

Symbiosis Therapy: The Potential of Using Human Protozoa for Molecular Therapy

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The concept of symbiosis is proposed as a disease therapy model. It is hypothesized that protozoa that live naturally in human tissues can be genetically modified for the production and delivery of therapeutic proteins. Approximately 30 identified species of protozoa live in a variety of human tissues, both intracellularly and extracellularly. *Leishmania*, one species of human protozoa, has been genetically altered for conditional auxotrophy and has shown no pathology in both mouse and nonhuman primate safety tests. Several species of protozoa have been transfected with a variety of genes and have successfully manufactured active foreign proteins. Protozoa have biochemical mechanisms to glycosylate proteins. Human protozoa have evolved sophisticated mechanisms for evading immune rejection and can sometimes persist for the lifetime of the host. Symbiosis therapy does not involve genetic alteration of the host and is potentially fully reversible. Research on treating genetic diseases is currently ongoing. For example, there is a group of more than 40 genetic diseases, lysosomal storage diseases, which result from defects in lysosomal enzymes primarily in macrophages. *Leishmania* specifically targets the lysosomal compartment of the macrophage and therefore may be the optimal vector for treatment of many of these diseases.

THE NEED FOR NEW APPROACHES IN MOLECULAR THERAPY

With the completion of the human genome, and the genomes of many model organisms, life scientists now have the tools to understand the mechanisms of all disease in the next several decades. In fact, the causes of many genetic diseases are already well understood today. Protein abnormalities are responsible for many, if not most, human diseases. In large measure, the ability to permanently replace defective, deficient, or absent proteins can potentially cure the disease. Given the large variety of genetic diseases, an equally large variety of therapeutic technologies will be needed to treat them as we move into the 21st century. This report will discuss why genetically engineered human protozoa offer an additional route to overcoming the challenge of manufacturing and delivering therapeutic proteins for the treatment of human disease.

USING SYMBIOSIS AS A MODEL FOR MOLECULAR THERAPY

Symbiosis, literally "to live together," is defined as two organisms that live to each other's mutual benefit.

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In fact, symbiosis is a common solution for biological problems in nature. Metazoans have often relied on single-celled microorganisms to compensate for biochemical deficiencies. For example, it is well known that in plants bacteria supply the function of nitrogen fixation, in termites cellulose is digested by flagellated protozoa for nutrition, and even in humans bacteria in the intestines help with the proper operation of the GI tract. Symbiosis has not proven to be a guiding principle in medicine to date; however, we propose that advances in genetic technology may alter this perspective. For example, Steidler *et al.* (1) have reported that genetically engineering *Lactococcus lactis* can be used to manufacture and deliver interleukin-10 for the successful *in vivo* treatment of murine colitis.

RELEVANT CHARACTERISTICS OF HUMAN PROTOZOA

Protozoa that live in the human body have several characteristics that suggest that they may be a relevant tool for treating disease (2). First, some background on these microorganisms. Protozoa are single-celled, independently living eukaryotic organisms. More than 45,000 species have been cataloged in nature. Most invertebrates and vertebrates have protozoan species that are adapted at living in or on them. Protozoa range in size from 1 to over 150 μm ; however, the larger forms are usually free-living varieties in the environment.

TABLE 1
Some Characteristics of Selected Human Protozoa

Species	Body location	Cellular location	Transmission route	Pathology
<i>Acanthamoeba</i>	Eye, nose, throat, skin	Extracellular	Ingestion, soil and dust	None to serious in i.c. ^a
<i>Endolimax</i>	Gut	Extracellular	Ingestion	None
<i>Entamoeba</i>	Gut, liver, mouth	Extracellular	Ingestion	None in 90% of infected patients to severe
<i>Chilomastix</i>	Gut	Extracellular	Ingestion	None
<i>Cryptosporidium</i>	Gut	Extracellular	Ingestion	None to diarrhea
<i>Giardia</i>	Gut	Extracellular	Ingestion	None to diarrhea
<i>Iodamoeba</i>	Gut	Extracellular	Ingestion	None
<i>Leishmania</i>	Skin, liver, spleen	Cytoplasm	Insect bite	Cutaneous—mild; visceral—severe
<i>Plasmodium</i>	Red blood cells, liver, CNS ^b	Intracellular	Insect bite	Severe
<i>Toxoplasma</i>	All cells, CNS	Cytoplasm	Ingestion	None to serious in i.c.
<i>Trichomonas</i>	G.U., gut, mouth ^c	Extracellular	Direct contact	Mild in G.U. forms, none in gut forms
<i>Trypanosoma</i>	Bloodstream, muscle	Extracellular	Insect bite	Severe

^a i.c.; immune-compromised patients.

^b CNS; central nervous system.

^c G.U.; genital-urinary system.

At least 30 species of protozoa are known to infect the human body (Table 1). Some of these species are pathogenic while others have no detectable adverse effects or pathologies. Most humans have at least one species of protozoa inhabiting their bodies. These organisms live in a variety of ecological niches from the gut to the brain. Some species live intracellularly while others are extracellular. Of the intracellular types, some are known to enter and live primarily in one specific cell type while others will enter most cell types. Because they are independent organisms, protozoa do not require any genetic interaction with the host genome or protein synthesis apparatus to survive and reproduce, such as occurs with viruses.

Protozoa spread from one host to another through a variety of methods. In the United States, approximately half of the population is infected with the species of *Toxoplasma*, primarily through direct physical contact with the domestic cat. *Entamoeba*, which live in the human gut, are transmitted through drinking water. Other protozoa, such as the malaria *Plasmodium* and *Leishmania*, are transmitted via insect vectors. In the absence of those specific insect vectors, the disease cannot be transmitted from one individual to another.

All human protozoa have developed sophisticated

mechanisms to escape immune rejection. Strategies involve hiding inside cells and rapidly altering surface antigens. In fact, there is evidence of protozoan persistence for years in the host. Immune responses to protozoan infection are primarily through the TH2 system.

PRIOR USE OF PROTOZOA IN DISEASE THERAPY

It is little known today, but there is precedence for the use of protozoa in medical practice. Before the advent of antibiotics, patients in the end stages of syphilis (neurosyphilis) were sometimes treated by malarial therapy (3). It was determined that syphilis was susceptible to high fevers. One way to generate high fevers in patients was through contracting malaria. The case fatality rate in untreated patients usually exceeds 80% within 4 years of neurosyphilis onset while that of malaria-treated patients ranges from 5 to 10%. The malaria was then treated with antimalarial drugs as needed. With the availability of penicillin in the 1940s, malarial therapy was abandoned, but not completely until the 1960s in the United States and the 1970s in Britain (often used in combined therapy with penicillin). In the end, thousands of lives were saved as a result of this cost-effective treatment.

FOREIGN PROTEIN MANUFACTURE BY PROTOZOA

One crucial issue in the use of protozoa for molecular therapy is the capability of these organisms to manufacture foreign proteins. A number of research laboratories have reported on this point. Bacterial genes such as β -galactosidase (4, 5) and β -glucuronidase have been expressed in *Trypanosoma cruzi* (4) and *Leishmania* (5). The neomycin phosphotransferase gene was expressed in *Trypanosoma brucei* (6, 7). Chloramphenicol acetyltransferase was expressed in *Leishmania* (8). Optical marker genes such as luciferase (9) in *Leishmania* and *Trypanosoma brucei* (10) and green fluorescent protein in *Leishmania* (11), *Toxoplasma* (12), and *Plasmodium* (13) have been successfully expressed. Some mammalian genes, like interferon- γ (14), have been produced in *Leishmania*. There are unpublished claims for the successful expression of interleukins 2 and 12 as well. In terms of the quantity of protein produced in protozoa, while a concerted effort to maximize protein production has not been made, foreign protein production of up to 1% of total cell protein has been reported (15). It is clear that human protozoa can make a variety of proteins and that many species have the genetic tools to successfully transfect genes. Furthermore, many species of protozoa have the glycosylation machinery for posttranslational modification of proteins (16, 17).

For some diseases, protein secretion is necessary for symbiosis therapy. Fortunately, the protein secretory mechanisms of protozoa are an area of active research. It has long been known that human protozoa secrete proteins to modify and enhance their environment. Malaria *Plasmodium*, for example, digests hemoglobin in the red cell in order to provide the amino acids necessary to reproduce. Secretory pathways are being studied in detail (for example, see Ref. 18). Tobin and Wirth reported that murine interferon- γ was secreted from *Leishmania in vitro* (14).

There are other suggestions that protozoa can manipulate the host through molecular trafficking. In a recent review article (19), *Toxoplasma gondii* infection of rats resulted in the reduction of the fear of cats. Rats are an intermediate host of *Toxoplasma* while cats are the final host. It appears that even in their benign cyst form (half of all people on Earth carry its cysts in their brains without visible effects) they are able to modify host behavior, in a detectable fashion, to optimize their ability to reach their final host. This also suggests a means to deliver proteins to the brain, therapeutically, without the need for major surgery or drug treatment to break down the blood-brain barrier.

LARGE-SCALE PRODUCTION

Some species of human protozoa can be grown *in vitro*. Standard microbial/mammalian cell culture growth conditions and equipment have been used. For example, in their insect form, *Leishmania major* can be cultured in

Medium 199 supplemented with fetal calf serum at 26°C. Human clinical trials have been performed with *Leishmania* (20). Other species, such as *Toxoplasma*, can only be cultured by growth inside of a host cell.

SAFETY ISSUES AND PROTOZOA

All therapeutic technologies need to be safe as well as efficacious. Fortunately, because of the long history of studying human protozoa, much can be said about the potential safety of symbiosis therapy with human protozoa. First, several species of human protozoa are known to be nonpathogenic. For the species, such as certain forms of *Leishmania*, that are mildly pathogenic, genetic controls have proven effective. For example, Titus *et al.* (21) tested a conditional auxotrophic mutant (*dhfr-ts⁻*) of *L. major* for pathogenicity in BALB/c mice. This null mutant was obtained by gene targeting and is marker free (22). Grimaldi *et al.* reported that this mutant line was unable to cause disease in both susceptible and immunodeficient mice (23). Mutant *Leishmania* organisms were found to persist for at least 3 months before decreasing to undetectable levels. This conditional mutant was further tested by Grimaldi *et al.* for safety in the Asian rhesus macaques (23). Following the subcutaneous injection of 10^8 organisms, they were found to persist for 3 months and the animals did not show signs of disease. It is extremely unlikely that these conditional auxotrophs can revert to wild type, because the entire gene is deleted from the organism. Additional levels of control on protozoa can be accomplished with the use of antimicrobials and suicide genes (24). It is therefore possible for the clinician to control or even to completely eliminate the therapeutic protozoa from the patient. Thus, symbiosis therapy with protozoa is potentially a fully reversible technology, unlike gene therapy.

One impediment to transplantation technologies is the possibility of transferring pathogenic viruses to the patient. No known human pathogenic viruses have their origins in protozoa and no protozoa act as a reservoir for human pathogenic viruses, presumably because protozoa are phylogenetically separated from humans by millions of years of evolution. Also, since protozoa are much more genetically stable than viruses, mutation to undesirable forms is much less likely to occur. Finally, protozoa are independently living organisms; they do not enter or alter the human genome as with gene therapy. Questions of activating oncogenes or altering the germ line are irrelevant to symbiosis therapy.

One final point relates to using protozoa in a more permanent treatment. While it has been proposed that auxotrophic mutants be used in the first phase of the technology, in the long term it would be desirable to have a single treatment last years or even decades in a patient. There is evidence reported in the literature (25) that species of *Leishmania*, for example, can have a life-long persistence in the human and mouse. Certainly long time periods between administration of therapeutic protozoa are potentially possible.

PROSPECTIVE USE OF SYMBIOSIS THERAPY IN A MODEL SYSTEM

As an example of how the biology of protozoa can quite closely match genetic disease physiology and pathology, the lysosomal storage diseases (LSDs) provide an ideal model system. This group of more than 40 diseases has a prevalence of 1 per 7700 births (26). These diseases result from a deficiency of either a lysosomal protein or proteins involved in lysosomal biogenesis. All LSDs result from either an inherited autosomal recessive gene or, in two cases, an X-linked recessive gene. The severity of the disease varies greatly from one individual to another. Disease results from the accumulation of normally degraded substrates within the lysosome. While all cells in the body have the LSD mutation, it is mostly a lack of activity in macrophage lysosomes that accounts for disease pathology.

Leishmania is the current leading candidate for symbiosis therapy in LSDs. Besides the safety benefits reported in the previous section, *Leishmania* species reside in the lysosomal compartment of macrophages. Thus, *Leishmania* specifically targets, enters, and lives in not only the correct cell type, but also the correct compartment within that cell type. This remarkable coincidence of biology is being taken advantage of for developing a new therapeutic technology. This technology can be tested in mouse LSD models since human *Leishmania* can survive in murine systems.

FUTURE POTENTIAL OF SYMBIOSIS THERAPY WITH PROTOZOA

Taking *Leishmania* as a model, it is possible to produce conditional auxotrophs of *Leishmania*, without markers, that could also incorporate suicide genes. This provides a redundant system for the elimination of the transgenic protozoa from the patient, if desired. *Leishmania* can manufacture foreign proteins including enzymes and hormones. This organism has had preliminary safety testing in mice and nonhuman primates and has been found to result in no apparent pathology in those cases. In addition, millions of humans have long-term *Leishmania* infections without chronic symptoms. LSDs appear to be the best disease group to target first because *Leishmania* live in the most desirable location for manufacturing and delivery of the therapeutic protein. Experiments to determine whether symbiosis therapy can work for LSDs are ongoing at this time in the author's company.

Given that there are thousands of genetic diseases in both humans and important animal species, a variety of therapeutic technologies will undoubtedly be needed to address all the possible types of defects. Symbiosis therapy with protozoa offers another approach that could

potentially be optimal for some of these diseases. Given that the human genome need not be altered, as with gene therapy, that new viral diseases are not likely to be transferred from protozoa, unlike xenotransplantation, and that large quantities of protozoa can be prepared, unlike stem cells, this technology may be the most optimal in some disease niches.

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