BIOTECHNOLOGY IN THE STUDY OF PROTOZOAN PARASITES

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ABSTRACT

Over the past decade extraordinary advances have been made in the study of protozoan parasites. Particular progress has occurred in areas such as cultivation of protozoan parasites, immunobiology of protozoan parasitic diseases, the biochemistry of protozoa and molecular genetics of these organisms. The application of sophisticated culture, monoclonal antibody and recombinant DNA technologies has resulted in elucidation of many of the biochemical and molecular bases of such phenomena as antigenic variation in African trypanosomas, the autoimmune basis of the pathogenesis of Chagas' disease, protective immunity in malaria and parasite evasion of host defense mechanisms. As a result a new generation of diagnostic procedures have to provide more accurate detection of protozoan infections and thus improved epidemiological information. Vigorous vaccine development efforts are underway which will lead to molecularly defined vaccines taylored to specific applications and will provide new weapons to combat protozoan diseases. Perhaps most importantly the molecular bases of host-parasite interactions are being established and will allow identification of unique biochemical aspects of the biology of protozoa thereby revealing appropriate targets for development of vaccines, accurate detection procedures and more efficacious chemotherapeutic agents.

INTRODUCTION

The term "biotechnology" is frequently used in today's literature yet appears to have no definition by consensus. Although a variety of meanings are apparent, common elements embodied in the term biotechnology seem to include several recently developed biochemical technologies which allow the identification, characterization and production of biological macromolecules. this suggests biotechnology could be broadly defined in the context of 4 groups of technologies: 1. cell culture, 2. nucleic acid technologies, 3. biochemistry (other than nucleic acids, especially protein/peptide biochemistry), and 4. hybridoma/monoclonal antibody (mAb) methods. Collectively these could be argued to represent the majority of technologies that come to mind when we hear the term biotechnology.

If we consider this collection of technologies, then what important issues regarding parasitic protozoa of animals are being, or can be addressed by biotechbology? In a general sense, a comprehensive issue being addressed is the molecular basis of the host-parasite relationship. What are the molecules of the host and parasite that participate in this

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interaction and what are the results of this interaction ? Frequently we approach this broad issue with the goal of intervention in this interaction, often in a very restricted way, in order to disrupt the host-parasite interactions that lead to disease in the host. Clearly the development of a vaccine is such an example. In other cases the new fundamental knowledge gained by studying a unique feature of a particular parasite-host combination is a driving force behind some of the investigations. The molecular genetics of antigenis variation in African trypanosomes has been an example of both phenomena.

With this introduction in mind I would like to offer some examples of the application of how one or more of these technologies has been applied effectively to general problems of current interest in the parasitology of parasitic protozoa of animals.

Antigenic Variation-Novel Gene Regulation and Expression

African trypanosomes are important parasites of domestic livestock in Africa and prevent the raising of livestock on much of the continent. Antigenic variation in African trypanosomes has been known for a long time and has recently been recognized in other parasitic protozoan species sich as *Giardia lamblia* as well.[1] The mechanisms of variation have been most extensively studied in African trypanosomes where protein biochemistry, hybridoma/mAb and recombinant DNA technologies have been applied extensively to this problem. Since the initial identification and partial analysis of the variant-specific surface glycoprotein (VSG) as the major variant antigen by Cross[2] a great deal of structural and genetic information about the VSG, the genes which code for VSG and their activation has been published[3,4,5] Employing a recombinant DNA approach Boothroyd et al [6] obtained the complete sequence of a complementary DNA (cDNA) coding for VSG 117. These workers demonstrated that the translation product had hydrophobic extensions on both the NH_2 and COOH ends and an unusual glycosylated aspartate on the COOH end.

One of the most striking features of African trypanosomes is the presence of genes for several VSG's with only one being expressed at a time[7], except for a very small percentage at the time of antigen switching[8]. This is because although multiple VSG genes are located on several chromosomes and in intrachromosomal as well as telometric sites, gene rearrangement mechanisms are necessary for non-telomeric genes to be expressed.[9] Thus in order for a VSG is accomplished for genes outside this site by duplicative transposition [10] to what is termed an expression-linked (extra) copy (ELC) gene. It is the ELC gene in the telometric site (transposed or already located here) which is then transcribed to give rise to the VSG molecule.[11]. Other structural genes for VSG's not transcribed. Such duplication (or lack of duplication) of basic copy genes to ELC was demonstrated by Southern blot (DNA-DNA hybridization) analysis of genomic trypanosome DNA's from clones expressing one VSG. These experiments showed that an extra copy a different size was present when the DNA was probed with a cDNA corresponding to the homologous, expressed VSG, but no extra copy was found when DNA was probed with a heterologous (non-expressed) VSG cDNA. Thus the ability to derive cDNA corresponding to the expressed VSG (ie, its mRNA) bya recombinant DNA methods, was essential to the establishment of this system to analyze the

molecular basis of VSG gene expression. The initiation of VSG gene transcription is not understood although recent evidence indicates that expression site-associated, non-VSG genes[12] and perhaps unusual RNA polymerases[13] may be involved, and this process is under active investigation.

A second type of *antigenic* variation has recently been observed in the lumen-dwelling protozoan, *Giardia lamblia*. Earlier work had shown that different isolates of *G. lamblia* displayed differences in antigenic profiles[14].

Employing an interesting nuclease from the mung bean which cleaves segments of genomic DNA of protozoans before and after genes[15], Adam and colleagues[1] prepared a genomic library of *G.lamblia* in gtll and selected clones expressing a 170.000 (170k) MW surface antigen using antisera specific for the 170k antigen. The DNa from this vector was subcloned into M13 phage and sequenced. This DNA hybridized in Northern blots to RNA from *G.lamblia* clones expressing the 170k antigen did not hybridize to clones lacking the 170k. antigen. Using the DNA sequence information these authors deduced a partial amino acid sequence for the 170k antigen and determined it contained 12% cysteine, confirmed by labeling the native 170k antigen with ^{35}S-cysteine. Employing different, additional *G.lamblia* clones and two mAbs specific for a 200k MW antigen and a 70k MW antigen, respectively, clones were selected from two isolates which expresed these antigens (or did not express them) by growth in the presence of these mAbs (ie, antigen deletion mutants)[16]. These results confirmed the variation in expression of two additional surface antigens of *G.lamblia*.

Pathogenesis of Parasitic Protozoa-Mechanisms

The mechanisms of pathogenesis of protozoan infections are not well understood and until recently presented an almost intractable problem. With the application of newly developed technologies to this problem, however, considerable progress is being made in understanding the molecular basis of how protozoan parasites damage their hosts.

The pathogenesis of Chagas' disease, a prevalent zoonotic disease in Central and South America caused by *Trypanosoma cruzi,* has long been thought to be partially due to autoimmune responses of the host triggered somehow by the parasite. The evidence has been that 1) mononuclear infiltration of cardiac muscle in the abscence of parasites 2) antibodies in the sera of patients which reacted with normal nerve and muscle tissue and 3) mononuclear infiltration and destruction of neuronal tissue in the absence of large numbers of parasites.

Two examples of anti-host antibodies induced by infection with *T.cruzi* are now well recognized: anti-laminin antibodies and antibodies which react with mammalian neurones. Early work with serum from patients with Chagas' disease showed that antibodies reacted with endocaria and vascular tissues[17]. Szarfman and colleagues later showed that murine laminin was the major antigen recognized by the antibodies[18] and recent data indicate a specificity for murine (but not human) laminin in convalescent human serum[19] suggesting a heterophile antibody response.

Autoimmune responses to neuronal tissues in patients with Chagas' disease have been known for some time[20]. Wood et al (1982) showed that a murine mAb elicited against rat dorsal root ganglia was toxic to

mammalian neurones in the presence of complement and reacted with viable amastigotes of *T.cruzi* but not a protective 90k MW antigen found in epimastigotes[21]. Recently antibodies against acetylcholinesterase have been detected in chagasic sera and a mAb specific for *T.cruzi* was shown to react with the neuromuscular junction of human skeletal muscle as well as acetylcholinesterase[22]. An anti-*T.vespertilionis* mAb, crossreactive with *T.cruzi*, has also been shown to react with sulfated lipids in mammalian brain and *T.cruzi*. These data add support to the autoimmune hypothesis of pathologic changes in cardiac and nervous tissue associated with chronic Chagas' disease.

Protozoan parasites which live primarily on secretory surfaces are in a somewhat unique niche and range from being highly pathogenic and invasive (eg, *Entamoeba histolytica)* to strictly surface-lumen dwelling (eg, *Giardia lamblia*). The mechanisms by which this important group of parasitic protozoans cause disease are only now being clearly established and continue to present unique challenges to parasitologists.

The gut protozoan *Entamoeba histolytica* has been long recognized to cause tissue damage locally and to be highly invasive in some instances. How *E. histolytica* causes such tissue damage, however, has only recently been addressed in a manner that the molecular basis of the pathogenic mechanism can begin to be understood.

The ability to culture *E. histolytica* [23] was crucial to obtaining sufficient quantities of each of these molecular species to perform biochemical analysis[24,25,26,27] and functional studies [28,29,30]. The use of biotechnology has now allowed a clearer understanding of the structural nature and function of these molecules. Three entities have been suggested as integral in the cytopathic mechanism of *E. histolytica:* a lectin involved in adhesion to mammalian cells, thiol proteases and a pore-forming molecule termed amoebapore[31]. The issue of adhesion molecules was addressed by Petri et al[27] who used mAbs raised against an N-acetyl-D-galactosamine-specific lectin of *E. histolytica* to affinity purify this lectin, raise additional mAbs against it, and purify sufficient quantities of the lectin to characterize the molecule biochemically. They showed that lectin was 260k MW molecule on non-reducing gels and was heterodimer consisting of a 170k and 35k[32]. Adherence of amebae to Chinese hamster ovary cells could be completely inhibited by mAbs directed against the 170k MW subunit thus establishing the functional role of this part of the molecule in adherence to host cells. Interestingly, mucin binds to this lectin and this binding is inhibited by N-acetyl-D-galactosamine an oligosaccharide moiety of mucin. In the presence of exogenous mucin adherence and cytotoxicity of *E. histolytica* against rat colonic epithelial cells could be inhibited[33].

Adhesion requirements are important in the parasite-host cell interaction between many other species of protozoan parasites including an 83kd MW molecule of trypomastigotes of *Tripanosoma cruzi* which binds to heart myoblasts (Lima and villalta 1980)[34], laminin-binding proteins in the membranes of *Trypanosoma vaginalis* and *Tritrichomonas foetus*[35], mucin-binding lectin in *Tritrichomonas mobilensis* (Demes et al, 1989. Parasitol Res in press; D.J. Wells per comm), and a modified erythrocyte membrane molecule, present in human erythrocytes infected with *Plasmodium falciparum,* which may play a role in infected erythrocyte adhesion to nucleated cells of blood vessels.[36]

The molecular basis of cytotoxity of parasitic protozoans is also being established by the application of biotechnological methods. With *E. histolytica* it was well-recognized that this parasite can be cytotoxic toward mammalian cells [37,38,39] and that cell-free extracts could mediate this effect[40]. More recently some of the molecules responsible for this cytotoxicity have been purified and biochemically characterized. Bos et al[41] demonstrated that a partially purified cytotoxin was enhanced by cysteine, inhibited by iodoacetate and antibody againts *E. histolytica* and secreted into the growth medium. In other studies Lushbaugh and colleagues purified a 16-20k Mw cytotoxic molecule which displayed properties of a cysteine proteinase by ion exchange and affinity chromatography. The proteinase was reversibly inhibitable by P-chloromercuribenzoate if free sulfhydryl groups were present and displayed cytotoxic activity in HeLa targets. In other studies Young and colleagues and Gitler and colleagues[25] purified a lytic factor from plasma membrane fractions or whole lysates (respectively) of *E. histolytica*. This toxin results in the formation of ion channels in target cell membranes and purified toxin molecules of 30k MW and 14k MW[42] were purified. Differences in function were reported for these two lytic molecules in that the 30k MW factr lysed rabbit erythrocytes while the 14k MW factor did not.

Protective Antigens-Malaria Vaccine Efforts

A critical development in this regard has been the development of culture procedures for *Plasmodium falciparum* and *Eimeria* spp.[43,44,45] which allowed growth of the erythrocytic and exoerythrocytic of host cells by sporozoites and subsequent development in vitro.

Early efforts demonstrated that the sporozoite antigens of *P. berghei* could protect mice from challenge with sporo zoites[46,47,48]. These results suggested antigens of the sporozoite could be part or perhaps the only component of a protective vaccine for malaria and stimulated an enormous amount of work to determine what the esential sporozoite antigens were for successful immunization. Later work in which mAbs were prepared against a single molecule of approximately 44,000 MW[48], the circumsporozoite (CS) antigen, showed that it contained the dominant epitope involved in blocking penetration of cells in vitro as well as passively protecting mice against challenge with *P. berghei* sporozoites. Subsequently the dominant epitope of the CS antigen molecule was found to be composed of 37 repeats of a tetrapeptide (ASN-ALA-ASN-PRO = NANP)[49,50]. This work was facilitated by the discovery by McCutchan et al[15] that mung bean nuclease cleaved genomic *Plasmodium* DNA before and after genes at sites of naked DNA allowing the excision of a complete genomic CS gene and its cloning from erythrocytic stages of *P.falciparum* derived from culture. Thus culture methods, hybridoma and recombinant DNA technologies allowed the determination of the primary structure of the CS antigen.

Since the key epitope was a short, repetitive sequence NANP, synthetic peptides were prepared and shown to elicit antibodies in mice and rabbits if linked to a protein carrier[51]. Subsequent work, however, showed these responses were restricted to certain major histocompatibility haplotypes in the mouse[52,53,54] and that variation in the T cell epitope of the natural CS protein led to a lack of anamnestic response due to a lack of

crossreactivity between CS proteins from different *Plasmodium* isolates[55]. These results raised serious questions as to the feasibility of a vaccine composed solely of CS antigen components since T cell memory is necessary. This question together with the need to alleviate the requirement of a heterologous protein carrier (eg, PPD, vaccinia virus) are currently the subject of vigorous research efforts.

Parallel investigations have been done on the erythrocytic stages of *Plasmodium* and have identified additional antigens with vaccine potential. Experiments employing serum from patients with *P. falciparum* infections have shown that their antibodies recognized an antigen of 150.000 MW on the surface of infected erythrocytes (from culture)[56] and inhibited merozoite reinvasion of erythrocytes. Subsequently the antigen, termed Pf155/RESA, was shown to also contain repetitive sequences comprising immunodominant human B cell epitopes and non-variant T helper cell epitopes[57,58]; reviewed in Troye-Blomberg et al, 1988[54]). A genomic library was used to obtain DNA segments coding for the C-terminal end of the Pf155/RESA antigen and the combinant polypeptide of PF. 11.1 was expressed (in gt11) and used to elicit antibodies. An additional antigen from a second, distinct (gene termed 332) has been similarly developed[59]. These workers have shown that when the recombinant polypeptides were used to affinity purify human antibodies from the sera of *P.falciparum* patients, the anti-PF 11.1 antibodies reacted with 7 antigens, while the anti-332 antibodies reacted with 5 antigens, of cultured schizonts of *P. falciparum*. By obtaining the predicted amino acid sequence of this antigen from the PF 11.1 DNA sequence synthetic peptides were prepared representing these sequenced and the peptide-specific antibodies reacted with native *P. falciparum* schizont antigens as well. These results suggest the Pf155/RESA antigen is a second molecule worthy of further development as a malaria vaccine component. They further indicate a multivalent vaccine is likely to be needed for effective prophylaxis against malaria.

Evasion of Host Defenses-Survival

Protozoan parasites have evolved many ways to evade the defenses of the host organism. They can evade natural defenses such as barriers like the skin, enzymes such as proteases of the mammalian digestive tract and the otherwise lethal products of the immune system.

One important way parasitic protozoa can evade destruction is to in a sheltered location such as inside a host cell. In order for intracellular protozoan parasites to survive and complete their life cycle they *must* spend part of this life cycle inside the host cell(s). Members of the Apicomplexa are good examples, notably *Plasmodium* and *Eimeria* species. Both genera produce sporozoites which invade nucleated host cells; *Plasmodium* targeting hepatocytes while *Eimeria* invades enterocytes. Thus the molecular basis of penetration is a crucial aspect of the biology of genera such as *Eimeria* and *Plasmodium*.

Hollingdale and colleagues have shown that mAbs directed against the CS antigen of sporozoites of *P. falciparum* and *P. vivax* prevented attachment of sporozoites to human hepatoma cells in vitro[60]). Further work indicated that anti-CS mAbs could hinder development of sporozoites that did manage to penetrate target cells[61] indicating the mAbs damaged the sporozoites in some manner. Similarly, when sporozoites of *Eimeria bovis* are treated with a mAb specific

for a 20,000 MW surface antigen, termed P20, these sporozoites are prevented from penetrating host cells in vitro or hindered in their development in vitro. if they manage to penetrate[63]. Employing mRNA-cDNA-expression vector strategies Jenkins and colleagues have produced merozoite- and sporozoite-derived antigens of *E. acervulina* which were reactive with sera from hyperimmunized rabbits[64] and a sporozoite antigen reactive with an anti-sporozoite mAb which identified a 22k MW surface antigen of sporozoites[65]. The recombinant antigen from sporozoites, MA1, was also able to stimulate avin T cells from chickens immune to *E. acervulina* in an in vitro proliferation assay[66]. These results indicate the marked effect of antibody on parasite stages which may not see antibody in an initial infection of a nonimmune host and give insight into a potential point at which the immune response could interrupt the life cycle. If, however, the sporozoites do avoid contact with the crucial antibody they would likely escape destruction in this intracellular location since neither hepatocytes nor enterocytes are known to have any effector function (cf. macrophages). In any event these results show that the antigenic and biological properties of *Plasmodium* and *Eimeria* spp. are being effectively studied at the molecular level and these investigations are rapidly providing insight into the immunology and cell biology of these parasites.

Another method of host defense evasion is attachment of host molecules to the surface of the parasite, a "cloaking" maneuver.

This allows the parasite to mask its own surface and to present to the host the host's own molecules thus being perceived by the host as self. Several parasitic protozoa have been shown to bind host molecules[66,67] or possess receptors for host molecules[32,68] (Demes et al 1989 *Parasitology Research* in press). While some of these interactions such as the attachment of fibronectin to a parasite ARG-GLY-ASP-SER sequence[22], laminin-binding receptors of trichomonads[68], and receptors in *E. histolytica* and trichomonads for mucin[32] (Demes et al *Parasitology Research* in press) must function in adhesion of the parasite to host surfaces and cells they could also allow the parasite to bind free host molucules (eg, plasma fibronectin dimers) thereby partially masking their surfaces from recognition by the host. Similarly suface dwelling parasites such as *Entamoeba, Trichomonas* and *Tritrichomonas* could bind molecules such as mucin to avoid recognition and attack by host products such as secreted proteases.

SUMMARY

Considerable effort is being directed at establishing the molecular basis of host-parasite interactions of protozoan parasites and their animal hosts. We have witnessed enormous progress in the past 10-15 years in our understanding of several of these parasitic relationship yet much remains to be done. The powerful collection of technologies we term biotechnology is rapidly changing as many procedures are being automated or replaced by faster, more powerful ones. For example the combination of the ability to perform automated oligonucleotide synthesis coupled with the recently developed thermocycling polymerase chain reaction synthesis of DNA (via Taq polymerase) employing olinucleotide primers now allows the amplification of nanogram quantities of DNA from native sources into microgram quantities overnight. This makes it possible to effectively study genes in low copy numbers, facilitates cloning of these genes and may allow develop-

ment of sensitive and rapid detection methods for parasitic infections.

Sequencing of polypeptides is now automated and automated DNA sequencing technology will soon be widely available. With these technologies we will soon be able to obtain the sequences of large areas of the genomes of parasitic protozoa which will allow us to establish the genetic basis of many of their life processes at the molecular level. Such knowledge will provide us with the tools to develop more effective diagnostic procedures and control measures such as chemically-defined vaccines and targeted chemotherapeutic agents.

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