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Review Article

A Paratransgenic Strategy for the Control of Chagas Disease

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Chagas disease results from infection with the parasite *Trypanosoma cruzi*. This disease remains a significant cause of morbidity and mortality in central and south America. Chagas disease now exists and is detected worldwide because of human migration. Control of Chagas disease has relied mainly on vector eradication however, the development of insect resistance to pesticides, coupled with cost and adverse health effects of insecticide treatments, has prompted our group to investigate novel methods of transmission control. Our laboratory has been instrumental in the development of the paratransgenic strategy to control vectorial transmission of *T. cruzi*. In this paper, we discuss various components of the paratransgenic approach. Specifically, we describe classes of molecules that can serve as effectors, including antimicrobial peptides, endoglucanases, and highly specific single chain antibodies that target surface glycoprotein tags on the surface of *T. cruzi*. Furthermore, we address evolving concepts related to field dispersal of engineered bacteria as part of the paratransgenic control strategy and attendant risk assessment evaluation.

1. Chagas Disease

American trypanosomiasis, or Chagas disease, is caused by the protozoan *Trypanosoma cruzi.* Between 8–11 million people worldwide are infected, and, of these, approximately 50,000 will die annually [1]. In 2000, the annual cost of morbidity and mortality attributed to Chagas disease in endemic countries was estimated to be close to US$8 billion [2]. Two years later, the WHO estimated the burden of Chagas disease to be as high as 2.7 times the combined burden of malaria, schistosomiasis, leishmaniasis, and leprosy [3]. Though traditionally a disease endemic to Mexico, central, and south America, human migration has resulted in reported cases of *T. cruzi* infection worldwide [4]. For example, cases of Chagas disease have been reported in Portugal [5], Spain [6, 7], France [8, 9], and Switzerland [10], countries that are favored for immigration from Latin America. Reports from Australia estimate *T. cruzi* infection in 16 of every 1,000 Latin American immigrants [1, 5], and Chagasic heart disease was reported in Brazilian immigrants of Japanese origin in Japan [6].

There have been numerous reported cases of Chagas disease resulting from unscreened blood transfusions and organ donation. Further, the parasite can be congenitally passed from mother to child. However, this disease is most often transmitted to humans by *T. cruzi*-infected blood-sucking triatomine bugs. These insects are members of the heteropteran family Reduviidae. The major vectors for Chagas disease in central and south America are *Rhodnius prolixus* (Figure 1(a)) and *Triatoma infestans* (Figure 1(c)), respectively. These bugs thrive in thatch and adobe of poorly constructed homes during the heat of the day, coming out in the cooler hours of the night to feed. Carbon dioxide emanating from the breath of the sleeping vertebrate victims as well as ammonia, short chain amines, and carboxylic acids from skin, hair, and exocrine glands are among the volatiles that attract triatomines. These insects are often dubbed "kissing bugs," from their common habit of biting the face, which is often exposed during sleep. The bite of triatomine bugs is painless, allowing the insect to feed without interruption. As the insect engorges, it defecates. If the insect is infected with *T. cruzi*, the parasite will be in the

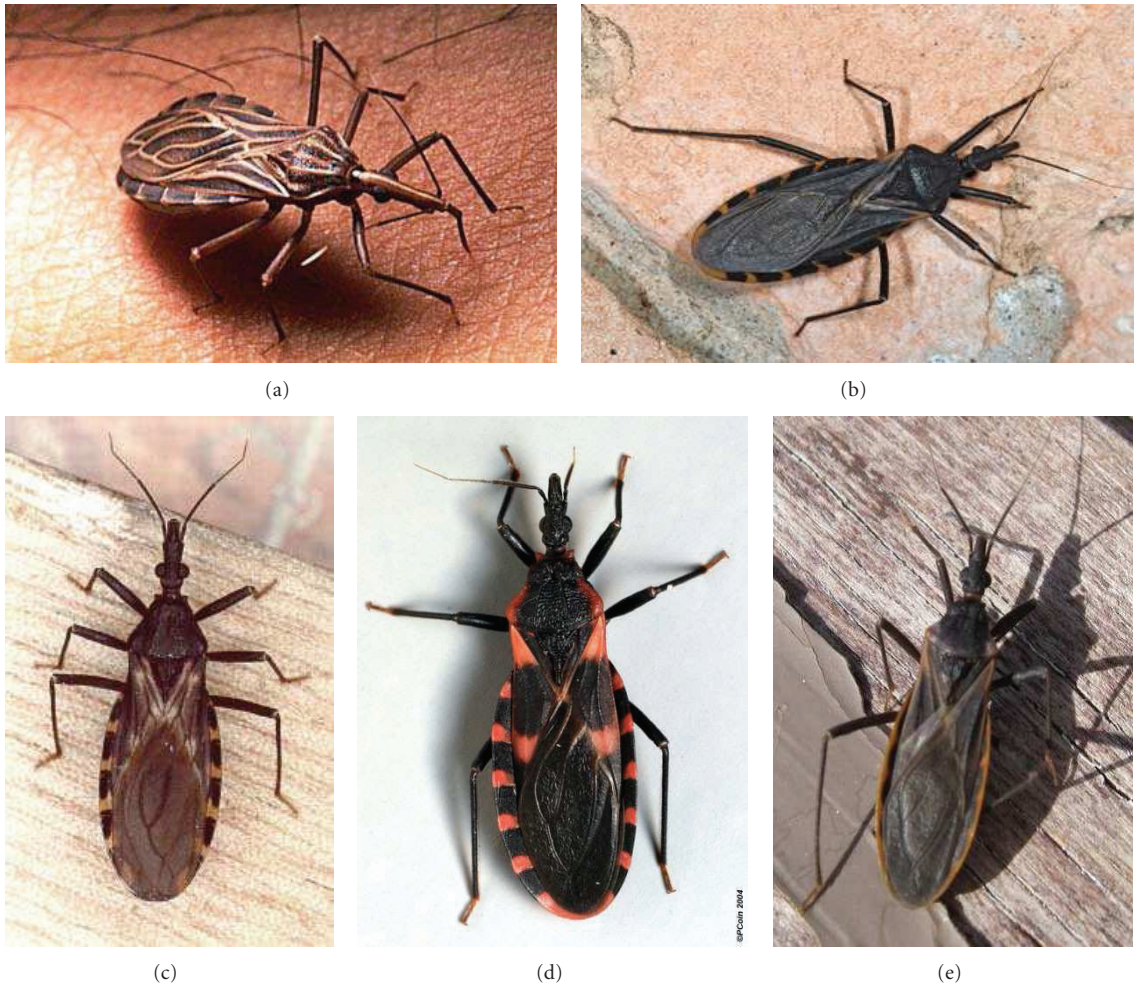

Figure 1: Triatomine bugs. (a) *R. prolixus*, picture adapted from http://www.jyi.org/articleimages/185/originals/img0.jpg; (b) *T. gerstaeckeri*, picture adapted from http://theearlybirder.com/insects/hemiptera/reduviidae/index.htm; (c) *T. infestans*, picture adapted from http://www.k-state.edu/parasitology/546tutorials/ARTHFIG01.JPG; (d) *T. sanguisuga*, picture adapted from http://bugguide.net/node/view/5164; (e) *T. rubida*, picture adapted from http://bugguide.net/node/view/185220.

fecal material and will then enter the bloodstream when the victim scratches the irritated bite wound.

There are currently more than 300,000 people infected with *T. cruzi* living in the United States. Most acquired the disease while residing in endemic areas [11]. Although *T. cruzi*-infected vectors and animals are found in many parts of this country [12], there have only been 5 documented cases of autochthonous (indigenous) transmission in the US [13]. Surveys of Reduviid bugs in the American Southwest have shown high rates of *T. cruzi* infection in *T. rubida* (Figure 1(e)), *T. protracta, T. sanguisuga* (Figure 1(d)), and *T. gerstaeckeri* (Figure 1(b)) [12, 14–16]. Species-specific behavior of the north America triatomines may explain why more autochthonous transmission is not observed. In a study by Klotz et al. [17], field caught *T. protracta* and *T. rubida* were allowed to feed on live immobilized white mice and the defecation pattern was observed for one hour. Of the 71 triatomines observed, only 30 (42 percent) produced a fecal droplet within one hour after feeding. Sixty-seven percent of these defecated 1.5–6 cm from the mouse, whereas 33 percent defecated 7–10 cm away from the mouse. None of the bugs defecated on the mouse.

The transmission cycle of *T. cruzi* is complex. Reduviid vectors become infected with *T. cruzi* when they feed on vertebrate blood containing bloodstream trypomastigotes (Figure 2). The trypomastigotes differentiate into epimastigotes in the midgut and then to infective metacyclic trypomastigotes as they move further into the hindgut of the insect. When the triatomine bug takes its next blood meal, the trypomastigotes are defecated onto the bite wound of its victim. Transmission of the parasite therefore occurs through contamination of the bite site. Upon entry into the blood stream of the vertebrate host, the trypomastigotes colonize muscle and neuronal tissue where they form intracellular amastigotes. These proliferating cells will form pseudocysts, which after several successive cell divisions, will asynchronously transform into trypomastigotes. The trypomastigotes then escape from the pseudocysts into the blood

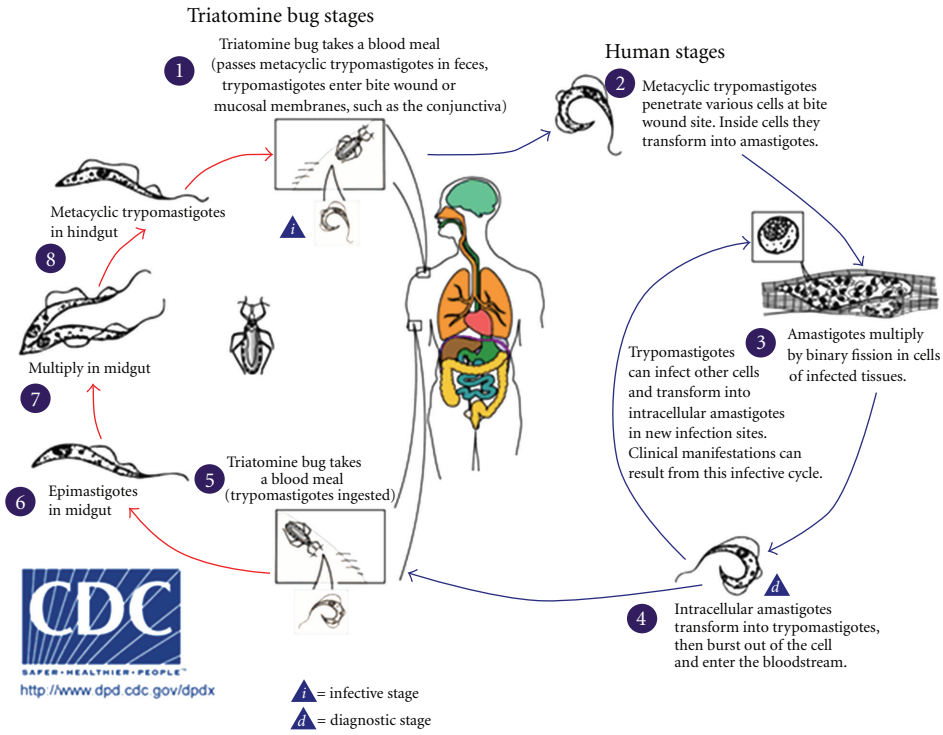

Figure 2: An infected triatomine bug takes a blood meal and releases trypomastigotes in its feces near the site of the bite wound. Trypomastigotes enter the host through the wound (1). Inside the host, the trypomastigotes invade cells near the site of inoculation, where they differentiate into intracellular amastigotes (2). The amastigotes multiply by binary fission and form pseudocysts (3). Following several cycles of division, these cells will asynchronously differentiate into trypomastigotes, which are then released into the circulation as bloodstream trypomastigotes where they can start infecting cells from other tissues (4). The cycle of infection continues as another triatomine bug becomes infected by feeding on human blood containing the circulating parasites (5). The ingested trypomastigotes transform into epimastigotes in the vector's midgut (6). The parasites multiply in the midgut (7) and differentiate into infective metacyclic trypomastigotes in the hindgut (8). This figure is adapted from http://www.dpd.cdc.gov/dpdx.

and lymph to invade new cells. The cycle of transmission continues when another triatomine bug takes a blood meal from the infected vertebrate.

Chagas disease is characterized by three successive stages. The acute stage, often characterized by generalized malaise, fever, swelling of the lymph nodes, and enlargement of the liver and spleen, is minimally symptomatic and lasts from 4 to 8 weeks. The hallmark of acute infection is the chagoma, an inflammatory skin lesion that develops at the site of a triatomine bug bite. The lesion is a result of lymphocytic infiltrate, intracellular edema, and adjacent reactive lymphadenopathy due to the intramuscular presence of *T. cruzi* at the site of inoculation. When the bite is on the face near the eye, the characteristic Romana's sign occurs (Figure 3). The acute phase is then followed by an indeterminate phase that can last between 10 to 20 years. During this period, there are few clinical manifestations but active replication of *T. cruzi* and periodic release of bloodstream forms of the parasite into the circulation occurs. Approximately, 30 percent of these patients progress to symptomatic chronic Chagas disease, which often manifests in the 4th or 5th decade of life [18]. Hallmarks of chronic infection include inflammation of the heart muscles, enlargement of the esophagus, and enlargement of the colon. For the most part,

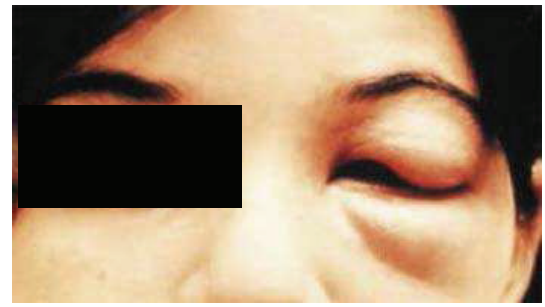

Figure 3: Romana's sign is characterized by unilateral palpebral edema, conjunctivitis, and lymphadenopathy. Photograph is adapted from WHO/TDR 2011.

the gastrointestinal manifestations of chronic Chagas disease are geographically restricted and play a lesser role in the overall disease burden of Chagas disease. However, progressive heart disease is a leading public health concern throughout much of central and south America. The chronic phase of Chagas disease is incurable and on average is associated with a ten-year shortening of life span.

Whereas acute Chagas disease is treatable with appropriate and timely antiparasitic medication, the chronic phase of

this debilitating disease often goes undiagnosed. Antiparasitic treatment of chronic disease is of questionable clinical benefit and is often limited by adverse drug reactions and side effects. In the absence of vaccines and effective drug therapies, control of Chagas disease had relied largely on measures aimed at vector eradication. To date, several large-scale insecticide-based efforts have been undertaken with considerable success. In 1991, an international coalition of governmental agencies from Argentina, Bolivia, Brazil, Chile, Paraguay, Uruguay, and Peru started The Southern Cone Initiative to minimize transmission of *T. cruzi* by *T. infestans*. This coalition developed educational programs aimed at reducing human contact with *T. infestans,* as well as blood bank screening programs to eliminate transmission of *T. cruzi*. Successes of this program include a Pan-American Health Organization awarded certificate for the disruption of Chagas disease transmission by *T. infestans* to Uruguay, Chile, and Brazil [19]. However, sustainability of this program has been called into question due to the need for continued application of pyrethroid insecticides and possibility of reinfestation of domestic structures [19]. The poor effects of pyrethroid insecticides are mainly caused by their short-lasting residual effects in outdoor sites exposed to sunlight, high temperatures, rain, and dust [20, 21]. The reduced effectiveness of pyrethroids was further compounded by the development of triatomine populations that were resistant to a variety of insecticides in Chagas disease endemic areas [22]. This, coupled with recent surveillance data indicating resurgence of human infections, particularly in the Argentinan Gran Chaco [23], would suggest that current insecticidal programs for control of vectorial transmission of Chagas disease are failing, and that novel, effective, and sustainable supplementary tactics are critically needed to maintain suppression of Chagas disease transmission.

2. The Paratransgenic Approach, an Alternative Strategy to Control Vectorial Transmission of *T. cruzi*

Triatomine bugs subsist on a blood-restricted diet. To supplement their basic nutritional and developmental needs, these insects have developed a symbiotic relationship with nocardioform actinomycetes [24]. These soil-associated bacteria are thought to aid in the processing of B complex vitamins and are essential to the survival of the bug. The symbiosis of these bacteria with several triatomine species and the amenability of these cells to genetic manipulation are the cornerstones of the paratransgenic strategies aimed at interrupting vectorial transmission of *T. cruzi*.

Rhodococcus rhodnii is a soil-associated nocardioform actinomycete. It also lives extracellularly in the gut lumen of *R. prolixus* in close proximity to *T. cruzi*. This microbe is transmitted from adult triatomine bugs to their progeny through coprophagy, the ingestion of fecal material from other bugs. *Rhodococcus rhodnii* is critical to the growth and development of *R. prolixus* [24]. *Rhodnius prolixus* nymphs that lack gut-associated symbionts (aposymbiotic) do not reach sexual maturity and most die after the second developmental molt. Introduction of the bacteria to the first or second instar nymphs permits normal growth and maturation. In 1992, we transformed *R. rhodnii* with pRr1.1, a shuttle plasmid containing a gene encoding resistance to the antibiotic thiostrepton, to support the hypothesis that a transgene-carrying symbiont could be introduced into *R. prolixus* [25]. These transformants were introduced into aposymbiotic first instar *R. prolixus* nymphs via artificial membrane feeding and reared in the presence and absence of thiostrepton. Examination of bacteria from the gut of insects carrying the transformed symbionts demonstrated that thiostrepton resistant colonies could be recovered from these vectors, regardless of antibiotic presence, for up to 6.5 months after infection. These studies demonstrated that genetically modified *R. rhodnii* symbionts expressing a selectable gene product could be stably maintained in *R. prolixus* without adverse effects on insect survival and fitness, thereby substantiating the paratransgenic approach.

For the paratransgenic strategy to work, it is imperative that a population of symbiotic bacteria, that is, amenable to culture and receptive to genetic manipulation, be identified within a given disease-transmitting vector. The fitness of these symbionts should not be compromised nor should their normal functions within the vector be affected following genetic manipulation. The transgene products, when expressed in proximity to the target pathogen, should interfere with pathogen development in the vector, and should not be detrimental in any way to the vector. Finally, the dispersal technique used to spread the genetically modified symbiont/commensal to naturally occurring vector populations should minimize the spread of the transgene to other organisms in the vector's environment, which include both the nontarget microbiota inside the vector and other organisms that live in the same ecological niche.

Since our initial experiments, we have adapted the paratransgenic strategy to numerous other vector-borne disease systems, including sand-fly-mediated leishmaniasis [26, 27] and sharpshooter-mediated Pierce's disease [28]. The strategy is also being developed and extrapolated into shrimp mariculture [29]. In this paper, we will focus on work relating to the paratransgenic control of Chagas disease with a number of effector molecules, specifically, antimicrobial peptides (AMPs), recombinant endoglucanases that disrupt the surface of the parasite, and functional transmission-blocking single-chain antibodies.

2.1. Antimicrobial Peptide Genes. Cecropin A is an AMP that was isolated from the giant silk-worm moth *Hyalophora cecropia* [30]. This AMP is 38 amino acids in length. Cecropin A lyses cells by binding to and covering the parasite membrane surface, effectively dissipating transmembrane electrochemical gradients [31]. We cloned the DNA sequence for cecropin A into the pRr1.1 shuttle vector to produce pRrThioCec. This plasmid was then used to transform *R. rhodnii* [32]. Paratransgenic *R. prolixus* were generated with the cecropin A-expressing symbionts and allowed to engorge on *T. cruzi*-laden human blood until they reached sexual maturity. Hindgut contents from paratransgenic insects carrying pRrThioCec-transformed *R. rhodnii* were

either devoid of *T. cruzi* trypomastigotes (65 percent) or maintained markedly reduced titers of the parasite (35 percent) [32]. In contrast, all control insects harboring untransformed *R. rhodnii* or *R. rhodnii* transformed with pRr1.1 (original shuttle vector without cecropin gene) that were infected with *T. cruzi* in the same manner carried mature trypomastigotes. This study provided proof of concept for the paratransgenic strategy, and suggested that other AMPs might be employed singly or in concert to elicit complete elimination of infective parasites in the hindgut of *T. cruzi* vectors.

A large number of AMPs have been and are being discovered that function in a variety of ways, including disruption of cell membranes similar to cecropin A, interference with host metabolism, and inactivation of cytoplasmic components [31]. Many AMPs are also capable of discriminating between host and invading organisms, thereby permitting the expression of recombinant AMP's from certain cell lines without deleterious effects to a host insect. *In vitro* studies were carried out with six AMPs selected from different insect sources to determine their differential toxicity profiles against host bacterial strains and *T. cruzi* parasites [33]. AMP's were identified that displayed high toxicity against *T. cruzi* (LC_{100} < 10 μM) compared to *R. rhodnii* (MBC > 100 μM) in single synthetic peptide treatment regimens. These peptides; apidaecin, cecropin A, magainin II, and melittin, were employed in pairwise treatment protocols against *T. cruzi.* Dual peptide treatments of *T. cruzi* showed synergistic or additive effects between different AMP's resulting in increased toxicity over any single AMP treatment. The best example for this was observed with apidaecin. When administered alone to *T. cruzi*, apidaecin killed the parasite at the 10 μM dose, but when used in combination with melittin, magainin II, or cecropin A, complete lethality to *T. cruzi* was seen at 1.0 μM—a tenfold decrease in the necessary lethal concentration. While all combinations exhibited additive activity compared to single AMP treatments, synergistic activity was observed when magainin II was applied in combination with apidaecin or melittin (Figure 4). It has been inferred from the pair-wise treatment data that the additive and synergistic effects observed could improve the 65 percent rate of *T. cruzi* elimination seen in the initial *in vivo* studies with cecropin A. Furthermore, the use of peptides in combination could reduce the development of peptide resistance in target *T. cruzi* populations.

Rhodococcus rhodnii has been transformed with expression plasmids for the four peptides (melittin, magainin II, apidaecin, and cecropin A) and expression of these molecules was confirmed by ELISA and western blot (Fieck et al., in prep.). The shuttle vector employed for these studies expressed AMP gene sequences from the *Mycobacterium kansasii-α* antigen promoter and export signal sequence. Selection of positive transformants was achieved for both the *E. coli* cloning host and *R. rhodnii* symbiont by growth on carbenicillin and confirmed by colony PCR. Cell lysates from AMP-transformed *R. rhodnii* have been shown to be toxic to *T. cruzi* in single- and pairwise *in vitro* toxicity assays. *In vivo* experiments are currently underway at the Centers for Disease Control (CDC) to test the toxicity of products from single and dual peptide-carrying symbionts to *T. cruzi* in aposymbiotic *R. prolixus* nymphs.

2.2. Single-Chain Antibodies. Antimicrobial peptides act as direct effectors in the paratransgenic model by physically damaging cell structure or metabolic function, resulting in parasite death. Single-chain antibodies (scFv) comprise a second class of effector molecules selected to negatively impact *T. cruzi* development and transmission by acting through an indirect mechanism. In this design, scFvs with binding specificity to *T. cruzi* surface proteins interfere with the physical contact between trypanosomes and the vector that is essential for parasite development. This interference model predicts that the activity of the effector scFv molecules would be specific to parasite development and elicit fewer negative effects on the vector or transformed symbiont. Parasite maturation, which involves metacyclogenesis of *T. cruzi* from noninfective epimastigotes to infective trypomastigotes, occurs in the gut of the triatomine bugs and is an important step in the transmission of Chagas disease [34]. This maturation process is dependent on interactions between the surface epitopes of *T. cruzi* and the gut lumen of the insect vector [35] and would be the target of scFv effector activity.

Single-chain antibodies usually consist of variable regions of heavy and light chains of immunoglobulins connected by a flexible linker, (Gly4Ser) n = 3–5, that permits the two protein domains to interact effectively with their corresponding antigen [36]. The fact that DNA sequences for scFvs can be cloned into expression plasmids and expressed from bacterial transformants renders these molecules uniquely suited to this system of insect paratransgenesis.

To test the ability of a scFv to be expressed and functional within the gut of the Reduviid vector, the pRrMDWK6 expression shuttle plasmid was constructed with a marker gene coding for a murine antiprogesterone antibody fragment, rDB3 [37]. Constitutive expression of rDB3 from pRrMDWK6-harboring symbionts was under control of the *M. kansasii-α* antigen promoter/signal sequence and could be quantified by ELISA for progesterone binding activity. Aposymbiotic *R. prolixus* nymphs were exposed to DB3-expressing *R. rhodnii* symbionts and allowed to develop on blood meals. Subsequent examination revealed that the rDB3 antibody fragment was synthesized by the transformed *R. rhodnii* and secreted into the gut lumen throughout the development of the nymphs to the adult stage (6 months). Protein extracts from the gut of paratransgenic *R. prolixus* bound progesterone suggesting that the presence and activity of scFvs could be maintained in the environment of the insect gut [37]. Further evidence for this was provided by similar experiments carried out with *T. infestans*, another major vector of Chagas disease. A *Corynebacterium* sp., a bacterium closely related to *R. rhodnii*, was identified as a symbiont in this vector. We successfully transformed this bacterium to express rDB3 from the pRrMDWK6 shuttle vector, and generated paratransgenic *T. infestans* lines [38]. ELISA analysis of gut extracts from these paratransgenic bugs

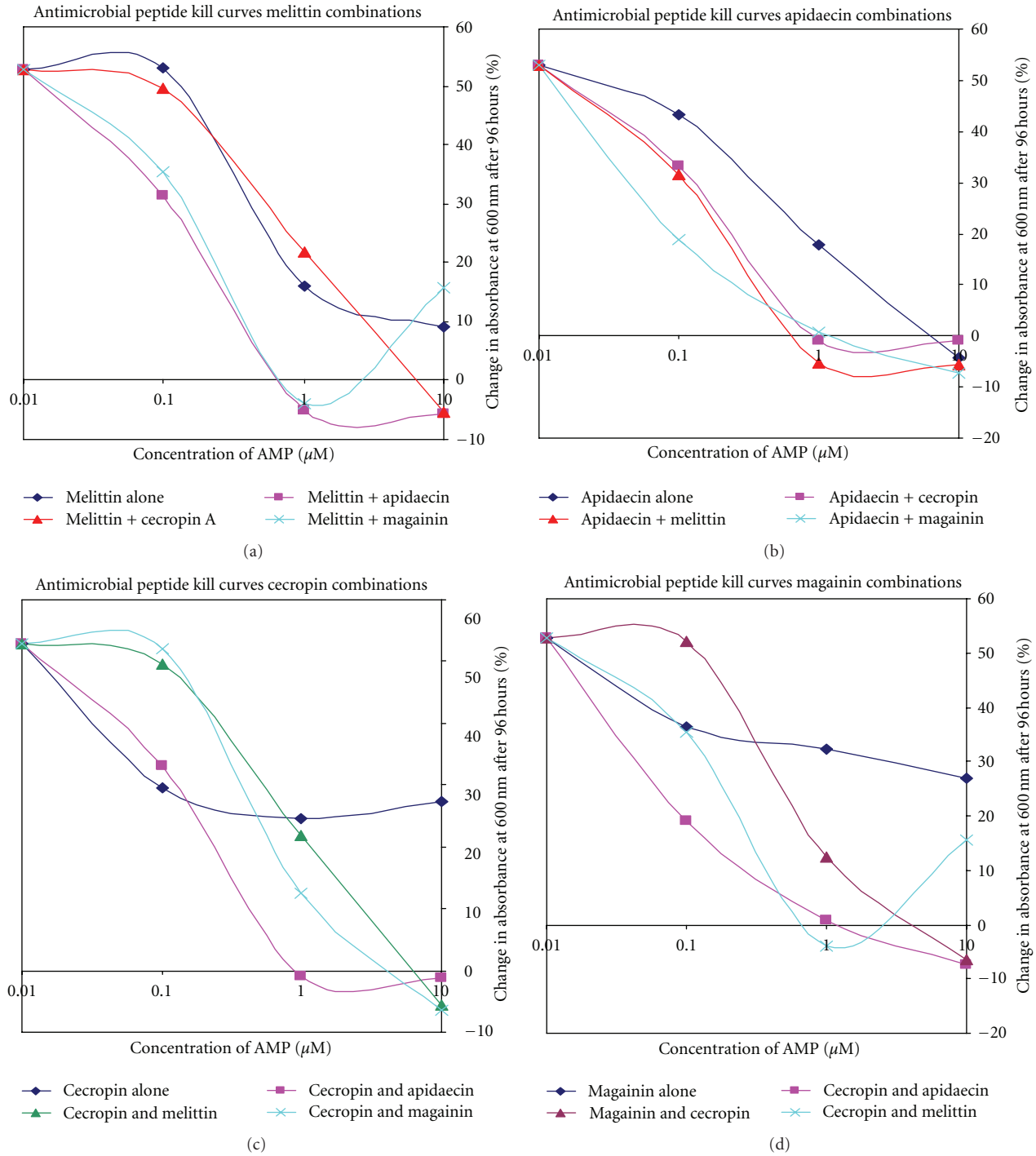

FIGURE 4: Antimicrobial peptide kill curves for dual-combination treatments of *T. cruzi* cultures. Results averaged from triplicate samples in three separate experiments and displayed as the percent change in absorbance at 600 nm compared to untreated controls. Figure is adapted from Fieck et al. *Trypanosoma cruzi*: synergistic cytotoxicity of multiple amphipathic antimicrobial peptides to *T. cruzi* and potential bacterial hosts [33].

showed the presence of rDB3 antibodies capable of binding to progesterone [38].

Progression from the paratransgenic system employing a marker scFv to one utilizing effector scFv's required the development of antibodies with strong binding affinities to parasite surface coat proteins. *Trypanosoma cruzi* does not synthesise or catabolise free sialic acid, but expresses a developmentally regulated sialidase which is used for surface sialylation by a trans-sialidase mechanism [39]. If the appropriate galactosyl acceptor is available the sialyltransferase activity of the *T. cruzi* sialidase is greater than its hydrolytic activity. This implies a mechanism that is capable of remodelling the *T. cruzi* glycan surface using host glycoconjugates as the sialyl donor. Such sialylation might

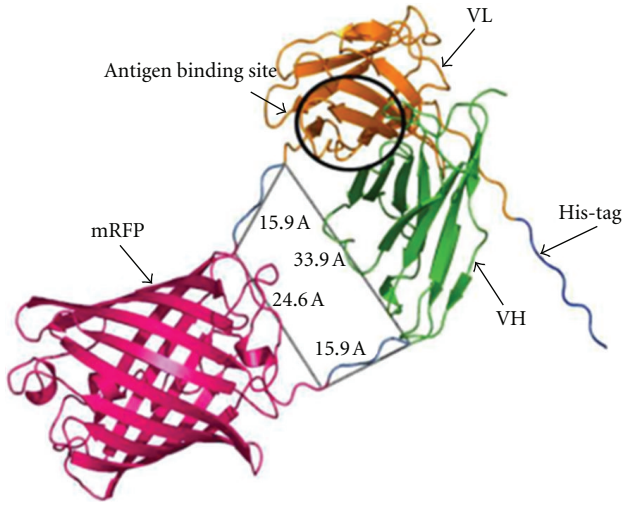

Figure 5: Molecular assembly of an affinity fluorescent protein. The REDantibody shown here has mRFP engineered between the VH and VL domains of the scFV, resulting in a highly stable red fluorescent targeted affinity probe. Figure adapted from Markiv et al. Module based antibody engineering: a novel synthetic REDantibody [36].

provide protection for *T. cruzi* from the innate immune responses. This large family of cell surface sialyated mucin-like glycoproteins plays an essential role in the parasite's life cycle [35], and, therefore is excellent targets for scFv binding. Two well characterised murine monoclonal antibodies, B72.3 [40] and CA19.9 [41], that bind sialyl-Tn and sialyl-(le)a surface glycans, respectively, were selected for application to the paratransgenic system incorporating scFv's for control of Chagas disease transmission [36]. Synthetic DNA sequences encoding the variable regions of the heavy and light chains of these monoclonal antibodies were used to assemble the complete coding sequences for the scFvs. In place of the standard 15 amino acid linker between the heavy and light chain fragments, a monomeric red fluorescent protein (mRFP) derived from the red fluorescent protein cloned from the *Discosoma* coral, DsRed [42], was inserted as a rigid linker that conferred extra stability and fluorescence to the scFvs (Figure 5). Binding to fixed *T. cruzi* epimastigotes and fluorescent optical properties of these scFvs were demonstrated using confocal microscopy (Figure 6). The gene sequences for these scFvs are currently being inserted into the *E. coli/R. rhodnii* shuttle vector for the generation of traceable scFv-expressing symbionts and subsequent generation of effector scFv containing paratransgenic *R. prolixus*.

2.3. β-1,3-Glucanase. Endoglucanases comprise a third class of trypanocidal molecules expected to function as effector molecules in the paratransgenic system for Chagas disease control. *β*-1,3-glucanase is part of an endoglucanase complex isolated from *Arthrobacter luteus* called lyticase. This molecule functions by breaking the 1–3 and 1–6 glycosidic linkages of surface glycoproteins [43]. The surface of *T. cruzi* is covered by a thick coat of glycoproteins proposed to play a role in the binding of the cell body and flagellum

Figure 6: Confocal image of anti-sialyl-Tn REDantibody targeting glycan structures on the surface of *T. cruzi* epimastigotes. Figure adapted from Markiv et al. Module based antibody engineering: a novel synthetic REDantibody [36].

to membranes in the vector gut [44]. This binding is necessary for *T. cruzi* to complete its development and, as a consequence, is essential for its transmission. In unpublished work, we showed that *A. luteus* lyticase was efficient in lysing *T. cruzi* while being nontoxic to *R. rhodnii* and *R. prolixus*. We inserted the cDNA for *β*-1,3-glucanase into our shuttle plasmid and isolated extracts from the transformed *R. rhodnii* for use in toxicity assays against *T. cruzi*. Although *β*-1,3-glucanase was originally described as being effective only as a part of the lyticase complex in the presence of a complementary alkaline protease [43], we have demonstrated that recombinant *β*-1,3-glucanase is biologically active and clears *T. cruzi* at low concentrations even in the absence of the protease (Jose et al., in prep.). Toxicity of the recombinant *β*-1,3-glucanase against *T. cruzi* is comparable to *A. luteus* lyticase complex. These results emphasize the potential for use of *β*-1,3-glucanase as another effector molecule in a paratransgenic strategy to control Chagas disease transmission. The *in vivo* toxic effects of recombinant *β*-1,3-glucanase expressed from symbiotic *R. rhodnii* transformants in *R. prolixus* will be determined using the previously described experimental approach.

The three classes of molecules described above target *T. cruzi* differentially. An effective paratransgenic strategy for field application would involve the delivery of these effector molecules in combination, for example: AMPs with scFvs or AMP's with endoglucanases. This strategy should reduce not only transmission of *T. cruzi*, but also the development of resistance resulting from prolonged treatment with a single effector.

3. Preparations for Field Trials

In anticipation of field trials, we have tested the efficacy of a simulated triatomine-fecal preparation, CRUZIGARD, consisting of an inert guar gum matrix dyed with India ink, as a method for delivery of engineered *R. rhodnii* to closed colonies of *R. prolixus* [45]. The CRUZIGARD preparation was mixed with 10^8 colony forming units (CFU)/mL of genetically modified *R. rhodnii* and used to impregnate cages constructed of thatch and adobe building materials

from Chagas-endemic regions of Guatemala (Olopa) [45]. In these experiments, field caught adult *R. prolixus* from the Olopa district were placed in the cages and removed after eggs were laid. Nymphs were allowed to mature in the CRUZIGARD-treated cages. Nine months later, genetically altered *R. rhodnii* were detected in approximately 50 percent of F_1 adults and comprised nearly 95 percent of total CFUs in these bugs, demonstrating that CRUZIGARD may be useful as a gene dispersal strategy even in environments where competing microbes are present. To increase the volume and duration of CRUZIGARD ingestion, and consequently increase rates of vector inoculation with transformed symbiont, on-going collaborations to develop triatomine attractants and semiochemicals to supplement the current CRUZIGARD formulation are underway.

We realize that deployment of genetically altered *R. rhodnii* into the field may have profound environmental consequences. In a recent publication, we evaluated the risks of horizontal gene transfer (HGT) between R. rhodnii and Gordona rubropertinctus, a closely related nontarget Gram-positive actinomycete [46]. We developed a model that treats HGT as a composite event whose probability is determined by the joint probability of three independent events: gene transfer through the modalities of transformation, transduction, and conjugation. Genes are represented in matrices, with Monte Carlo method and Markov chain analysis used to simulate and evaluate environmental conditions. The model is intended as a risk assessment instrument and predicts an HGT frequency of less than 1.14×10^{16} per 100,000 generations at the 99 percent certainty level [46]. This predicted transfer frequency is less than the estimated average mutation frequency in bacteria, 10^{-1} per gene per 1,000 generations. These predictions were further substantiated when laboratory studies that involved coincubation of R. rhodnii and G. rubropertinctus in conditions highly conducive to HGT resulted in no detectable HGT. These results would suggest that even if HGT were to occur between *R. rhodnii* and *G. rubropertinctus*, the transgene would likely not persist in the recipient organism, and that the likelihood of these unwanted events is vanishingly small [46].

To further minimize gene spread to nontarget arthropods, we are engaged in the development of a strategy to determine the minimum amount of transformed symbionts necessary to prevent *T. cruzi* transmission to humans, and an effective method of CRUZIGARD application suitable for at-risk domiciles in the endemic region. We are also engaged in the development of second-generation paratransgenic delivery systems that utilize microparticle encapsulated, genetically altered *R. rhodnii* for targeted release in the gut of the triatomine bug. Finally, we realize that the field release of engineered bacteria cannot occur until a risk assessment framework is in place. Because such information will not be readily available through field release trials, we are working with collaborators to develop a framework involving rigorous mathematical modeling and simulations. Outputs of these models will be integral to informing risk assessment and regulatory oversight of the paratransgenic program, and, ultimately, to permit field trials of the paratransgenic strategy.

4. Conclusion

Chagas disease affects the lives of millions of people worldwide and remains a major cause of mortality and morbidity, as well as economic loss [47]. Increased attention from the World Health Organization and interest from governments of endemic regions have yielded desirable results for control of Chagas disease transmission. However, success of disease control with large-scale insecticide-based approaches, as demonstrated through the Southern Cone [48], central American [49], Andean Pact [50], and Amazonian Initiatives [49], has been dimmed by the looming possibilities of environmental toxicity, human health impacts, cost of repeated applications, and development of vector resistance.

We describe a novel and potentially environmentally friendly method to control vectorial transmission of Chagas disease. This paratransgenic approach is based upon genetically manipulating symbionts of the triatomine vectors to express effector molecules that would kill or prevent the development of the parasite within the gut of the insect. We have demonstrated the feasibility of this approach in a number of laboratory-based experiments, using effector molecules such as AMPs, endoglucanases and highly specific scFvs. A number of collaborations are underway to evaluate environmental risk related to field release of the genetically modified symbionts. We believe that eventual field application of the paratransgenic approach could provide a more effective and feasible alternative to current strategies of Chagas disease control in endemic regions of the world.

Conflict of Interests

The authors declare that they have no competing interests.

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