AN IMMUNOGENETICALLY DEFINED AND IMMUNODOMINANT TRYPSANOSOMA CRUZI ANTIGEN

RODRIGO RAMOS, MARIA JURI, ALICIA RAMOS, GUSTAVO HOECKER, SERGIO LAVANDERO, PEDRO PENA, ANTONIO MORELLO, YOLANDA REPETTO, JUAN CARLOS AGUILLON, AND ARTURO FERREIRA

Universidad de Chile, Ministerio de Salud, Servicio de Salud Metropolitano Sur. Santiago, Chile

Abstract. Two strains of mice, A. SW (H-2') and A.CA (H-2'), were immunized with live trypomastigotes or epimastigotes of the Tulahuen strain of Trypanosoma cruzi or with their sonicates. By immunowestern blotting, sera from A.SW mice, but not from A.CA, recognized, in an immunodominant fashion, a 45 kDal polypeptide (Tc45) present in both epimastigotes and trypomastigotes. Since A.SW and A.CA strains are congenic for the major histocompatibility H-2 complex, recognition of Tc45 seems to be controlled by this genetic region or by gene(s) located in its immediate vicinity. Subcellular fractionation revealed that Tc45 is mainly present at the cytoplasmic compartment.

Humans are highly variable in the ways they react to Trypanosoma cruzi invasion. Initial infection can be fatal, although most people survive this phase, remaining asymptomatic throughout their lives or developing the chronic form of the disease.

Inbred and congenic strains of mice are excellent tools for analyzing the genetic basis for the variability of the immune response to many pathogens. Indeed, inbred strains of mice infected with T. cruzi vary from highly resistant to highly susceptible, a situation similar to the behavior of humans. The extensive variation in susceptibility within these two species suggests the existence of a genetic basis for natural resistance to this disease.

Among the host factors contributing to resistance, participation of antibodies has been clearly demonstrated by protecting experimental animals with immune serum or immunoglobulin fractions, by antibody-dependent cell-mediated cytoxicity and by enhancement of T. cruzi infection in rats neonatally suppressed in their antibody production.

It is most likely that the parasite contains many antigens with different degrees of immunogenic, protective and diagnostic values. Among these, the best characterized are glycoproteins of MWs: 90,000 (GP90), 72,000 (GP72), 25,000 (GP25), 57,000/51,000 (GP57/51) and 85,000.

The A.SW (H-2') and A.CA (H-2') strains of mice, congenic for the H-2 major histocompatibility complex, differ dramatically in their susceptibility to the acute infection mediated by the intraperitoneal (i.p.) injection of 10,000 blood trypomastigotes of the Tulahuen strain of T. cruzi. All A.CA mice die about 12 days post inoculation, while the A.SW counterparts survive indefinitely.

The immune response against an organism of such complexity as a protozoan parasite is most likely controlled by genes dispersed in the host's genome. Among them, the H-2 complex is a candidate whose role should be ascertained, since it contains several genes controlling important features of the immune response. The experimental model represented by the A.SW/A.CA combination of congenic strains provides an opportunity to assess this role at the molecular level of antigenic recognition. We report here that the A.SW strain has the capacity to generate antibodies against a 45 kDal polypeptide present in epimastigotes and trypomastigotes of the Tulahuen strain of T. cruzi. The prominence of this polypeptide, judged by immunowestern blotting (IWB), and the fact that it is recognized by the A.SW but not by the A.CA strain, allows us to characterize it as an immunodominant and immunogenetically defined moiety.

MATERIALS AND METHODS

Parasites

Blood trypomastigotes from the Tulahuen strain of T. cruzi, maintained by passage in irradiated (500 r) A/Sn mice, were obtained at the peak of parasitemia by collecting the blood in 3.5% w/v sodium citrate and immediately centrifugating (150 × g for 30 sec). The parasites
present in the supernatant were washed and re-
suspended in phosphate-buffered saline (PBS). 
Epimastigotes were grown in liver infusion try-
ptose (LIT) liquid culture medium, harvested at 
the exponential phase of growth, washed and re-
suspended in PBS. Total antigenic extracts were 
obtained separately from epimastigotes and try-
pomastigotes, by subjecting them to five cycles 
of freezing and thawing in liquid nitrogen, fol-
lowed by sonicication (250 W for 30 sec). After 
measuring the total protein concentration and 
adding a protease inhibitor cocktail (antipain 25 
µg/ml, leupeptin 25 µg/ml, apronitin 2 TIU/ml, 
PMSF 1 mM and TLCK 1 mM), the samples 
were aliquoted and frozen in liquid nitrogen. 
Freshly collected live epimastigotes and trypo-
mastigotes were also used for immunization of 
experimental animals.

**Experimental animals**

Three-month old A.CA (H-2*) and A.SW (H-
2*) female mice, bred in our colony, were used 
in all experiments. The H-2 haplotype of these 
strains is routinely confirmed in our laboratories 
by standard serological procedures. Three ex-
perimental groups per strain, 7–8 animals each, 
were immunized following different protocols (A, 
B, C). Preimmune blood was obtained from all 
animals.

Protocol A involved four immunizations per 
animal, one week apart, with 50 µg of total pro-
tein from sonicated trypomastigotes per injec-
tion. The first one was administered in the rear 
foot pads, the following two, subcutaneously, and 
the last one i.p. Starting two weeks later, the 
animals were bled five times, one week apart, 
through the ventral tail vein and artery. Protocol 
B involved an i.p. immunization with 6 x 10³ 
cultured epimastigotes per mouse. After immu-
nization, serum samples were obtained five times, 
one week apart. No parasitemia or symptoms of 
disease were detected in the animals immunized 
according to these protocols. Protocol C con-
sisted of injecting i.p. 10⁴ bloodstream trypo-
mastigotes, with blood taken at 12, 35, 60 and 
90 days post inoculation. The series of bleedings 
could not be completed in A.CA animals which, 
in agreement with previous findings,16 died from 
acute infection before the second one scheduled. 
Parasitemia was assessed in 5 µl of fresh blood, 
at a 400 times magnification.17 Sera were stored 
at −20°C until tested.

**Immunoradiometric Assay (IRMA)**

Twenty five µl of sonicated bloodstream try-
pomastigotes or epimastigotes (20 µg total pro-
tein per ml), in 0.1 M carbonate buffer (pH 9.6), 
were placed in the wells of polyvinylchloride 
(PVC) plates. The plates were then incubated 
overnight at 4°C; washed three times with PBS-
0.05% v/v Nonidet P-40 (PBS-NP40); saturated 
for 1 hr with PBS with 1% w/v bovine serum 
albumin; and incubated at room temperature in 
triplicates for 1 hr with immune and preimmune 
sera from individual mice. Next they were washed 
three times with PBS-NP40; incubated for 60 
min at room temperature with 10⁵ cpmp of a goat 
IgG (affinity purified and radiolabeled with ¹²⁵I), 
anti-mouse IgG, and washed three times with 
PBS-NP40, after which the radioactivity asso-
ciated with the individual wells was measured. 
Negative controls included wells without anti-
body and/or antigen.

**Immunowesternblotting**

One hundred µg (total protein) of sonicated 
trypomastigotes and epimastigotes were electro-
phoresed in a continuous front, in 10% SDS-
PAGE slabs, and the proteins present in the en-
tire track were transferred to nitrocellulose pa-
paper. The nitrocellulose sheets were then blocked 
with 5% (w/v) non-fat powdered milk in PBS; 
washed three times with PBS-NP40; cut into 0.5 
cm wide strips and incubated for 2 hr with 1: 
100 dilutions of sera from A.CA and A.SW mice; 
washed three times with PBS-NP40, and incu-
bated for 2 hr with an anti-mouse IgG (made in 
goats, affinity purified and radiolabeled with ¹²⁵I); 
washed three times, dried and exposed for ra-
dioautography for 10 hr at −70°C with intensifier 
screens. In all cases, the radioautographic pat-
terns shown corresponded to the electroblotted 
polypeptides present in the whole SDS-PAGE 
track.

**Partial purification of Tc45 by electroelution**

Three mm SDS-PAGE slices, cut from analy-
tic gels, were electroeluted overnight, at 0.5 
volts. The presence of Tc45 in the eluted ma-
terial was monitored by IWB against immune 
A.SW sera.
FIGURE 1. Immunization of A.SW, but not of A.CA mice, with a trypomastigote sonicate (see protocol A, Materials and Methods) induces the production of antibodies against a MW 45,000 (Tc45) antigen, present in trypomastigotes and epimastigotes of *T. cruzi*. Epimastigote (tracks 1–5) and trypomastigote (tracks 6–10) sonicates were separated in a 10% SDS-PAGE slab under denaturing conditions, transferred to nitrocellulose paper and reacted with a 1/100 dilution of sera from: immune A.SW (5th bleeding) (tracks 1–2; 6–7), immune A.CA (5th bleeding) (tracks 3, 8), preimmune A.SW (tracks 4, 9) and preimmune A.CA (track 5, 10). Binding of mouse IgG was detected with a second radiolabeled antibody, followed by radioautography. Similar results were obtained with the 2nd–4th bleedings while the first one was consistently negative (not shown). The weaker bands of MW, similar to Tc45, observed in tracks 8–10, correspond to the heavy chain of mouse (A/Sn) IgG contaminating the electroblotted blood trypomastigote sonicate (unpublished data). This heavy chain probably underlies the Tc45 band detected by the A.SW strain (tracks 6–7). Since they are grown in vitro, epimastigotes (tracks 1–5) are not contaminated with mouse IgG.

FIGURE 2. Immunization of A.SW, but not of A.CA mice, with live epimastigotes (See protocol B, Materials and Methods) induces antibodies to Tc45. A *T. cruzi* epimastigote sonicate was electrophoretically analyzed as described in the legend for Fig. 1. Antigenic detection was performed with a 1/100 dilution of sera from individual immune A.SW (tracks 1–5) and A.CA (tracks 6–9) animals. As positive control, a pool of sera from A.SW animals (4th bleeding), immunized according to protocol A, was used (track 10). Similar results were obtained with the 2nd and 3rd bleedings (unpublished data). The first bleeding was negative.
Presence of Tc45 in subcellular fractionation of epimastigotes

This was performed as previously described, with minor modifications. Epimastigotes were suspended in TRIS-HCl pH 7.5 buffer, containing MgCl₂ and sonicated for 15 sec (5 sec pulses with 1 min interval between pulses) (in the cold). This was followed by a series of centrifugations: 750 g × 10 min, 5,000 g × 30 min, 14,000 g × 30 min and 123,000 g × 60 min, yielding 4 pellets (P₁-P₄, respectively) and a supernatant (S₄), corresponding to that obtained after the 123,000 × g centrifugation. In agreement with previous reports, by using enzymatic markers (succinic dehydrogenase for mitochondria, acid phosphatase for lysosomes and plasma membrane, glucose-6-phosphatase for microsomes; all of them from Sigma Chemical Corp.), we could determine that P₁ and P₂ corresponded to nuclei, flagella, kinetoplasts and mitochondria; P₃ to plasma membrane; P₄ to microsomes and S₄ to cytoplasm. To identify Tc45, the fractions were subjected to IWB, loading equal amounts of protein per well. The IWB was developed with a rabbit antiserum raised against whole sonicated epimastigotes. This antiserum recognized in IWB (in an immunodominant fashion) Tc45 present in a whole parasite sonicate as well as Tc45 electroeluted from SDS-PAGE gels.

RESULTS

Immunizations of A.SW and A.CA congenic strains of mice were performed according to protocols A, B and C (see Materials and Methods), corresponding to sonicated trypomastigotes, cultured epimastigotes and infective trypomastigotes, respectively.

When epimastigote or trypomastigote sonicates were analyzed in IWB against sera from animals immunized according to protocols A or B, the A.SW strain consistently and specifically recognized a 45 kDa, immunodominant polypeptide (provisionally designated as Tc45) (Fig. 1, tracks 1–2, 6–7; Fig. 2, tracks 1–5). The A.CA strain invariably failed to recognize this polypeptide (Fig. 1, tracks 3, 8; Fig. 2, tracks 6–9). When immunization protocol (live infective trypomastigotes) was used, comparison between the strains was not possible, since A. CA animals
died around 12 days post inoculation, a point at which circulating anti-*T. cruzi* antibodies were not detectable by IWB or by IRMA (unpublished data). The A.SW strain did, however, recognize Tc45 (Fig. 3, tracks 1–4) in a way similar to mice immunized with sonicated trypomastigotes (protocol A) (Fig. 1, tracks 1–2; 6–7) or with a large dose of live cultured epimastigotes (Fig. 2, tracks 1–5). In all experiments, the molecular weight of Tc45 was determined by comparison with markers for SDS-PAGE.

Evidence that the whole range of polypeptides separated in the SDS-PAGE was transferred to the nitrocellulose sheet was obtained by staining it with Amido Black (Fig. 4). Basically, the same banding pattern observed in the Coomassie Blue-stained SDS-PAGE (tracks 1–3) is also observed on the stained nitrocellulose (track 5). In the 45 kDa region no predominant stained band is observed.

Among the best characterized *T. cruzi* glycoproteins, that of MW 57,000/51,000 (GP 57/51) is the closest to Tc45 in terms of apparent molecular weight. Since the resolution limit of our SDS-PAGE system is close to 5,000 MW units, it was necessary to rule out the possibility that these two polypeptides are identical. We performed an IWB with whole sonicated epimastigotes and GP 57/51 was detected with a monoclonal antibody (212BH8, kindly donated by Dr. J. Scharfstein, Department of Biophysics, Federal University of Rio de Janeiro, Brazil). This monoclonal antibody, used cold or radiolabeled with $^{125}$I, recognized GP 57/51 which displayed a clearly different molecular weight from Tc45 (unpublished data).

Tc45 was partially purified from analytic SDS-PAGE, starting from whole sonicated epimastigotes (see Materials and Methods). The immunogenetically defined antigenic properties of Tc45 were preserved in the eluted material as judged by its reactivity with immune sera from A.SW animals (Fig. 5, tracks 1, 2) and its lack of recognition by immune sera from A.CA mice (track 3). The relative purity of the electroeluted material was assessed by its reactivity in IWB...
IMMUNODOMINANT T. CRUZI ANTIGEN

Since protozoan parasites such as T. cruzi are antigenically complex, they may induce in the vertebrate host a complex immune response involving simultaneous, interrelated or independent branches of the immune system. For this reason, immunogenic antigens have been difficult to detect and many reports focus on in vivo responses against intact infective organisms or against crude antigenic preparations. Additional difficulties derive from host heterogeneity contributing to the variability of an immune response whose control probably lies with several genetic elements.

The murine model may contribute to understanding complex immune responses in experimental vertebrate hosts. It is conceivable that clues as to the immunology of the host- T. cruzi relationship may be obtained by comparing defined parameters of the immune response of mouse strains. That ideally should fulfill three important requisites. First, they should display clear-cut differences in their capacity to resist the T. cruzi infection. Second, it should be possible to assign the control of this differential capacity to genes with a defined location in their genomes,

### Table 1

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Succinic dehydrogenase</th>
<th>Acid phosphatase</th>
<th>Glucose 6-phosphatase</th>
<th>Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total homogenate</td>
<td>6.8 (100)</td>
<td>7.6 (100)</td>
<td>15.0 (100)</td>
<td>(100)</td>
</tr>
<tr>
<td>P1</td>
<td>18.6 (89)</td>
<td>6.9 (32)</td>
<td>26.4 (57)</td>
<td>(57)</td>
</tr>
<tr>
<td>P2</td>
<td>7.8 (8)</td>
<td>13.4 (12)</td>
<td>22.0 (17)</td>
<td>(6)</td>
</tr>
<tr>
<td>P3</td>
<td>2.3 (0.8)</td>
<td>27.6 (13)</td>
<td>35.4 (9)</td>
<td>(3)</td>
</tr>
<tr>
<td>P4</td>
<td>1.7 (0.9)</td>
<td>40.0 (17)</td>
<td>102.5 (11)</td>
<td>(5)</td>
</tr>
<tr>
<td>S4</td>
<td>0.0 (0.0)</td>
<td>7.0 (24)</td>
<td>0.0 (0.0)</td>
<td>(29)</td>
</tr>
</tbody>
</table>

P1-P2: Nuclei, flagella, kinetoplasts, mitochondria.
P3: Plasma membranes.
P4: Microsomes.
S4: Supernatant after centrifuging at 123,000 × g.

In all cases the specific enzymatic activity is expressed as nmol/min mg.

Values between parentheses represent percentage of the total.
and third, it should be possible to identify the molecular target of their immune response.

According to a previous study from our laboratories the A.CA/A.SW congenic pair fulfills two of these requisites. First, they show a dramatic difference in their sensitivity to an acute infection mediated by the i.p. injection of 10,000 trypomastigotes of the Tulahuen strain of T. cruzi and second, given that these two strains are congenic for the H-2 complex, the control of this differential sensitivity can be assigned to this genetic segment or to its immediate vicinity.

The data presented here show that at the humoral level these two strains respond differently to a parasitic challenge with live or sonicated trypomastigotes or epimastigotes. Using Tc45 seems to be the only polypeptide recognized by the A.SW strain and not by the A.CA, thus placing the control of this response in association with the H-2 complex. This restriction has been consistently substantiated by numerous experimental observations. As soon as a humoral immune response (monitored by IRMA against a total epimastigote sonicate) is shown, Tc45 is always detected by A.SW animals, as shown throughout Figures 1, 2, 3, 5. In contrast, and with no exception, A.CA mice failed to detect this polypeptide (Figs. 1—2, 5).

Immunowestern blotting, our criterion for recognition of Tc45, is a technique that imposes rather stringent conditions to the antigen (presence of methanol during the transference, presence of SDS in the gel and denaturation of the proteins previous to the SDS-PAGE). The possibility that there are other proteins whose recognition, under physiological conditions, is also immunogenetically restricted, remains to be determined.

There are precedents for the fact that at the humoral level animals immunized with a whole unicellular parasite respond mostly against a single polypeptide. One example is malaria, another intracellular parasite, in which experimental animals respond almost exclusively against a single polypeptide (circumsporozoite protein) when immunized with whole sporozoites.

Since Tc45 is present in epimastigotes, it will be possible to attempt its semipreparative purification using relatively large numbers of this parasite stage. The strategy for this purification.

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**Figure 6.** Subcellular location of Tc45. 4 x 10^10 epimastigotes of the Tulahuen strain were sonicated and subjected to differential centrifugation. The subcellular composition and the presence of Tc45 in each fraction were monitored with enzymatic markers. The presence of Tc45 was detected by immunowestern blotting using a 1/100 dilution of a rabbit anti-T. cruzi antiserum raised against a whole epimastigote sonicate. Track 1, total homogenate; tracks 2 and 3, nuclei, flagella, kinetoplasts and mitochondria; track 4, plasma membranes; track 5, microsomes; track 6, cytoplasmic components.
depends on its cellular location. Immunization procedures of susceptible strains with Tc45 or, alternatively, immunization of resistant ones followed by passive transfer of their immune sera to sensitive animals are contingent on the success of this purification. Such studies may establish if the correlation between resistance to the acute infection and capacity to mount a humoral immune response against Tc45 is causal rather than spurious. Most relevant to this possibility is the fact that passive transfer of Sepharose-Protein purified IgG obtained from A.SW animals, 35 days post infection, protected 100% of A.CA animals against an otherwise lethal challenge with 10,000 trypomastigotes.

Subcellular fractionation of epimastigotes, monitored with enzyme markers (Table 1) and by IWB of the fractions (Fig. 6), indicates that Tc45 is present mainly in the cytoplasmic compartment. This finding is compatible with the idea that the Tc45 molecule displays an overall hydrophilic behavior, and that its purification strategy should be developed accordingly. No variations were observed in the stained patterns obtained by comparing SDS-PAGE of whole epimastigote extracts with the nitrocellulose after electrotransference of the polypeptides (Fig. 4, tracks 1–3 versus track 5).

Pilot experiments, recently performed in our laboratories, show that Tc45, purified by analytic electroelution, is recognized by some sera from humans serologically positive for T. cruzi. Considering our results with the murine model, it will be important to establish if this recognition in humans is also controlled by the HLA system and if it correlates with the course of the disease.

Finally, the congenicity for H-2 of the A.CA/A.SW strain pairing has been formally established, based on ample serologic evidence and on the fact that they derived from the same inbred partner (A/WySn). Our assignment of the capacity to recognize Tc45 to the H-2 region, based on these two strains, should be supported by studies underway on segregation analysis of this trait. Studies on other congenic and congenic recombinant strains carrying the relevant haplotypes may confirm this finding and may allow us a more precise mapping of the gene(s) involved.

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Authors’ address: Rodrigo Ramos, Sergio Lavandero and Arturo Ferreira, Departamento de Bioquimica y Biologia Molecular, Facultad de Ciencias Quimicas; Maria Juri, Alicia Ramos, Gustavo Hoecker, Departamento de Biologia Celular y Genetica, Facultad de Medicina; Antonio Morelio, Yolanda Repetti, Departamento de Bioquimica, Facultad de Medicina; Juan Carlos Aquillon, Departamento de Medicine Preventiva Animal, Facultad de Cienicas Veterinarias; Universidad de Chile, Santiago, Chile. Pedro Pena, Ministero de Salud, Metropolitana Sur, Santiago, Chile.

REFERENCES

4. Castello Branco AZCL. Protecao mediada por im.


