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BACTERIAL SYMBIONTS OF THE TRIATOMINAE AND THEIR POTENTIAL USE IN CONTROL OF CHAGAS DISEASE TRANSMISSION*

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■ **Abstract** Chagas disease is caused by the parasitic protozoan *Trypanosoma cruzi* and transmitted by insects in the family Reduviidae, subfamily Triatominae, commonly known as kissing bugs. Because these insects feed throughout their entire developmental cycle on vertebrate blood, they harbor populations of symbiotic bacteria in their intestinal track that produce nutrients that are lacking in the insects' limited diet. It is possible to cultivate these bacteria, genetically modify them, and place them back into their insect host, thus generating a paratransgenic insect. This procedure has allowed the expression of antitrypanosomal gene products in the insect gut, thereby resulting in insects that are incapable of transmitting Chagas disease. A method has been developed that would allow introduction and spread of genetically modified symbionts into natural populations of kissing bugs, thus leading potentially to a transgenic intervention tool for use as a part of an integrated vector control approach.

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INTRODUCTION

Despite great advances in public health worldwide, insect-transmitted infectious diseases remain a leading cause of morbidity and mortality. Malaria continues to afflict hundreds of millions of people annually, with nearly two million deaths per year recorded primarily in Africa (58). Leishmaniasis, Chagas disease, and lymphatic filariasis remain important human diseases that affect millions (35, 37, 38, 77). Furthermore, newly emerging vector-borne diseases such as West Nile encephalitis, ehrlichiosis and multidrug-resistant malaria underscore the impact of arthropod-borne human illness.

Currently, the best methods for control of many insect-borne diseases involve the use of chemical pesticides. Such campaigns may, in the short term, yield spectacular results (69). Malaria was reportedly eliminated from the Indian subcontinent with aggressive use of DDT in the 1950s, but it has resurged with vengeance, with close to 3 million cases reported annually. However, insecticide campaigns are limited in several significant ways. Environmental toxicity and adverse effects on human health can restrict the use of some chemical pesticides. Additionally, emergence of insect resistance to a wide variety of insecticides has in some cases significantly compromised their efficacy (43, 78). The cost of repeated applications of pesticides is often prohibitive in terms of manpower and other operational costs. Therefore, the wholesale elimination of insect pests by chemical control is neither practical nor probable.

Among the current methods that are being evaluated for potential control of vector-borne diseases are efforts that target modification rather than elimination of insect populations (13). These strategies primarily involve either direct genetic transformation of an insect genome via mobile DNA elements, resulting in a transgenic insect (19, 21, 51–53), or expression of a gene product in the host insect via transformed symbiotic microbes, resulting in a paratransgenic insect (6–12, 31–33). It is the latter of these strategies, the application of paratransgenesis for the control of Chagas disease transmission by triatomine bugs, that is the focus of this review.

SYMBIOSIS IN BLOOD-FEEDING INSECTS

Insects and other arthropods that feed throughout their entire life on single food sources often, if not always, harbor populations of symbiotic microorganisms that supplement the insects' restricted diet. This phenomenon, which has been the focus of numerous books and review articles, holds true for aphids and other homopterans that feed on plant juices, various stored-grain pests, insects that feed on the woody parts of plants, and insects that feed on vertebrate blood (3, 17, 18, 24, 29). Mosquitoes do not fall into this category, from a nutritional perspective, because only the adult female feeds on blood, solely for the purpose of producing eggs. Insects such as sucking lice, bed bugs, wingless dipterans, and triatomines, however, all feed exclusively on blood and therefore harbor symbiotic microorganisms. The precise role of these microbes is often unclear, which is the case with triatomine vectors of Chagas disease, but symbiotic microorganisms in blood-feeding insects are generally thought to function in vitamin B biosynthesis (18, 24, 29). In triatomines, it may be that, rather than produce specific vitamins or nutrients that are used directly by the bugs, they themselves are digested, and the various cellular components provide the missing nutrients (18, 40, 44, 60). In this way, the insects essentially cultivate microorganisms within their intestinal tract for their own dietary use. This hypothesis fits with observations in various *Triatoma* spp. of a diversity of gut flora in field-collected individuals (24). It is also consistent with data for *Rhodococcus rhodnii* in its host *R. prolixus*, in which the number of bacteria in the gut fluctuates over time, reaching the highest number at approximately 5 days following the ingestion of a bloodmeal, then decreasing in number as time passes, with some being shed in feces and others apparently being digested (24).

PARATRANSGENESIS

Paratransgenesis is a Trojan horse approach to control of disease transmission by insects. It employs the interactions between disease-transmitting vectors, bacterial symbionts of the vectors, and the pathogenic agent (8). Symbiotic bacteria are isolated and genetically transformed in vitro to export molecules that interfere with pathogen transmission. The genetically altered symbionts are then introduced into the host vector, where expression of engineered molecules affects the host's ability to transmit the pathogen, i.e., its vector competence. This approach attempts to decrease pathogen transmission without adverse effects on the vectors themselves. Furthermore, it employs, as a gene delivery mechanism, bacterial flora native to the host vector. There are several requirements for such an approach to be successful. These are shown in Table 1 and include, among other things, the existence of a suitable symbiotic relationship, a symbiont that can be cultured and genetically modified, an appropriate refractory gene, and a method for introducing the gene into field populations of insects.

TABLE 1 General requirements of a paratransgenic method for potential vector control applications

An appropriate symbiotic association must exist within a given disease-transmitting vector.
Bacterial symbionts should be amenable to culture and genetic manipulation.
Genetically altered symbionts should remain stable.
Fitness of the genetically altered symbionts to re-infect host vectors should not be compromised. Furthermore, their normal symbiotic functions should not be altered.
Transgene products released from the genetically altered symbionts should interact effectively with the target pathogen(s).
A method must exist for dispersal of the genetically altered symbionts amongst naturally occurring populations of vectors.

Several current projects aim to develop a paratransgenic approach for control of disease transmission. Indeed, this approach looks promising for the potential manipulation of arthropod vectors of African sleeping sickness, leishmaniasis (R.V. Durvasula & K. Ghosh , unpublished data), and several plant diseases. The most significant progress to date, however, has been achieved in the area of Chagas disease vectors (6–12, 31–33). These studies serve not only as models for paratransgenic work in other vectors, but may in the future also provide a viable component of an actual Chagas disease control program.

CHAGAS DISEASE

Chagas disease is an important parasitic disease of humans throughout much of Central and South America (62, 63, 65–70, 77). It is caused by the protozoan *Trypanosoma cruzi* and is transmitted to humans by blood-sucking insects in the family Reduviidae, subfamily Triatominae, frequently referred to as reduviids, triatomines, or kissing bugs. These insects infest thatch and adobe homes that are common in the rural tropics, and they transmit the parasite to humans via fecal droplets deposited during feeding. Close to 90 million people live in areas where transmission of *T. cruzi* may occur, and between 12 and 15 million cases are estimated (67, 78). Although most acute infections are minimally symptomatic, between 10% and 30% of cases progress to a chronic debilitating life-threatening illness of the cardiac and gastrointestinal systems. Approximately 50,000 deaths per year are attributable to the chronic sequelae of Chagas disease, making it a leading cause of morbidity and mortality in tropical America (20, 57).

Neither a vaccine for prevention nor an effective cure for chronic Chagas disease currently exists. Three multinational control programs are presently underway, however, all of which focus on eliminating domestic populations of triatomines through insecticide use and on screening of blood banks to eliminate contaminated blood (66, 69). In the Southern Cone nations of Uruguay, Argentina, Brazil, and Chile, the results have been dramatic, and new cases of Chagas disease have

been virtually eliminated (26). In other countries, however, significant problems persist. Some of the specific concerns include control of peridomiciliary vector species such as *Triatoma dimidiata* in Central America (1, 62–65). This species not only lives in homes, but also frequently inhabits structures around homes, such as storage sheds, chicken coops, and piles of wood or unused building materials. Populations of bugs inhabiting these peridomiciliary sites allow reinfestation of insecticide-treated homes. A similar situation is true for *Triatoma brasiliensis* in Brazil, which is now considered the most important domestic vector of Chagas disease in this country, in the aftermath of the successful control efforts focused against *Triatoma infestans* (22, 23, 61).

In addition to the difficulties in control caused by peridomiciliary triatomine populations, the emergence of insecticide resistance presents another potential obstacle. Whereas resistance has not yet been a widespread problem for the control of Chagas disease vectors, it may be developing as one. Vassena and colleagues report resistance to synthetic pyrethroids in populations of *Rhodnius prolixus* from Venezuela and *T. infestans* from Argentina (74).

One of the most important obstacles to the success of regional insecticide-based control programs is the extremely difficult task of spraying every infested home within the region. The goal of these programs is elimination of the two key domestic species, *R. prolixus* and *T. infestans*. Consequently, success hinges on a highly coordinated effort to treat every infested home effectively with insecticides (69). *R. prolixus* is the primary domestic vector species in northern South America and in parts of Central America (70). It is thought to be indigenous to northern South America and to have been introduced into Central America in the early 1900s from a single laboratory escape that may have occurred in El Salvador (30, 70, 81). This hypothesis is supported by independent DNA-based studies that show that the populations of *R. prolixus* from Central America are highly homogeneous, indicative of a founder effect (30). If this species has spread through most of Central America in approximately 80 years following a single release event, one might ask the question, "What happens in a control program if a single home is missed, or a single village, or a few homes in several villages?" How long will it take for the region to become infested again? Furthermore, can some of the poorer countries in these regions mount sufficient political will to bear the economic burden of sustaining a long-term insecticide control program with subsequent posttreatment surveillance? Both are necessary to insure that homes are not reinfested from peridomiciliary vector populations or insects from homes that were not treated. For these reasons, despite recent success achieved using insecticides, a commitment must be maintained to the development and evaluation of alternative methods aimed at controlling Chagas disease transmission.

PARATRANSGENESIS OF CHAGAS DISEASE VECTORS

Although there are only a dozen or so species of triatomines that are considered important vectors of *T. cruzi* in humans, the work on paratransgenesis has focused primarily on *R. prolixus*. This species maintains a symbiotic relationship with a

nocardiform actinomycete, *Rhodococccus rhodnii* (24, 40, 44, 60). *R. rhodnii* is a ubiquitous soil organism that reaches concentrations of up to 10^8 colony-forming units/ml in the hindgut of *R. prolixus*. As discussed previously, it is thought to be involved somehow in the metabolism or sequestration of B complex vitamins in the diet of *R. prolixus*. This microorganism is acquired by early nymphal stages through coprophagy, i.e., the ingestion of feces of other *R. prolixus* individuals. Newly emerging nymphs of *R. prolixus* are transiently aposymbiotic (lacking in gut-associated symbionts). Through coprophagy they populate their gut with the symbiont and can thus continue development (3, 24, 40, 44, 60). Because *R. rhodnii* remains extracellular and reaches its highest concentrations in the hindgut lumen in close proximity to infective forms of *T. cruzi*, it is a favorable candidate for paratransgenic studies for potential vector control applications.

If aposymbiotic nymphs of *R. prolixus* emerge from surface-sterilized eggs and are reared in sterile chambers without access to *R. rhodnii*, they fail to develop to mature adults, despite regular blood meals from most host animals (3, 24, 40, 44, 60). If symbiotic bacteria are provided to the aposymbiotic nymphs, either in blood or via fecal contamination, the insects are able to complete their normal sexual development, without loss of fitness. The environmental hardiness of *R. rhodnii*, its amenability to genetic manipulation, and the relative ease of symbiont transfer among laboratory populations of *R. prolixus* provided the basis for selecting this particular symbiont-host system.

Establishment of Paratransgenic *Rhodnius prolixus*

In the initial studies the symbiont *R. rhodnii* was transformed with the *Escherichia coli-Rhodococcus* shuttle plasmid pRr1.1 (10). This plasmid was based on a pBR322 family plasmid, pIJ30, which contained the *Streptomyces*-derived thiostrepton antibiotic resistance marker gene (72, 73). A restriction enzyme-generated DNA fragment containing the plasmid replication origin from an uncharacterized low-copy number endogenous plasmid of *R. rhodnii* strain ATCC 35071 was cloned into pIJ30. The resulting shuttle plasmid could be maintained and modified in *E. coli* and then used for stable transformation of *R. rhodnii* (10). The transformed bacteria were then introduced into newly emerged aposymbiotic first instar nymphs of *R. prolixus* in blood provided to the bugs through an artificial feeding apparatus with a synthetic membrane. Bug colonies were maintained with and without thiostrepton selection. The transformed bacterium allowed sexual maturation of the experimental bugs at a rate comparable to the maturation of the untransformed bacterium. Thiostrepton-resistant *R. rhodnii* were detectable in the gut of experimental insects for the 6.5-month duration of the study, irrespective of selection. This study supported the hypothesis that a transgene-carrying symbiont could be introduced into *R. prolixus* and maintained without adverse effects on insect survival and fitness. Furthermore, it suggested that an engineered symbiont could be maintained in a stable fashion throughout the life cycle of the bugs (10).

Expression of the Antitrypanosomal Peptide, Cecropin A, in *Rhodnius prolixus*

Once a successful transformation system was developed, the next studies involved attempts to express an actual antiparasitic peptide to render the insect incapable of transmitting *T. cruzi*. It has long been known that insects are capable of mounting an effective immune response against invading organisms, although it is considered relatively crude in comparison to that of mammals. This topic has been the focus of numerous reviews (16, 34, 56, 75, 76). Certain groups of these factors have significant impacts on the ability of insect vectors to become infected with and subsequently transmit human disease pathogens (5, 28, 53). One group of insect immune peptides is the cecropins, which derive their name from the saturniid *Hyalophora cecropia*, from which they were originally described (14, 15, 79). Cecropin A, a member of this class of naturally occurring peptides, has been studied extensively, and the gene that encodes it has been cloned, sequenced, and expressed (39, 49).

In studies aimed at evaluating its potential use for paratransgenic applications, cecropin A had a minimal bactericidal concentration of 23 μM for *E. coli*, a range of 150–240 μM for different strains of *T. cruzi*, and 500 μM for *R. rhodnii* (31). Additionally, nonvegetative forms of *R. rhodnii* were completely resistant to any amount of cecropin A. Consequently, these results suggested that cecropin A could be potentially useful as an antitrypanosomal peptide to be expressed in a paratransgenic system. After the preliminary feasibility of the approach was established, efforts were made to establish a paratransgenic line of insects. In these studies pRr1.1 was modified to contain a gene encoding the 38–amino acid pore-forming peptide L-cecropin A, and the resulting construct was used for transforming *R. rhodnii*. Aposymbiotic first instar nymphs of *R. prolixus* were then colonized with either wild-type *R. rhodnii* or cecropin A–producing *R. rhodnii*. Colonies were maintained without thiostrepton selection. However, stability of pRrThioCec was favorable with a plasmid decay rate of 0.5% per generation of *R. rhodnii*. Initial published studies involved 7 insects in each group; experiments were repeated with 100 insects in each group. All insects that carried wild-type or thiostrepton-resistant symbionts were successfully infected with *T. cruzi* at the fourth instar stage and developed mature trypomastigotes in the hindgut. In 65% of insects that carried cecropin A–producing symbionts, *T. cruzi* were eliminated. In the remaining 35%, *T. cruzi* counts were reduced by 2–3 orders of magnitude. These studies provided initial evidence that a genetically altered symbiont could be used to eliminate transport of an infectious agent from a host vector (9, 31).

Expression of a Functional Antibody Fragment in Paratransgenic *Rhodnius prolixus*

Whereas insects have the capacity, at least in some limited fashion, to recognize "nonself," their immune response is not as sophisticated as that seen in mammals, in

which there is the capacity to mount a vast repertoire of antibodies against individual antigenic epitopes of an invading organism. In recent years, however, through phage display technology, it has become possible to clone single-chain antibody-encoding genes that can be expressed in heterologous organisms (4, 41, 42, 45, 50). These single-chain antibody fragments are composed of a variable region of a heavy chain and a kappa light chain (VH-Kappa), which can be selected from libraries of heavy and light chain genes, expressed via phage, and screened for binding specificity for target antigens. This powerful technology can be utilized for expression of mammalian-derived antipathogen antibodies in an insect disease vector.

In studies with Chagas disease vectors, a single-chain antibody, rDB3, was actively expressed by genetically altered *R. rhodnii* in paratransgenic *R. prolixus* (32). The antibody fragment, a murine VH-Kappa chain that binds progesterone (41, 42), was used as a model for single-chain antibody expression. A shuttle plasmid, pRrMDWK6, was constructed that contained the gene encoding rDB3 under control of a heterologous promoter/signal peptide element derived from *Mycobacterium kansasii* alpha antigen gene (59) and utilizing a kanamycin resistance marker. Following confirmation of secretion of rDB3 by western blot analysis, second instar nymphs of *R. prolixus* were infected with either rDB3-producing *R. rhodnii* or wild-type *R. rhodnii*. Sampling of successive stages of nymphs revealed that kanamycin-resistant *R. rhodnii* could be detected in the experimental group. Furthermore, progesterone binding activity in gut extracts of the paratransgenic bugs was demonstrated. This activity could be partially inhibited by addition of free progesterone in the detection assay (32).

These experiments established the feasibility of expressing active single-chain antibodies within the gut of Chagas disease vectors for potential transmission-blocking activity against *T. cruzi*. Since this time, a gene encoding a single-chain transmission-blocking antibody fragment directed against the rodent malaria parasite *Plasmodium gallinaceum* has been successfully expressed in the mosquito *Aedes aegypti*, using a Sindbis viral transducing vector (25). These studies in such different insect vectors demonstrate the broad potential applicability of this approach for generating disease refractory or incompetent insects.

Use of Integrative Plasmids for Transformation of Bacterial Symbionts

One of the potential shortcomings of paratransgenic insects generated using plasmid transformation elements is genetic instability and the subsequent loss of the plasmid transformation vector. Although transformation stability has been calculated in some cases and appears to be generally adequate over relatively short periods of time (10, 31), any potential field application would require high stability rates for long-term sustainability. Consequently, much effort has gone into developing and evaluating other approaches for genetic transformation of bacterial symbionts. One such approach, which appears to be quite stable and promising, involves the use of DNA integration elements that are compatible with the symbiotic bacteria. Several integrase elements have been identified in mycobacteria and have

been used for transformation and mutagenesis studies (54, 55). DNA integration vectors derived from mycobacteriophage L1 integrase–containing plasmids have been used successfully in paratransgenetic studies with several triatomine species and their respective symbionts. Stability of these elements in the absence of selection and adaptability of mycobacterial sequences to actinomycete genomes make these attractive choices for stable transformation of reduviid symbionts. Preliminary studies demonstrated stable single-copy integration of the element, pBP5, that contained the L1 integrase gene and a kanamycin selection marker, in *R. rhodnii* (E.M. Dotson, B.B. Plikaytis, T.M. Shinnick, R.V. Durvasula & C.B. Beard, manuscript submitted). Furthermore, the results showed that this construct remained stable in the absence of selection for over 100 generations, which was the duration of the study, with no loss or movement of the element. These data suggest that DNA integration element transformation systems are superior to shuttle plasmids and will probably replace them as the preferred method for generating stable paratransgenic triatomines.

Dispersal of Transformed Symbionts in Vector Populations

The aforementioned studies describe some of the molecular machinery and methods employed in the generation of paratransgenic insects. Two different classes of potential antitrypanosomal genes have also been discussed: insect immune peptides and single-chain antibody fragments. Before these methods can be evaluated for potential deployment and testing, an effective method for field introduction must be developed (8). Field use of this approach requires a robust method for delivery of foreign genes to vector populations and subsequent spread of genetic material. Such a method should approximate naturally occurring modes of transfer, should not adversely affect fitness of transgene-carrying insects, and should permit selective transfer of recombinant microbes to target arthropods. Natural symbiont dispersal and acquisition among hatching nymphs of *R. prolixus* involves coprophagy. Hatching first-instar nymphs are transiently aposymbiotic, subsequently acquiring their symbiont flora through probing of feces; the acquired flora rapidly populate their gut, thereby providing the required supplementary dietary needs.

This natural mechanism for dispersal has been exploited as a potential means for dispersing genetically modified (GM) symbionts (33). A synthetic paste, termed CRUZIGARD, has been developed. This substance is composed of guar gum and India ink to which cultured GM *R. rhodnii* has been added. Following the addition of a small amount of ammonium sulfate, the material can be dispensed in droplets that simulate natural feces of *R. prolixus*, thereby stimulating coprophagy. In a set of studies aimed at evaluating this material for its potential use as a carrier for introduction of GM symbionts into natural populations, first-instar nymphs of *R. prolixus* were exposed in the laboratory to CRUZIGARD that contained either rDB3-producing GM *R. rhodnii* or wild-type *R. rhodnii*. Assays of gut contents of successive nymphal stages revealed the presence of genetically altered bacteria and progesterone-binding activity in the experimental group only. In the first set of assays the average number of recombinant *R. rhodnii* in paratransgenic individuals

increased with successive stages, which suggests successful establishment of the transformed bacteria. In this particular assay, however, recombinant *R. rhodnii* comprised less than 1.0% of the total number of microbial colony-forming units in the bug gut, presumably because the GM symbionts were introduced to nymphs already populated with wild-type bacteria.

In the second part of the study, cages were constructed to simulate the actual adobe and thatch building materials used in huts in Chagas disease–endemic regions of Central America. Thatch and adobe panels were impregnated with CRUZIGARD and fresh applications were made monthly. Adult field-collected *R. prolixus* were placed in the cages and removed after eggs were laid. Newly hatching nymphs were thus exposed subsequently to CRUZIGARD and the native feces from adult bugs that had been removed earlier. Soil from outdoors was placed in the cages to add competing microbes, and anesthetized rabbits were used as a source of monthly bloodmeals. In approximately 50% of sampled bugs in the experimental group, GM *R. rhodnii* were detected. None of the sampled adult bugs in the control group carried recombinant *R. rhodnii*. More importantly, GM *R. rhodnii* comprised nearly 90% of colony-forming units in the bugs that tested positive. These studies, while serving as the first effort to develop and evaluate a method for field introduction, suggest that CRUZIGARD may be useful as a dispersal strategy for employing GM symbionts for paratransgenic vector control applications (33).

Paratransgenic Studies in Other Triatomine Species

Bacterial symbionts have been identified and partially characterized for several additional triatomine species. A *Corynebacterium* sp. has been isolated from *T. infestans* and its symbiotic properties confirmed (R.V. Durvasula, O. Kruglov, J. Taneja, M. Goodwin, A. Kroger, E.M. Dotson, F.F. Richards & C.B. Beard, manuscript submitted). Transformation success has been achieved, similarly utilizing the pRrMDWK6 shuttle plasmid. As in *R. rhodnii*, the single-chain antibody fragment rDB3 is functionally expressed in paratransgenic *T. infestans*.

Similar studies are ongoing in *Gordonia rubropertinctus* and *Gordonia terrae*, which are putative symbionts of *Triatoma dimidiata*, an important domestic and peridomestic vector of Chagas disease in Central America. Both bacterial species have been genetically transformed using an L1 mycobacteriophage DNA integration element (P.M. Pennington & C.B. Beard, unpublished data). This family of integration elements appear to be broadly adaptable for use in most coryneform bacteria. Because the vast majority of symbiotic flora of triatomines fall into this bacterial group, paratransgenesis should be relatively easy to accomplish in many if not all triatomine species.

PARATRANSGENESIS AND VECTOR CONTROL

A hypothetical way that paratransgenic technology might be utilized for vector control of Chagas disease has been discussed elsewhere (7, 8, 12). Rather than replacing insecticide control, it could be incorporated into an integrated pest

management program. Where the targeted vector species is one that lives both in the home and in the peridomicile, a bait formulation of the bacteria (i.e., CRUZIGARD) could be applied in potential hiding places and resting sites of bugs, following insecticide treatment of the house. Consequently, as homes become reinfested, the nymphs hatching from eggs laid by adult migrants would ingest the GM symbionts that had been applied in the bait formulation. The key to successful introduction would be insuring that the bait is applied in ample concentration so that it out-competes the native symbionts that are present in feces of the adult bugs that reinfest the home. Once the symbiont has been acquired by neonates, these paratransgenic nymphs would disperse the amplified GM symbionts, resulting ultimately in a population of insects incapable of transmitting disease.

Paratransgenic control might also be used in prevention efforts targeted against domestic vector species. This could be accomplished by applying GM symbiont bait formulations, as above, but in new homes or otherwise uninfested homes, in areas where infestation is a significant threat due to infestations that exist in nearby homes.

SAFETY CONCERNS AND REGULATION

The prospects for transgenic or paratransgenic control of insect vectors of animal diseases are promising; however, as with all other initiatives that involve release, consumption, or exposure to genetically modified organisms, there is significant concern. Potential risks associated with the release of GM insects have been reviewed recently (2, 7, 8, 36, 46–48, 80). In addition to the ecological concerns that would be associated with environmental release of any genetically modified organism (summarized in (Table 2), because insect vectors of human diseases are closely associated with humans and rely on them as a blood source, there are additional potential health risks involved. The magnitude and probability of these

TABLE 2 Framework for evaluating ecological risks associated with the release of a genetically modified arthropod[a]

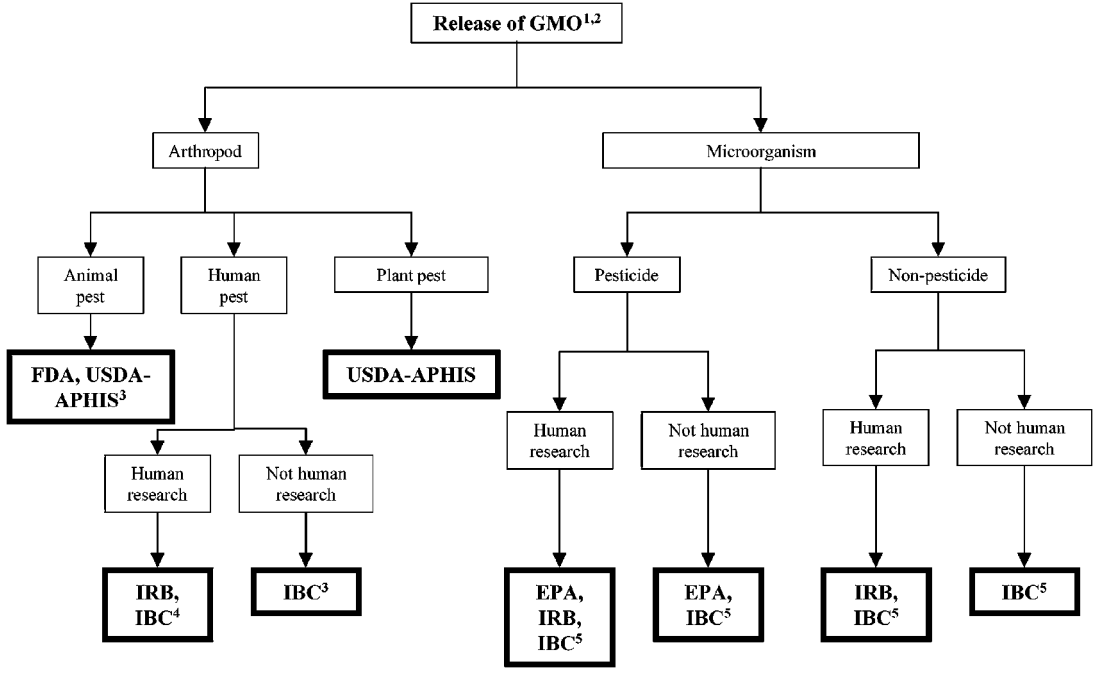
How the recipient organism was transformed through recombinant DNA technology, to include characteristics of the donor, vector, and recipient organisms and a description of the methods employed
The characteristics of the modified organism, to include the stability of the new genotype and the probability of gene transfer to other organisms with resultant consequences
Potential impact of the transgenic arthropod on native populations, communities, and ecosystems
Methods for evaluation of the safety of the transgenic organism in field trials before unrestricted release

[a]The information above is requested on the USDA-APHIS-BBEP Form 2000, "Application for Permit or Courtesy Permit under 7 CFR 340 (Genetically Engineered Organisms or Products)," accessible at http://www.aphis.usda.gov/biotech/arthropod/tgendisc.html

risks depend on a number of factors that include the nature of the genetic modification, the study design, and the potential impact on local disease transmission. Some of these issues are discussed in the draft guidelines for laboratory containment of medically important arthropods, which have been developed recently under the auspices of the American Society of Tropical Medicine and Hygiene, American Committee of Medical Entomology (ACME). This document addresses containment of transgenic and paratransgenic insects and provides a framework for evaluating some of the potential risk to humans associated with these studies, both in laboratory and to a lesser degree in field situations. The ACME draft guidelines can be accessed on the World Wide Web, where they are available for public comment at: http://www.astmh.org/subgroup/acme.html.

The question of who currently maintains the authority to regulate research on and environmental release of GM insects is complicated (see Figure 1) and has been reviewed recently (80). In general, if the organism is a vector of a plant disease, any release occurring in the United States would be regulated by the US Department of Agriculture, Animal and Plant Health Inspection Service (APHIS). Information on this process, as well as permits that have been issued or are under current consideration, are available on line (http://www.aphis.usda.gov/biotech/arthropod/). Oversight regarding the release of GM insects that feed on humans and/or vertebrate animals can sometimes be less clear. In most cases, APHIS would take the lead in oversight; however, there are areas of overlap of authority that are shared by APHIS, the Food and Drug Administration, and the Department of Health and Human Services, with clarification residing in the exact interpretation of the Coordinated Framework for Regulation of Biotechnology of the U.S. Office of Science and Technology Policy. Additionally, in cases in which the release involves an insect of public health importance, guidance is provided in part by the National Institutes of Health Recombinant DNA Advisory Committee (RAC) Guidelines (obtainable at http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html). If aspects of the study involve human subjects, as defined in the U.S. Code of Federal Regulations 45CFR46 (http://ohrp.osophs.dhhs.gov/humansubjects/guidance/45cfr46. htm) of the National Institutes of Health, Office for Human Research Protections, then institutional review board approval is required, in addition to other requirements. Environmental releases that are conducted or funded by U.S. government agencies are subject to environmental assessment as outlined by the National Environmental Policy Act (NEPA). The authority of U.S. agencies involved in oversight and

Figure 1 Flow chart demonstrating the current view on regulatory oversight for potential field studies involving transgenic and paratransgenic insects. Abbreviations: GMO, genetically modified organism; FDA, U.S. Food and Drug Administration; USDA-APHIS, U.S. Department of Agriculture, Animal and Plant Health Inspection Service; IRB, institutional review board for research involving human subjects; IBC, institutional biosafety committee; RAC, National Institutes of Health Recombinant DNA Advisory Committee; EPA, U.S. Environmental Protection Agency.



[1]This decision tree applies only to a field release that would occur on U.S. soils and/or a release on foreign soils sponsored by U.S. federal funds. It also assumes that final approval must be granted ultimately at the local level.

[2]All environmental releases that are conducted or funded by U.S. government agencies are subject to environmental assessment as outlined by the NEPA (40 CFR Parts 1500–1508 and Executive Order 12114).

[3]Current federal guidelines suggest that USDA-APHIS share regulatory authority for a field release of a genetically modified insect that feeds on certain groups of animals. Currently, however, they are not reviewing proposals of this nature.

[4]Regarding the release of a genetically modified insect that feeds on both humans and vertebrate animals, clarification of the review process may be required, based on interpretation of the Coordinated Framework for Regulation of Biotechnology, of the U.S. Office of Science and Technology Policy.

[5]Depending on the nature of the transgene and the particular experimental design, approval may be required by the National Institutes of Health Recombinant DNA Advisory Committee, in addition to IBC approval.

regulation is primarily limited to field releases conducted on U.S. soils or research in foreign countries supported by federal funding. This limitation highlights the need for additional guidelines and policy that would lead ultimately to an international review process.

In the case of field research involving paratransgenic triatomines, in which the experimental design does not involve the actual release of an insect but rather a GM microorganism, the situation is more similar conceptually to the use of a biological pesticide, such as *Bacillus thuringiensis*. In this case, conceivably, the precise nature of the genetic modification becomes important. In its natural nongenetically modified condition, *R. rhodnii* is a nonpathogenic symbiotic microorganism. If *R. rhodnii* were modified to be trypanocidal, it would probably then be considered a microbial pesticide and therefore fall under U.S. Environmental Protection Agency oversight. If the genetic modification involved introduction of an antimicrobial resistance gene or toxin gene, then RAC and/or National Institutes of Health, Office of Biotechnology Activities approval would be required. If the genetic modification involved introduction simply of a gene that encodes a biochemical or colorimetric marker, such as the algal green fluorescence protein or beta-galactosidase, or a transmission-blocking antibody fragment that simply interferes with the parasite-host relationship of the pathogen but is neither antitrypanosomal nor insecticidal, it is unclear what regulation would be required other than approval by the institutional biosafety committee, as dictated in the RAC guidelines.

If the release were to take place inside a human dwelling, as suggested above, the initial studies would most certainly involve human subjects, thus requiring institutional review board approval. It should be emphasized, similarly, that an environmental release of any genetically modified organism must be approved ultimately at the local level by authorities in the community affected by the release and with the informed consent of any persons affected by the release. Finally, it is important to note that the issues relating specifically to the process of oversight and regulation are currently being evaluated and clarified and will no doubt change as the prospect of an actual field release of a transgenic or paratransgenic insect becomes a reality.

SUMMARY

A paratransgenic approach to the modification of triatomine vectors of Chagas disease has been developed. The symbiont, *R. rhodnii*, has been genetically transformed to express cecropin A and a functional single-chain antibody in the gut lumen of the host vector, *R. prolixus*. Methods for integrative transformation of actinomycete symbionts have been developed. A strategy for introduction of genetically altered symbionts into field populations of Chagas disease vectors that employs the synthetic fecal preparation, CRUZIGARD, is being tested. This approach is far from the field application stage. Many questions remain regarding ideal gene constructs, optimal delivery mechanisms, and safety concerns about

release and containment of genetically modified organisms. Additionally, there are still many questions regarding regulations and the review process, especially as they relate to releases that might be conducted in other countries.

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