Genetically attenuated *Trypanosoma cruzi* parasites as a potential vaccination tool

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Chagas disease is the clinical manifestation of the infection produced by the parasite *Trypanosoma cruzi*. Currently there is no vaccine to prevent this disease and the protection attained with vaccines containing non-replicating parasites is limited. Genetically attenuated trypanosomatid parasites can be obtained by deletion of selected genes. Gene deletion takes advantage of the fact that this parasite can undergo homologous recombination between endogenous and foreign DNA sequences artificially introduced in the cells. This approach facilitated the discovery of several unknown gene functions, as well as allowing us to speculate about the potential for genetically attenuated live organisms as experimental immunogens. Vaccination with live attenuated parasites has been used effectively in mice to reduce parasitemia and histological damage, and in dogs, to prevent vector-delivered infection in the field. However, the use of live parasites as immunogens is controversial due to the risk of reversion to a virulent phenotype. Herein, we present our results from experiments on genetic manipulation of two *T. cruzi* strains to produce parasites with impaired replication and infectivity, and using the mutation of the *dhfr-ts* gene as a safety device against reversion to virulence.

Introduction

A century after the discovery of Chagas disease, there is still no effective drug treatment or vaccine to fight this affection. This disease is the clinical manifestation of the infection by the parasite *Trypanosoma cruzi*. Currently, there is no vaccine to prevent this disease and the protection attained with vaccines containing non-replicating parasites is limited. Genetically attenuated trypanosomatid parasites can be obtained by deletion of selected genes. Gene deletion takes advantage of the fact that this parasite can undergo homologous recombination between endogenous and foreign DNA sequences artificially introduced in the cells. This approach facilitated the discovery of several unknown gene functions, as well as allowing us to speculate about the potential for genetically attenuated live organisms as experimental immunogens. Vaccination with live attenuated parasites has been used effectively in mice to reduce parasitemia and histological damage, and in dogs, to prevent vector-delivered infection in the field. However, the use of live parasites as immunogens is controversial due to the risk of reversion to a virulent phenotype. Herein, we present our results from experiments on genetic manipulation of two *T. cruzi* strains to produce parasites with impaired replication and infectivity, and using the mutation of the *dhfr-ts* gene as a safety device against reversion to virulence.

### Generation of *Trypanosoma cruzi* Lines Carrying a Deletion of the *dhfr-ts* Gene

Several human parasites lacking specific genes have been successfully generated,
including the deletion of the ThiLG3 gene
in Trypanosoma brucei, or the gene encod-
ing the Oligopeptidase B in Leishmania
major and genes involved in Plasmodium
cell-infection. In our experience, mono-
allelic mutants of T. cruzi genes can read-
ily be obtained, but complete biallelic
abrogation of a targeted gene is not an easy
task. Indeed, few genes have been either
partially or completely deleted from the
genome of this intriguing parasite. 7,12-25

The dhfr-ts gene of trypanosomatids,
is a single copy gene which encodes the
bifunctional enzyme dihydrofolate reduc-
tase-thymidylate synthase (DHFR-TS). 26
Leishmania major and Trypanosoma bru-
cei parasites completely lacking the dhfr-
ts gene were generated by homologous
recombination. 22,23 However, our attempts
to obtain T. cruzi null mutants for this
gene were not successful. An endogenous
dhfr-ts copy was always detected after
the procedures, despite the selection marker
being in the correct locus replacing the cor-
responding dhfr-ts allele. Speculating that
the problem could be related to the genetic
background of the T. cruzi strain used
(naturally attenuated TCC strain); we also
tested the procedure with well character-
ized T. cruzi virulent strains, such as CL
Brienner and Tulahuen, but without success.
Alternatively, and as it has been shown for
Leishmania, the parasites may undergo
duplication events to avoid the loss of an
essential gene. 27,28 Cruz et al. 29 were able to
obtain dhfr-ts null mutant parasites from
an attenuated Leishmania strain, but not from
a virulent one. After transfections and
antibiotic selection, these authors
detected mainly tetraploid and polyploid
parasites, suggesting that in a natural
population of Leishmania exists differ-
ent levels of ploidy, and that those with
an increased number of chromosomes get
selected. 22 However, in our studies with
T. cruzi, irrespective of the phenotype of the
strain used in the experiments (viru-
rent or attenuated), at least one copy of the
dhfr-ts gene still remains, again suggest-
ing that it is essential for parasite survival.
Similarly, locus amplification was the
outcome of experiments aimed to obtain
null mutant T. cruzi parasites for the
enoyl-CoA hydratase (ech) and the UDP-
GlcP 49-epimerase (TcGALE) genes. 21,25
Assessing the frequency at which these
amplification/duplication events take
place could help to optimize targeted dele-
tion protocols for probing the plasticity of
the genome of this parasite.

Biological Behavior of dhfr-ts
Monoallelic Mutant Trypanosoma cruzi Parasites

Previous studies have shown that deletion of the
dhfr-ts gene could render trypano-
somatid parasites avirutotropic and lead to
parasite death. 21,22 The DHFR-TS enzyme
catalyzes sequential reactions in the bio-
synthesis of thymidine monophosphate
(dTMP), which is needed for DNA syn-
thesis and cell replication. Therefore inhi-
bition of this enzyme results in a deficit of
thymidine and consequently death. It has
been reported that L. major dhfr-ts para-
sites are unable to replicate in macrophages
in vitro and to cause permanent disease in
experimental animals, most likely attrib-
uted to the auxotrophism generated in this
mutant line. 30 It has also been shown that
DHFR-TS is essential for Trypanosoma brucei,
nulls of these parasites are completely unable to grow in vitro, and
lack the ability to establish and maintain
infections in mice. 31 Since we were not
able to generate null mutations for the par-
site in this gene, assays for auxotrophism
could not be performed. However, we
detected significant impairment in growth of
the dhfr-ts mutant compared with the
wild-type TCC parasites.

The Tulahuen strain of T. cruzi is
highly virulent, and the deletion of one
dhfr-ts allele has been proven to produce a
remarkable attenuation of its virulence for
a variety of experimental animals with dif-
ferent genetic background. The significant
loss of the ability of Tulahuen dhfr-ts-
parasites to develop blood parasitism in
immunocompetent mice suggests that
this gene may be considered as a virulence
factor of T. cruzi. In contrast, the wild-
type TCC T. cruzi strain was originally
attenuated, and infections were detected
only with highly sensitive methods
applied to very young, immunodeficient
or immunosuppressed mice. Moreover,
we were not able to recover parasites from
infected mice after two months of infec-
tion. Introduction of the dhfr-ts muta-
tion led to additional attenuation of this
subline, since prior to the parastrilological
and histological detection assays, animals
were submitted to an immunosuppres-
sion regimen. Despite this, no positive
PCR or hemoculture were found in the
population of animals infected with TCC dhfr-
ts mutant parasites. These results are in
agreement with previous data indicating
that parasite recovery in low level infec-
uations is considerably arduous. TCC dhfr-
ts parasites were not detected by sensitive
procedures used to confirm parasite clear-
ance by effective drug treatment, 32 sug-
gest that these mutant parasites, if not
totally eliminated, may be maintained at
extremely low numbers.

Use of Genetically Attenuated
dhfr-ts Trypanosoma cruzi
Parasites as Experimental
Vaccines

As a general rule, the success of live-
attenuated microorganisms as vaccines
against yellow fever, smallpox, measles,
rubella, tuberculosis, etc., suggests that
the more similar a vaccine is to the nat-
ual disease, the better is the protective
immune response. 33,34 The efficacy of
these vaccines depends on the use of live
naturally attenuated organisms and a true,
albeit self-limited infection is necessary to
attain strong and long lasting protection. 35
On this basis, it would not be unreason-
able to think that genetically attenuated
intracellular parasites could be tested as
experimental vaccines. Furthermore,
in Plasmodium and Leishmania, this
approach has met major advances. 3,4
However, in the case of T. cruzi, the evalu-
ation of genetically attenuated parasites as
experimental vaccines still deserves fur-
ther experimentation.

We have evaluated the protective effect
of dhfr-ts TCC parasites and the cellular
immune responses in short and long-term
immunization schemes. Paraise-specific
CD8+ T cells have been shown to be cru-
cial in the immunity against T. cruzi. 36 We
first investigated whether these mutant
parasites were able to trigger cellular and
humoral immune responses in infected
animals comparable to the induced by
infection of wild-type TCC parasites. The
use of an immunodominant T. cruzi pep-
tide encoded in trans-sialidase family genes

ADDENDUM

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has been utilized successfully to estimate the percentage of T. cruzi specific CD8+ T cells in experimental infections. This specific CD8+ T-cell response against a single epitope (TSKb20) was considerably lower in mice infected with TCC dhfr-ts/- parasites than in mice infected with TCC wild-type, even when using highly susceptible IFNγ- background mice. Among several factors, the development of specific CD8+ T cells is not only determined by the variety of available antigens but by the quantity. These results are most likely correlated with the limited infectivity of TCC mutant parasites and therefore with a late or inefficient antigen presentation by dendritic cells. The antibody response of non-infected or infected mice with TCC mutant or wild-type parasites did not differ significantly. Thus, antibody responses do not seem to be mediators of enhanced protective immunity induced by vaccination with TCC attenuated lived parasites.

The naturally attenuated TCC strain of T. cruzi has been successfully used by our group not only in vaccination assays involving laboratory mice but in protection field studies in guinea pigs and dogs. TCC inoculations protected these domestic reservoir animals against naturally transmitted infection. Moreover, the trafficking of parasites from hosts to vectors was significantly reduced, the TCC strain acting as a transmission-blocking vaccine. These results were promising for further prophylaxis trials but the risk of reversion to a virulent phenotype could not be excluded, since the basis of attenuation of the TCC strain is not known. The introduction of additional attenuating mutations, targeted to identified virulence genes could thus operate as a safety device and justified further efforts to genetically manipulate the TCC strain and evaluate its protective capacity in experimental animals. Both the dhfr-ts/- mutant and the wild-type TCC parasites generated a similar level of protection against virulent infection, either after short (15 d) and long (370 d) intervals between immunization and challenge (Figs. 1 and 2). In the immunized animals, CD8+ T-cells responses were monitored by determining TSKb-20 specific proliferation, 15 and 370 d after immunization, in parallel comparison of the mutant and the wild type. A significant proliferation was detected after 15 d in TCC wild-type infected mice which was no longer observed at 360 d and was not matched by dhfr-ts/- mutant infected mice. Taken together, protection and T-cell studies indicate that resistance to reinfection is long lasting and is sustained by immune cells probably responding to a set of epitopes broader than TSKb20.

**Final Conclusions**

Gene targeted deletion through homologous recombination has been one of the most important tools developed for the study of gene functions, especially for those organisms in which gene silencing via interference RNA is not possible. Targeted deletion of specific genes can also be regarded as a potential procedure to generate clones of pathogenic organisms capable of growing in vitro but less efficient to invade and persist in vivo. Therefore, this strategy provides the potential for mass production of attenuated pathogens with a built-in safety device against reversion to virulent phenotypes.

The first T. cruzi mutant strain lacking a complete functional gene was developed about 17 y ago. Despite this considerable passage of time, only a few T. cruzi strains carrying specific gene deletions have been developed. It seems that T. cruzi is an organism where gene-targeted deletion and gene silencing have so far not been applied as productively as in other trypanosomatids, such as Trypanosoma brucei and Leishmania. However, the few studies in which T. cruzi mutants were compared with wild-type parasites using infectivity measurements almost invariably revealed a change toward attenuation of virulence. The use of T. cruzi attenuated parasites in vaccine development since the infection induced is limited and may consequently lead to the establishment of a protective immune response. However, genome manipulation could lead to a loss of the protective immunity either because such genetically modified parasites cannot survive long enough to fully activate the immune system or, because they do not express antigens epitopes essential for triggering a good immune response, albeit less likely. For these reasons, a wide spectrum of specific individual genes or a combination of them (as well as different T. cruzi strains) should be evaluated.

In summary, genetically modified live parasites may be better and safer inducers of a protective immune response than non-replicating immunogens. They might be applicable to dogs and other mammals that act as natural reservoirs with...
the potential for reducing the intensity and spread of the disease. Therefore, this work sets a precedent, not only for the development of new improved molecular techniques for gene deletion but for the generation of genetically attenuated parasites which could be safely used as a potential vaccine against Chagas disease.

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