontal cortex and basal ganglia of *T. cruzi*-infected C57BL/6 mouse. H&E stains of sagittal sections of the brain from a C57BL/6 mouse 18 days after intranasal inoculation are shown. (A) Three cellular foci in the frontal cortex (CX). Original magnification, ×200. Enhanced magnification (×400) of the indicated areas reveals microglia (open arrowhead) surrounding infected cells (closed arrow). (B) Uninfected basal ganglia. Magnification, ×200. (C) One cellular focus in the striatal tissue of the basal ganglia (BG) showing a microglial nodule (open arrowhead) surrounding infected cells (closed arrow). Original magnification, ×400. Additionally, infected F4/80-positive macrophage-like cells (presumably microglia) were found within nodules (inset). Bar, 50 μm.
FIG. 5. Increased numbers of inflammatory foci in the basal ganglia of intranasally infected compared to subcutaneously infected mice. C57BL/6 mice were infected intranasally (i.n.) with \(25 \times 10^3\) or subcutaneously (s.c.) with \(5 \times 10^3\) T. cruzi parasites, and at 25 days PI, their brains were fixed in formalin, embedded in paraffin, sectioned both coronally and sagittally, and stained with H&E. Three noncontiguous brain sections (basal ganglia) were counted per mouse. From each section, 25 fields within the basal ganglia (magnification, \(\times 200\)) were counted. The average number of parasite nests per 25 fields for each mouse was determined. The numbers of T. cruzi nests (foci) were calculated from the averages for three mice. **, \(P < 0.001\).

**Discussion**

Our results show that nasal colonization with T. cruzi results in robust infection of the CNS that maxes out after the rise and fall of a modest parasitemia. This pattern is similar to but quantitatively the reverse of the parasite load produced by injecting T. cruzi into soft tissues (footpad). Because of the relatively subdued systemic infection follow-

ing intranasal administration, we could quantitate the rise and fall of brain invasion in susceptible BALB/c mice, which would otherwise be impractical if the parasites were injected subcutaneously, normally a lethal route of infection in this mouse strain. This suggests that it may be possible to study brain invasion over a reasonable time course in immunocompromised animals intranasally infected with T. cruzi, which would be a useful model of T. cruzi reactivation, a phenomenon that occurs in chagasic patients coinfected with human immunodeficiency virus or subjected to immunosuppressant drugs. Reactivation leads to robust parasite growth in the heart and severe myocarditis, while in the CNS tumor-like parasite masses form in the brain, resulting in an inexorably fatal disease progression (14, 24, 27, 34, 42). Because the brain is an area that is impenetrable for most therapeutics, this method could also be useful for targeted delivery of antiparasitic agents, such as in chagasic patients coinfected with human immunodeficiency virus.

Why does intranasally inoculated T. cruzi preferentially invade the brain? The olfactory neuroepithelium is the only site in the body that directly links the environment to the CNS, and thus it could serve as a vehicle for proteins and small particles to reach the CNS, thereby bypassing the blood-brain barrier. Such a possibility was proven experimentally about 70 years ago with the demonstration that intranasally administered Prussian blue migrated to the brains of mice and rabbits via the olfactory route (32). Growth factors and other proteins can, likewise, access the brain via the olfactory and trigeminal nerve pathways independently of the bloodstream (39). Olfactory nerve-dependent, circulation-independent brain entry is not restricted to inert particles or proteins. The microbial pathogen Streptococcus pneumoniae, which typically causes meningitis through the hematogenous route, invades the CNS via olfactory tissues, without detectable bacteremia, if inoculated into the nasal cavity (43).

Therefore, intranasally administered T. cruzi likely gains access to the brain via olfactory nerve tissues.

**TABLE 1. Absence of motor behavioral disorders in mice infected with T. cruzi**

<table>
<thead>
<tr>
<th>Group</th>
<th>Neurotoxin(^a)</th>
<th>No. of animals with disorder/total no. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hind-limb dystonia(^b)</td>
<td>Truncal dystonia(^b)</td>
</tr>
<tr>
<td>Control group</td>
<td>–</td>
<td>0/12</td>
</tr>
<tr>
<td>Infected mice</td>
<td>–</td>
<td>0/12</td>
</tr>
<tr>
<td>Uninfected mice</td>
<td>+</td>
<td>10/10</td>
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\(^a\) Animals were treated with the neurotoxin 3-NP (10 mice) or intranasally infected with \(25 \times 10^3\) T. cruzi organisms (12 mice) (see Materials and Methods).

\(^b\) Symptoms related to basal ganglion degeneration were assessed 1 day after the last injection of the neurotoxin or at the peak of brain parasitism (25 days postinfection).

\(^c\) Scores for hind-limb dystonia were as follows: 0, absent; 1, intermittent or increased hind-limb space, abnormal crouching, and poor hind-limb movement and coordination.

\(^d\) Scores for general locomotor activity were as follows: 0, normal; 1, slight or marked reduction in general activity: the mouse stayed in place and showed reduced displacement velocity, rearing, or grooming.
Additionally, it may also reach the CNS after invading cells in the nasal cavity, amplifying the infection locally, and subsequently migrating to the brain via the olfactory tissues. It is also possible that a smaller subset of intranasally administered parasites will invade distant sites and subsequently the brain via the circulation. Regardless, it is clear that *T. cruzi* administered intranasally on the mucosa in the nasal cavity preferentially invades the CNS. Interestingly, a recent study demonstrated that *T. cruzi* atraumatically deposited in the mouse conjunctiva migrated through the nasolacrimal duct to reach the nasal cavity, from where it invaded ductal and respiratory epithelia and then local lymph nodes and distant tissues via the bloodstream (18). Although it was not determined if the conjunctival/nasal cavity circuit led to brain invasion, we predict, based on our findings and the data in the literature about brain entry by proteins and bacteria administered into the nasal cavity, that conjunctival infection with *T. cruzi* results in robust brain invasion.

In the brain, *T. cruzi* targeted the basal ganglia regardless of whether the infection was initiated via the intranasal mucosa or subcutaneous tissues. In the basal ganglia, *T. cruzi* grew abundantly and triggered a strong inflammatory response. The strong inflammatory response is in accordance with reports by others (31, 36, 37). One would expect *T. cruzi* infection and the ensuing inflammatory response in the basal ganglia to cause abnormal control of movement and posture and changes in muscular tone, which normally accompany alterations in the striatum and substantia nigra,

FIG. 6. Absence of neurodegeneration in the brains of mice infected with *T. cruzi*. C57BL/6 mice were infected intranasally with 25,000 *T. cruzi* parasites for 25 days. Animals were sacrificed and perfused with PBS, and their brains were fixed with formalin. Slides were stained with H&E to detect parasites (arrows) or with Fluoro-Jade C to visualize degenerating neurons. (A) Basal ganglia. Erythrocytes (arrowheads) reacted with the Fluoro-Jade C dye. (Inset) Enlarged view of infected tissues to indicate infected cells and the absence of detectable neurodegeneration. Bar = 50 μm. (B) Large intestine. Magnification, ×600. Note the extensive neurodegeneration in colon myenteric neurons.
as in parkinsonism, Huntington’s disease, and African sleeping sickness (10, 22, 26). Puzzlingly, we did not find evidence of motor disorders in any of the T. cruzi-infected mice at the peak of brain invasion and inflammation, in contrast to the obvious signs of basal ganglion disorders in the mouse model of Huntington’s disease (16) (Table 1). The absence of motor disorders agrees with the lack of detectable neurodegeneration in the brains of T. cruzi-infected mice (Fig. 6). A similar paradox is common to acute chagasic patients, whose CNS are infected with T. cruzi and who nevertheless do not present symptoms normally related to brain infection (20).

The intranasal inoculation mouse model described here should be useful for studying the molecular basis of T. cruzi invasion of the brain, such as the binding of parasite-derived neurotrophic factor trans-sialidase to the nerve growth factor receptor TrkA (2, 9, 15) and of a trans gene expression in neuronal PC12 cells. Brain Res. 1217:195–202.


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