Toxoplasma gondii: 25 years and 25 major advances for the field

John C. Boothroyd
Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford CA 94305-5124, USA

Abstract
This article is an attempt to identify the most significant highlights of Toxoplasma research over the last 25 years. It has been a period of enormous progress and the top 25 most significant advances, in the view of this author, are described. These range from the bench to the bedside and represent a tremendous body of work from countless investigators. And, having laid out so much that has been discovered, it is impossible not to also reflect on the challenges that lie ahead. These, too, are briefly discussed. Finally, while every effort has been made to view the field as a whole, the molecular biology background of the author almost certainly will have skewed the relative importance attached to past and future advances. Despite this, it is hoped that the reader will agree with, or at least not disagree too strongly with, most of the choices presented here.

Keywords
Toxoplasma gondii; Cell biology; Population biology; Molecular biology; Genetics; Diagnosis; Vaccines; Treatment

1. Introduction
The period 1983–2008 was one of the most productive for the field of biology and the study of Toxoplasma gondii was no exception. When asked to write on this quarter-century of advances, I considered various ways to approach the problem. I could survey my colleagues for their views of the most important papers. But whom to ask and how to prevent a skewing based on which areas are most heavily populated today …? Alternatively, I considered using an algorithm that combines citation frequency, impact factor of the journal and years elapsed since publication. But some papers get frequently cited because they report a simple method or popular strain or, worse, because they were wrong in their conclusions and lots of people have published work documenting the errors. In the end, I decided that rather than using a method that has the pretense of objectivity, I will go with a process that is transparently subjective – the discoveries and papers that I personally feel have had the greatest impact on our field. To make the task manageable, I have set a limit of 25 – an average of one per year – on the number of advances. Of course, many people could and I hope will make a compelling case for why something that was number 26 or 38 on my list should supplant number 6 or 22 but, in the end, choices had to be made and I hope all will agree that the majority of these 25 achievements do, indeed, represent a list of at least most of the key milestones in our field over the past quarter-century.
In making my list, I have tried to identify discoveries that have most changed our thinking in the various categories ranging from molecular biology to clinical practice. The charge to me was, fortunately, not an abstract one: I entered the field in 1983, the year that this review conveniently dates back to. I have tried hard to remember what I first read back then and consider how common wisdom has changed since that time.

So, let’s consider where things stood in 1983. First, the complete life cycle was known, although for only about 13 years. In or around 1970, several investigators discerned that the sexual cycle of the parasite occurs in felines which can shed many millions of oocysts in their feces. These are highly infectious to the parasite’s intermediate hosts which include almost any warm-blooded animal, from birds to humans to sea otters. Within each intermediate host, the parasite disseminates as the rapidly growing tachyzoite stage before eventually entering the persistent form, the bradyzoite. These are encysted within various tissues, most notably the brain, and are infectious to another intermediate host or the cat if eaten. This basic biology, as well as the ultrastructure of these different developmental stages, were well established by 1983.

Also by 1983, fairly good methods for diagnosis and treatment were available but this was a pivotal year for infectious diseases in general. The AIDS epidemic was just erupting and Toxoplasma had recently been recognized as one of the most important causes of CNS infection in such patients. At about this time, critically important protocols emerged for diagnosing and treating this new “opportunistic infection” (although many would argue that, in the context of toxoplasmosis, the term “opportunistic” is misleading since most cases are a result of reactivation of an infection that predates the acquisition of AIDS). Diagnosis had relied on serology and/or symptomatology but the AIDS epidemic put the imperative on diagnosing toxoplasmic encephalitis and major efforts were launched using modern imaging methods.

Imperfect but moderately effective drugs were available in 1983 but no commercial vaccine existed. Here, again, the emergence of AIDS was to have an enormous impact. One could even argue that the most important advance in human toxoplasmosis during the past 25 years was the development of highly active anti-retroviral therapy or HAART. This cocktail of drugs that attack HIV replication has allowed the HIV/AIDS epidemic to be controlled, at least in countries with the infrastructure and means to provide the drug to most patients. As a result, toxoplastic encephalitis has all but disappeared in HAART-treated patients, presumably because their immune systems are sufficiently strong that they can control any reactivation of the parasite. I haven’t included HAART in my “top 25” advances, however, because while there can be absolutely no argument about its enormous impact on human toxoplasmosis, it is not an advance directly related to the parasite, as such.

Perhaps not surprisingly, no genes had been cloned or sequenced by 1983 and no proteins identified other than by virtue of their reactivity to antibodies and/or existence as bands on a gel. Genetic crosses had been performed with great promise for the future but genetic maps had yet to be produced.

Twenty-five years later, the field of Toxoplasma research is almost unrecognizable. What follows is a brief synopsis of one person’s view of the most significant 25 achievements. They are unranked and instead are presented in crude order of general biology, cell and molecular biology of the parasite, a dissection of its interaction with the host and ending with the clinical arenas of prevention, diagnosis and treatment. Undoubtedly the list is skewed by the author’s own interests but every effort has been made to take a holistic view of the field.
2. Twenty-five advances

2.1. Toxoplasma has a remarkably clonal population structure

For a nearly ubiquitous infectious agent with a tremendously broad host range and a well-described sexual cycle, there have been some notable surprises about the population biology of Toxoplasma. First, and foremost, is that a few strains have come to dominate in some very broad niches (Darde et al., 1992; Sibley and Boothroyd, 1992b). Within Europe and North America, for example, most strains isolated from humans and domestic/farm animals are one of just three clonal types, dubbed types I, II and III (Howe and Sibley, 1995). Equally surprising, these three strains are closely related with types I and III appearing to be the products of a cross between type II and one or other of two strains, alpha and beta (Saeij et al., 2005a). The three super-successful genotypes have swept across two continents and clearly have some enormous advantage over other strains that co-exist but on nothing like the same scale (Su et al., 2003). Some of the genes that may be responsible for this selective advantage are just beginning to be identified as discussed further below.

In other regions of the world and even in other niches within Europe or North America (e.g., in the sea otters of California (Miller et al., 2004)), types I, II and III do not predominate although many strains have obvious common ancestry with one or other of the “big three” (Lehmann et al., 2006). As is so often the case, we have the least amount of information on strains from Africa but the data appear to indicate that South America may have been the birthplace of Toxoplasma: it has the greatest diversity of strains of any region yet examined (considering both human and non-human hosts) but then so too does it have the greatest ecological diversity which might be the force that produced and/or maintained this diversity (Lehmann et al., 2006).

A tempting scenario for today’s situation is that the enormous ecological disruption that accompanied European colonization of the Americas and Africa made possible matings between previously isolated strains of Toxoplasma (e.g., type II, alpha and beta). Among the progeny were two strains with enormous fitness for the new environments that emerged, like Norwegian roof rats or European house sparrows that became ubiquitous citizens of the world.

2.2. In addition to their obvious structural differences, the various developmental stages of Toxoplasma are antigenically and biochemically well suited to their respective roles in transmission

As mentioned further below, there are many antigenic differences between the acute stage of the infection (tachyzoites) and the chronic stage (encysted bradyzoites), especially on their surface (Ware and Kasper, 1987; Tomavo et al., 1991). Perhaps even more importantly for drug development, they appear to have significantly different metabolisms with bradyzoites being notable for their generation of amylpectin (Coppin et al., 2003). Gene expression studies have identified different isoforms of several enzymes typified by enolase (Dzierszinski et al., 2001) and lactate dehydrogenase (Yang and Parmley, 1995), each of which has a tachyzoite- and bradyzoite-specific form (Wan et al., 1997). Whether these different forms serve to drive the metabolism in different directions or simply to operate more efficiently in a different intracellular environment has yet to be established.

2.3. Differentiation between the two asexual forms, tachyzoites and bradyzoites, is a dynamic process that is partially driven by stresses that can be mimicked in vitro

Unfortunately, the amount of bradyzoite material that can be obtained from an infected animal is limited, making their study difficult. This situation was greatly improved when stimuli were found that would induce differentiation in vitro (Soete et al., 1993). Since the initial description that alkaline conditions would cause such a switch, many additional stresses have been noted.
to induce bradyzoite development suggesting a relatively non-specific trigger is involved. It has even been suggested that the stress of the immune response elicited by tachyzoites (e.g., nitric oxide generation (Bohne et al., 1994)) might be sufficient but it is hard to know if this is the biological inducer in vivo.

Mutants that fail to efficiently differentiate have been identified (Matrajt et al., 2002; Singh et al., 2002) but the actual signals and molecules involved in transducing the stimulus for differentiation to the nucleus remain a mystery.

2.4. The Toxoplasma genome can be readily manipulated in vitro

It is hardly surprising that, like virtually all other organisms that are the objects of intense study, Toxoplasma can be genetically manipulated. Nevertheless, this is included in this “top-25” list because of the enormous boost such methods have provided in terms of the sophistication (and precision) of questions that can be addressed. There is now a very large number of selective markers that can be used for stable transformation ranging from chloramphenicol acetyl transferase (Kim and Boothroyd, 1993; Kim et al., 1993) to the far more versatile hypoxanthine-guanine-phosphoribosyltransferase (Donald and Roos, 1993).

Using such markers, the genome of Toxoplasma can be manipulated to delete genes, even clusters of several tandem genes in one step (Saeij et al., 2008). This has made it possible to explore the function of genes through analysis of specific “knock-out” mutants. Equally important, genes can be introduced into the parasite’s genome allowing the development of methods for detection of parasites in vivo (Hitziger et al., 2005; Saeij et al., 2005b), for probing the immune response in model, transgenic mice (Pepper et al., 2004) and for exploring the role of allelic variation in key virulence genes (Saeij et al., 2006, 2007; Taylor et al., 2006). It has even allowed Toxoplasma to be explored as a possible piggy-back vaccine vector, exploiting the powerful immune response it elicits to produce immunity to other antigens from other, less manipulable or more dangerous infectious agents (Charest et al., 2000; Shirafuji et al., 2005).

2.5. The nuclear genome of Toxoplasma is comprised of 14 chromosomes totaling 65–70 Mbp

A combination of traditional genetic mapping, physical separation of chromosomes and large-scale genomic sequencing (Khan et al., 2005b) have revealed the nuclear genome of Toxoplasma to consist of 14 chromosomes ranging in size from about 2 to 7 mega-bp (Sibley and Boothroyd, 1992a). Extensive analysis confirmed the early genetic work showing that the asexual stages are haploid. Initial large scale sequencing efforts were led by David Sibley at Washington University where a very large set of expressed sequence tags (over 100,000) from tachyzoites, bradyzoites and sporozoites, from various strains, was generated (Wan et al., 1997). This was followed by a tremendous effort from the Institute for Genomic Research (TIGR) that yielded an essentially complete genome sequence from the GT1, ME49 and VEG strains (representing types I, II and III, respectively). This has been complemented by work at the Wellcome Trust Sanger Centre on chromosomes 1A and 1B (the smallest two chromosomes which are so similar in size they are numbered A and B) from the type I strain RH (Khan et al., 2006).

Obviously, having a mass of sequence information is only as useful as the format in which it is accessible and fortunately, here too the field has benefited from a tremendous effort from the laboratory of David Roos at the University of Pennsylvania. The product has been ToxoDB (www.ToxoDB.org), an intuitive and readily accessible database with extensive information on each predicted gene (Kissingger et al., 2003; Gajria et al., 2008). Among other useful information on this website are the expression levels for essentially all genes in different strains, polymorphisms in graphic format, mass spectrometry information on peptides detected in
various proteomic efforts and histone-code data showing where modified histones are enriched and thus where transcription start sites occur. The data accessible through ToxoDB are a result of enormous effort by many laboratories.

Twinning of this enormous set of data with similar information from Toxoplasma’s cousin, Neospora, allows for many informative comparisons to be made; e.g., which genes are unique to one or other genus, implying a special function in that genus’ biology versus genes that are conserved in both but not found in genera outside the phylum, suggesting a phylum-specific role (e.g., in invasion, as discussed further below). The Neospora sequence is the product of the Wellcome Trust Sanger Centre and Jonathan Wastling at the University of Liverpool. The sequence and annotation are also available at www.ToxoDB.org and/or www.EuPath.org.

2.6. Laboratory crosses can be used to map important phenotypes and genes

Pioneering results showing the sexual cycle occurs in felines and that this involves classical meiotic recombination have been greatly expanded using molecular tools. Thus, a genetic linkage map has been generated (Sibley et al., 1992; Khan et al., 2005b) and, given the existence of essentially complete genome sequences for the three most commonly studied strains, there are now markers available at saturating density across the genome. The creation of Affymetrix microarrays for Toxoplasma that encompass not only markers for virtually all known genes but also the ability to discriminate many of the known polymorphisms, will make mapping progeny from future crosses a relatively simple process. These arrays are the result of a major bioinformatic effort led by Amit Bahl and David Roos at the University of Pennsylvania whose laboratory designed the microarrays with input from the broader Toxoplasma community.

Types I, II and III strains differ in many interesting and important biological properties, not least of which is the course of the infections they produce in mice (and maybe other species – see below). The genetic basis of these phenotypes can be mapped in F1 progeny of crosses between types I and III or II and III, the two pairings so far examined (Saeij et al., 2006; Taylor et al., 2006). Using a candidate gene approach to make an educated guess about which gene in a given genetic interval might be responsible for a particular phenotypic difference, two loci have been specifically identified. Both are rhoptry proteins and, as described further below, allelic variants of these two proteins appear to interact with a given host animal in dramatically different ways.

In addition to the gross biological phenotypes of disease severity, in vitro methods can be used to look for molecular differences in how a host cell responds to the invaded parasite. Microarray technology has given the field a way to interrogate the entire transcriptome of the host cell to determine which host genes might respond differently to different strains of Toxoplasma (Blader et al., 2001; Chaussabel et al., 2003; Knight et al., 2006; Fouts and Boothroyd, 2007; Kim et al., 2007a). The results have revealed a startling number of strain-specific differences in host response (Saeij et al., 2007). Mapping these differences back onto the parasite genome has been done and one polymorphic parasite gene, ROP16, has so far been found to be a crucial driver of differences in the host transcriptional response. This is discussed in more detail below.

2.7. The multi-membranous body in the anterior of the parasite is a devolved plastid

There have been several big discoveries concerning the cell biology of Toxoplasma. One of the biggest was the finding that the variously named multi-membranous body in the apical region is a vestigial plastid with an apparent common ancestry with algal plastids (Fichera and Roos, 1997; Kohler et al., 1997). This was based on the sequencing of its genome, building on the earlier work of Borst (Borst et al., 1984) and Wilson (Gardner et al., 1991a; Gardner et al., 1991b). Based on all this and the fact that it is present in most Apicomplexan species, the body was dubbed the “Apicoplast”, a name that has held up well. Based on an inventory of its
contents, the vast majority of which are encoded in the nucleus, the function of this unusual organelle has been at least partially deduced: it appears to play a key role in a number of metabolic pathways, as discussed further below.

2.8. Micronemes secrete an important adhesin involved in gliding motility and invasion

Invasion moved from phenomenological description to a moderately detailed molecular understanding. Micronemes were found to be the secretory organelles that deliver key adhesins to the parasite surface, most notably MIC2 (Wan et al., 1997; Carruthers et al., 2000; Huynh et al., 2003; Huynh and Carruthers, 2006). This protein appears to be the linchpin connecting the parasite’s actin/myosin motors to the parasite surface and, perhaps, to the host cell surface, as well (Huynh and Carruthers, 2006; Huynh et al., 2003). The C-terminal, cytosolic domain of MIC2 is believed to be attached to aldolase that serves as the bridge to short actin filaments (Jewett and Sibley, 2003) which in turn can interact with an unusual myosin anchored to the inner membrane complex (Meissner et al., 2002). The result is a molecular motor that, so long as it can grab onto something firm outside the parasite, can propel the parasite forward.

2.9. The moving junction formed during invasion is a collaboration between microneme and rhoptry neck proteins

During invasion, there is a ring-like circle of contact between the parasite and host plasma membranes known as the moving junction (MJ). This has been long recognized in Toxoplasma and its cousins extending as far as Plasmodium. The micronemes also contribute a key component of the MJ, the so-called AMA1 ((Alexander et al., 2005) named for its orthologue in Plasmodium, which is an apical membrane antigen (Marshall et al., 1989)). Not surprisingly, the MJ includes several additional parasite proteins. Unexpectedly, however, these derive not from the micronemes but from the most apical portion of the rhoptries, known as the rhoptry necks (Alexander et al., 2005; Lebrun et al., 2005). For this reason, these proteins were dubbed RONs (Bradley et al., 2005). Thus, two secretory organelles collaborate to produce the MJ.

2.10. The surface of the different stages is dominated by stage-specific subsets of a family of glycophosphatidyl-inositol(GPI)-anchored proteins

The many surface antigens that had been noted by surface labeling have now been largely identified. These are almost all members of a family of proteins defined by the first to be described, p30 (Kasper et al., 1983) or SAG1 (for surface antigen 1 (Burg et al., 1988)). The SAG1-related sequences (SRS) proteins are encoded by over 100 genes (Jung et al., 2004). They are generally expressed in only one developmental stage such that the antigenic surface of a bradyzoite and tachyzoite are almost completely different (Kasper, 1989; Tomavo et al., 1991; Lekutis et al., 2000). This appears to allow bradyzoites to develop at the same time as a very potent immune response is eliminating tachyzoites (Kim et al., 2007b).

Within a given developmental stage, the relative abundance of the different SRS proteins varies enormously with some being far more abundant than others. (Nagel and Boothroyd, 1989; Tomavo et al., 1989). This suggests very different functions for each although what those specific functions are has yet to be determined. It appears that some may serve as adhesins for the initial attachment to a host cell (Mineo et al., 1993) while others may serve to attract the immune response, possibly deflecting that response away from other, more important members (Rachinel et al., 2004; Kim and Boothroyd, 2005).

2.11. Rhoptries and dense granules deliver membrane-associated proteins into the host cell

During invasion, tachyzoites release the contents of their rhoptries and dense granules. Much of this material ends up in the host cell, possibly associated with structures that have been
dubbed “evacuoles” (Hakansson et al., 2001). These mysterious structures are likely but not yet proved to be the membrane-limited, vesicle-like entities observed by electron microscopy that appear inside the host cell near the tip of the invading parasite (Nichols et al., 1983). Eventually their components are found associated with the parasitophorous vacuole.

One such protein, ROP2 (Beckers et al., 1994), has been suggested to be involved in the long-observed association of mitochondria with the parasitophorous vacuole membrane (Sinai and Joiner, 2001). While initial thinking was that this protein was an integral trans-membrane protein, more recent data suggests that it in fact associates with the membrane through other means since the putative trans-membrane domain is likely instead to be a buried, hydrophobic alpha-helix (El Hajj et al., 2006). ROP2 is the prototypic member of a large family of related proteins (El Hajj et al., 2006) that have emerged through proteomic, genomic and genetic studies. Two members of this family have recently been proven to be essential to the host-pathogen interaction as discussed further below.

Dense granules also somehow introduce their contents into the host cell where they can reach myriad destinations including the reticular network inside the parasitophorous vacuole (Dubremetz et al., 1993). These proteins frequently possess an unusual property whereby they can switch from behaving as a soluble protein to one that is tightly associated with membranes, possibly through the impact of local conditions on an amphipathic helix (Mercier et al., 1998a; Mercier et al., 1998b).

Intriguingly, at least one dense granule protein, GRA7, has been detected on the surface of the infected host cell (Neudeck et al., 2002). As yet, this is the only report of a *Toxoplasma* protein stably expressed on the surface of infected cells. GRA7 may also be involved in the unusual process by which cholesterol is delivered to the parasites (Coppens et al., 2006).

2.12. Rhoptries also introduce soluble proteins directly into the host cell

While probably everyone has long suspected it, evidence that rhoptries inject freely soluble proteins into the host cell, independent of the evacuoles, has emerged only recently. The most extreme cases involve two proteins that reach the host nucleus, a protein phosphatase (PP2c-hn; (Gilbert et al., 2007) and a protein kinase (ROP16; (Bradley et al., 2005; Saeij et al., 2007). The prediction, obviously, is that the presence of these proteins in the host cell will have major impacts on the infected cell. While this has been shown for ROP16 (the protein interferes with STAT3 and STAT6 signaling), the function of the protein phosphatase has yet to be determined. These two proteins are likely the first of what will ultimately prove to be a long list of soluble proteins injected into the host cell.

2.13. Secreted rhoptry proteins are highly polymorphic and different allelic versions yield very different host responses

Given their role in direct negotiations with the infected host cell, and the fact that different strains of *Toxoplasma* have probably co-evolved with different hosts, it is perhaps not surprising that the secreted rhoptry proteins are among the most variable proteins in the parasite. Presumably, each variant evolved to interface, maybe literally through protein-protein interactions, with a species-specific version of a host target. In the example above, one allelic flavor of ROP16 may have evolved to efficiently phosphorylate a mouse protein while another was optimized for a bird version of the same protein. Whatever the evolutionary explanation for their differences, it is clear that these allelic variants have a major effect on the dialogue between the parasite and host. Although its target is not known, introducing a different allele of ROP18 into a strain that is normally not virulent in mice causes it to become over four logs more virulent in this host species (Saeij et al., 2006; Taylor et al., 2006). ROP18 is another member of the ROP2 family but, unlike the soluble ROP16, it tightly associates with the
parasitophorous vacuole membrane (El Hajj et al., 2006, 2007). It is an active kinase but how it interacts with the host cell and what its ultimate function is are unknown. Its importance is clear not only from its effects on virulence but also on the growth rate of the parasites in vitro (El Hajj et al., 2006, 2007). Whatever their specific functions, these proteins are clearly under enormously powerful selective pressures that presumably serve to find the optimum that allows an infection to be established without overwhelming the host before transmission can occur.

2.14. Many biochemical pathways exist that are plant-like and absent from their mammalian hosts

The “metabolome” of Toxoplasma is still a work in progress but many studies have revealed some unusual metabolic pathways. Perhaps not surprisingly, given the presence of three distinct metabolic compartments (nuclear, mitochondrial and Apicoplast) with three distinct ancestries, there is a richness to the parasite’s overall metabolism with many pathways that are highly different from their hosts. Among the more unusual pathways are three that occur within the plant-like apicoplast. First, there is a ferredoxin-NADP(+) -reductase that reduces ferredoxin, a key redox mediator for probably several pathways (Thomsen-Zieger et al., 2003; Seeber et al., 2005). Lipoic acid, interestingly, can either be made in the plastid or scavenged from the host cell (Crawford et al., 2006). This contrasts with the situation in many eukaryotes where the mitochondrion is the site of lipoic acid synthesis. Type II fatty acid biosynthesis, another plant-like pathway, also occurs in the apicoplast (Waller et al., 1998). All of these are extremely exciting potential drug targets since all are very different from pathways in the mammalian host. Although hardly surprising, the crucial importance of the plastid is well evident from the fact that it is the target of the clinically effective drug, clindamycin (Fichera and Roos, 1997; Camps et al., 2002).

2.15. Multiple pathways exist for stimulating innate immune response pathways

Among the first cells to raise the alarm in many infections are dendritic cells that constantly sample the body for intruders. This is the case for Toxoplasma infections, as well (Sousa et al., 1997; Bourguin et al., 1998; Seguin and Kasper, 1999). These cells may be the first cells to produce the crucial cytokine (IL12) that then initiates the all-important early inflammatory response to Toxoplasma infections.

Toxoplasma is sensed as foreign by the innate arm of the immune system via several different interactions. The fact that toll like receptors (TLRs) are involved is evident by the extreme dependence on one of the central players in TLR signaling, MyD88 (Scanga et al., 2002). TLR2 (Mun et al., 2003) and TLR9 (Minns et al., 2006) are clearly key, although the ligands from the parasite that stimulate these receptors are not yet known. TLR11 is an unusual TLR (it is present in mice but nonfunctional in humans) and it appears to be an important receptor for the parasite (Yarovinsky et al., 2005). Interestingly, it recognizes a protein that is relatively conserved in evolution, the actin-binding molecule profilin. Since profilin is only known to be a soluble cytosolic protein, how it makes contact with a host TLR is unclear unless it is through dying parasites. This seems an unsatisfying solution since if the parasite is dying, the innate immune responses are likely already activated. The parasite’s heat shock protein 70 (HSP70) presents less of a topological challenge as a key stimulator of TLR4 (Aosai et al., 2006) since HSP70 molecules often pass through the secretory system of eukaryotic cells and can be detected on the surface where conceivably their chaperone functions might continue. Likewise, Toxoplasma cyclophilin is easily envisaged as a ready stimulator of the chemokine receptor 5 (CCR5) on dendritic cells (Aliberti et al., 2003) since cyclophilins are small, secreted proteins with known ability to interact with other proteins.
2.16. IL12 induction in turn leads to IFN-γ, a key mediator of the immune response to Toxoplasma

One of the key cytokines in the response to Toxoplasma is IFN-γ (Nathan et al., 1983; McCabe et al., 1984; Omata et al., 1984). One of its more unusual mechanisms of action may be its impairment of tryptophan metabolism in the host, resulting in starvation of the parasite for this essential amino acid (Pfefferkorn, 1984). The most important sources of IFN-γ during infection appear to be natural killer (NK1.1+) and T-cells responding to IL12 (Denkers et al., 1993; Gazzinelli et al., 1994; Khan et al., 1994).

2.17. Relatively few antigens are targeted by the immune response

The immune response to Toxoplasma infection in mice is initially focused on a very few antigens. In the case of the B-cell response, there are two abundant and highly immunodominant surface antigens SAG1 (Rachinel et al., 2004) and SAG2A (Prince et al., 1990) as well as several dense granule proteins (Cesbron-Delauw et al., 1989; Mevelec et al., 1992; Lecordier et al., 1993, 1995; Mercier et al., 1993; Fischer et al., 1998). These antigens appear highly immunogenic in humans, as well.

On the T-cell side, GRA6 has been shown to be the major antigen in at least one mouse model (Blanchard et al., 2008) while others have observed GRA4 and ROP7 to be key, also in mice (Frickel et al., 2008). The major human T-cell antigens have yet to be identified.

2.18. Major histocompatibility complex (MHC) haplotype is an important variable in disease outcome

Studies in both mice and humans have revealed an important role for the major histocompatibility complex (MHC) genes in how the host responds to Toxoplasma infection. Interestingly, the effect in humans is considerably more subtle than in mice where the “d” allele at the MHC class I “L” locus has been shown to be a key determinant in the host response (Suzuki et al., 1991; Brown et al., 1995). This has now been found to be because a particular parasite protein, the GRA6 mentioned above, is efficiently presented by Ld (Blanchard et al., 2008). Presumably, mice of a different haplotype are unable to present this key epitope, resulting in a very different immune response.

In humans, there may also be an impact of MHC type although available data suggest this is a relatively minor aspect of the variation seen in disease in humans. The possibility is, however, supported by studies with transgenic mice expressing human leukocyte antigen (HLA) genes (Brown et al., 1994). Direct evidence from epidemiological studies in people, however, have been less clear. The available evidence suggests that, at least in congenital infection, there is some impact of class II haplotype: DQ3 is associated with increased susceptibility (Mack et al., 1999) whereas class I genes show no detectable correlation (Meenken et al., 1995).

2.19. Apoptotic pathways of host cells infected with Toxoplasma are disrupted

Apoptosis is a key mechanism used to eliminate cells, including those that are infected with Toxoplasma (Liesenfeld et al., 1997). Not surprisingly, therefore, many infectious agents appear to have evolved mechanisms for blocking apoptosis. Toxoplasma appears to have several means by which such an effect is accomplished (Hisaeda et al., 1997; Nash et al., 1998; Goebel et al., 1999), among them blocks in caspase function (Goebel et al., 2001; Nishikawa et al., 2002; Payne et al., 2003). NFkB is a key transcription factor that plays a central role in induction of apoptosis and its expression is severely disrupted in cells infected by Toxoplasma (Haque et al., 1998; Caamano et al., 1999; Butcher et al., 2001; Kim et al., 2001). While there is yet to be consensus around the means by which this disruption is
accomplished, NFκB is clearly one of the most important players in the host response to infection and so any perturbation of its function will have major effects downstream.

2.20. Toxoplasmic encephalitis in AIDS patients can be discriminated from other diseases by non-invasive imaging techniques

Prior to 1983, toxoplasmic encephalitis was a very rare condition but all that changed with the AIDS pandemic. It quickly became clear that *Toxoplasma* was an important cause of CNS disease in AIDS patients and protocols were developed for discriminating this from other etiologies such as cancer. Among these computed tomography (CT) and magnetic resonance imaging (MRI) were especially important and allowed for much more reliable diagnosis and, hence, more timely treatment (Kupfer et al., 1990; Laissy et al., 1994).

2.21. PCR is a sensitive method for detecting *Toxoplasma*

PCR brought something of a revolution to the diagnosis of infectious agents in general; however, because of cost and technical difficulty, plus the total absence of circulating parasites and *Toxoplasma* DNA in many patients, PCR has seen limited use in diagnosing *Toxoplasma* infection. Nevertheless, it has been extremely useful in detecting the parasite in the environment and/or in situations where diagnosing an acute infection is important to the clinical management of the patient. For example, PCR has been used for detecting whether the parasite has crossed the placenta and infected the developing fetus (Grover et al., 1990; Savva et al., 1990) or is the etiologic agent in AIDS patients with encephalitis (Grover et al., 1990; Savva et al., 1990). PCR is now a part of the diagnostic armamentarium available to the clinician although it is of little value in situations where one wishes to know whether a patient has a history of infection (i.e., is chronically infected) because parasite DNA will not be present in readily obtainable samples (e.g., peripheral blood).

In addition to simply answering whether parasites are present in acute disease, PCR can be used to determine which strain of *Toxoplasma* is responsible for an infection (Fuentes et al., 2001; Grigg et al., 2001; Khan et al., 2005a). As discussed further below, this may give the needed resolution to establish significant correlations between *Toxoplasma* infection and disease outcome.

2.22. Serology can be used to distinguish acute/chronic infection and maybe which strain of parasite is involved

In many instances, it is important to know whether an infection has been recently acquired or is a chronic infection that has been persisting for years. Clinically, this difference has a dramatic impact on the prognosis, especially for the developing fetus. Hence, tests that distinguish the two states with minimal invasiveness are an invaluable clinical tool. In most cases, it appears that a two-step process works best, consisting of an analysis of IgM titers followed by IgG avidity tests (Roberts et al., 2001).

Following up on the possibility that different strains of *Toxoplasma* produce different types and severity of disease in humans, there is a need for non-invasive means for distinguishing the type of strain responsible for an infection. Detecting the parasite by PCR is definitive but acquiring parasite material from the patient is difficult in all but the most acute cases. Instead, serological means have been attempted where strain-specific peptides are used to look for the presence of specific antibodies (Kong et al., 2003; Peyron et al., 2006). While still very rudimentary, this approach has been shown to be workable in a very limited study of humans where the strain type was known.
2.23. Organellar functions are an important source of new drug targets

Drug therapy for toxoplasmosis has long centered on inhibitors of parasite nucleotide metabolism, specifically the drugs pyrimethamine and sulfamethoxazole which inhibit dihydrofolate reductase and dihydropteroate synthase, respectively. One could argue that one of the most important advances in treating toxoplasmosis was the finding that combination therapy of trimethoprim and sulfamethoxazole used to treat Pneumocystis pneumonia in AIDS patients, also has a significant impact on *Toxoplasma* (Zangerle and Allerberger, 1991). While this certainly has saved many lives, it was not entirely unexpected as many studies had been performed previously showing significant efficacy of this pairing in treating toxoplasmosis.

Since 1983, however, several new drugs have been identified as having therapeutic potential in treatment of toxoplasmosis and these disproportionately interfere with organellar functions. Atovaquone is a ubiquinone analogue that was originally developed for treatment of malaria but has shown efficacy against *Toxoplasma* in limited human trials (Kovacs, 1992). This drug targets mitochondrial electron-transport, specifically cytochrome b, in both *Toxoplasma* (McFadden et al., 2000) and *Plasmodium* (Srivastava et al., 1999). Clindamycin is a well-studied antibiotic that inhibits prokaryotic translation machinery and has proven effective as a second-line drug against *Toxoplasma*. This macrolide targets ribosomes in the plastid (Fichera and Roos, 1997; Camps et al., 2002). Interestingly, the effect of the drug is delayed such that parasite growth is impeded only after several divisions (Pfefferkorn et al., 1992). This may be due to its effect being dependent on the complete loss of plastid DNA which, given its relatively high copy number to start (~8 identical DNA molecules/plastid), takes several parasite divisions to occur (Fichera and Roos, 1997).

2.24. Attenuated parasites make good vaccine prospects

Candidate strains for vaccines have been generated by several different means. The approach that yielded a commercially viable strain used serial passage through mice. This produced strain S48 that appears to have retained full infectivity for the acute stages but has lost the ability to differentiate into bradyzoites and thus is cleared after the acute phase of the infection (Buxton et al., 1991). This strain can be used to acutely infect an ewe before it is pregnant such that when it does mate with a ram, the fetus is protected by the maternal immune response. Fortunately, acute infection in adult sheep is not particularly severe.

Temperature-sensitive mutants have also been generated, using random chemical mutagenesis and screening (Waldeland et al., 1983). These are attenuated for growth in animals and have been explored as a potential vaccine but not yet to the point of commercial development (Waldeland et al., 1983; McLeod et al., 1988). More recently, specific genes have been targeted for deletion. The resulting auxotrophy is of course non-reverting, an enormous benefit over the other two approaches where reversion is always a possibility. The most dramatic success with the specifically engineered approach has been deletion of the carbamate phosphate synthetase II, an essential enzyme in the synthesis of pyrimidines (Fox and Bzik, 2002). The engineered parasites grow in vitro if uracil is provided in excess but they are severely crippled and unable to cause significant disease in vivo, where this nucleotide is not so abundant. The result is a strong protection from subsequent infection.

2.25. Host behavior may be altered by Toxoplasma infection

The possibility that infection with *Toxoplasma* changes the behavior of its host to increase transmission has long been recognized (Hay et al., 1984; Webster et al., 1994). Evidence expanding on this phenomenon has now been obtained in laboratory experiments where rats that are chronically infected with *Toxoplasma* lose their innate fear of cats, making them more likely to become prey (Berdoy et al., 2000; Vyas et al., 2007). Since cats are potentially a huge amplifier of the parasite (one cat can shed over $10^8$ oocysts into the environment), making an
intermediate host more likely to fall prey to a hunting cat would have considerable selective advantage to the parasite. But proving that this is the reason for this behavioral change is impossible and instead we must satisfy ourselves with argument based on circumstantial evidence. One piece of supporting evidence is that the behavioral effects are highly specific: the innate fear of canines is not affected and neither are other “hard-wired” or learned fears (Vyas et al., 2007).

In humans, there have been several reports of correlations between being sero-positive for *Toxoplasma* and certain behaviors (e.g., risk of being in a car accident (Flegr et al., 2002)) or psychiatric conditions (e.g., schizophrenia ((Destounis, 1966; Derouin et al., 2002; Hinze-Selch et al., 2007)), some dating back many years. Unfortunately, teasing out all other possible confounders will be an enormous challenge. For example, does a predisposition to risky behavior result in the increased probability of both reckless driving and reckless eating (leading to more *Toxoplasma* infection)? Even prospective studies that follow large cohorts to look for seroconversion versus development of psychiatric conditions will likely not yield definitive answers. Ethical consideration will require notifying those who seroconvert of this fact and this information could trigger a psychiatric/behavioral change.

3. And what are some of the challenges for the next 25 years?

Having laid out a remarkable list of successes in the last quarter-century, there remain a tremendous number of questions to be answered. Some of these have barely been the subject of even a handful of papers. Others have attracted huge effort and produced many leads but with a sense that only the surface of the problem has been scratched. Talented minds and adept hands are needed aplenty. What follows then is a list of some important goals for the coming years.

3.1. Determine the relative contribution of tissue cysts versus oocysts in human infection

Should we be more focused on oocysts or tissue cysts in public health programs? Is the guidance to pregnant women to avoid “kitty litter” a distraction from what might be the true source of most human infection (under-cooked meat)? We need a test that determines whether an infection originated with sporozoites (oocysts) or bradyzoites (tissue cysts). In theory, it might be possible to detect an immune signature (antibodies?) that differs depending on which stage initiated the infection but this is a tall order: the amount of oocyst/sporozoites antigen may not be enough to leave an immune signature and it will be very difficult to know whether an anti-bradyzoite response is from the initial inoculum or redifferentiation subsequent to invasion.

3.2. Invent accurate methods for easily determining the strain responsible for an infection

Before the question of whether strain type determines disease outcome can be fully addressed in humans, sensitive, reliable methods for determining the parasite’s genotype are needed. Unfortunately, obtaining sufficient parasite material to do this is extremely difficult unless the infection is in the acute stage, even with PCR. New methods are needed that are non-invasive, ideally using only a small blood sample. Doing this for infections that have long since entered the chronic phase represents a particular challenge. While there have been some promising beginnings, we are a long way from having a practical and reliable test for this.

3.3. Assess the importance of strain type on the severity and sequelae of Toxoplasma infection in humans

Once the methods described become available, it will be possible to address long-standing questions with much greater precision. Might the reason only a minority of women who become infected during the first trimester pass the parasite on to the fetus be because of strain type? Might the fact that ocular toxoplasmosis is more common and more severe in certain regions...
(e.g., Brazil) be because of strain type? The latter especially looks highly likely but has yet to be proved. And what of the tantalizing connection between *Toxoplasma* infection and other chronic diseases such as schizophrenia or even multiple sclerosis (Kaeser et al., 1977)? If we could determine which strain was infecting in each instance, stronger correlations might emerge.

Similarly, as the “hygiene hypothesis” gathers momentum (i.e., that being too clean has associated costs in terms of autoimmune diseases and the like), it could be that infection with some strains of *Toxoplasma* might even be a good thing. Again, only by being able to discriminate strains underlying infections in large numbers of people, might such correlations emerge.

**3.4. Determine what drove the emergence of the most successful clonal lines**

What combination of genes is behind the success of the types I, II and III lineages? Which lineages are on the ascendency and which should we be looking out for as the next wave? What were the natural hosts of these strains that provided the ecological niche in which they could flourish?

**3.5. Determine whether vaccination of humans is feasible and, if so, develop such a product**

At the recent congress held in Buzio, Brazil, that celebrated the centennial of *Toxoplasma*’s discovery in 1908, there was much debate about whether a human vaccine was feasible. Is the problem large enough to justify the cost? What about the risk associated with all vaccines? Who would be the target population; soon-to-be pregnant women, the immunocompromised or all people in regions, like Brazil, where infection of otherwise healthy adults can be severe? Pregnant women are among the most difficult patient groups in terms of the possibility of unintended adverse effects on the very delicate fetus growing inside. How would the efficacy of such a vaccine be tested? This is an enormously difficult problem since challenge studies would obviously not be ethical unless drugs were available that would provide a sterile cure for the infection (see below).

**3.6. Develop a vaccine for food animals and/or cats with the goal of preventing transmission to humans**

Instead of vaccinating humans directly, there is obviously an alternative and maybe simpler route to preventing human disease for any zoonosis: vaccinate the animals that are responsible for transmission to humans. As discussed above, the feasibility of such an approach has already been demonstrated for the commercial sheep vaccine based on the S48 strain. The challenge now is to adopt this approach for preventing tissue cysts from forming in the intermediate hosts, not just preventing problems in the developing fetus of those hosts. Perhaps a vaccine can be developed that provides a double benefit; it protects against abortion in the animal immunized and the humans who eventually eat tissue from that animal.

Immunizing cats is another attractive target but presents its own challenges – cats roam widely so immunizing your own cat may not be of much benefit unless other cats in the neighborhood are also immunized. At the least, however, such a vaccine might provide peace of mind and make it unnecessary to get rid of a valued pet when someone becomes pregnant or HIV-infected.

**3.7. Exploit the many new drug targets for development of better drugs, especially ones that will eliminate even the bradyzoite form**

We have only recently begun to understand the details of *Toxoplasma* physiology and the many unique targets that might be exploited for drug development. The apicoplast stands out as a
particularly rich collection of targets and metabolic pathways not found in humans. Developing new drugs is extremely costly, of course, and the worldwide magnitude of toxoplasmosis is generally not on a scale that will motivate pharmaceutical companies to invest the necessary resources. But the incentive does exist for Plasmodium and Eimeria and so Toxoplasma may be the test-bed for early drug-discovery and the field may reap some return when those drugs are shown to be effective in treating toxoplasmosis as well as malaria and coccidiosis.

3.8. Invent non-invasive means for determining whether infection has reached the fetus and, if so, what disease will result

Knowing whether Toxoplasma has reached the fetus is a crucial piece of information necessary in the decision-making process of how aggressively to treat, including the ultimate decision of whether to abort. Current methods involve indirect methods and/or amniocentesis with its own associated risks. Methods are needed to make this determination safely and accurately.

3.9. Determine whether behavioral changes in the host are of selective advantage to the parasite

It’s now clear that there are very specific behavioral changes in the infected intermediate host but are these of adaptive value or are they irrelevant and simply the result of looking very hard with very sensitive methods?

3.10. Discover the mechanisms that operate to produce those behavioral changes

How does Toxoplasma induce behavioral changes? Does it target specific cells? Does it release key molecules that affect the function of the CNS? Of course, determining the mechanisms involved will be dependent on advances in neurobiology that provide a solid understanding of these behaviors in normal, uninfected individuals. Ideally, we need a mutant parasite that doesn’t induce the changes; can Neospora help to dissect this phenomenon?

3.11. Identify the initial receptor recognized on a host cell to be invaded

What does Toxoplasma bind to on the surface of the cell and how does this transmit the signal to invade? It is presumably something generic and current data point to proteoglycans as a prime candidate but this point is far from proven. And what molecule on the parasite binds this host molecule? Is it one or more of the SRS surface antigens? They appear to have a receptor-binding groove that would accommodate a proteoglycan or another extended, polyanionic molecule but such an interaction has yet to be demonstrated as biologically relevant.

3.12. Identify what provides the “purchase” for the parasite during invasion

After the signal for invasion has been received and the actual invasion machinery deployed, how does the parasite grasp the host cell with the necessary “purchase” to pull itself in? The host molecules again must be nearly ubiquitous across vertebrates and highly conserved in structure. They cannot be free-floating in the host plasma membrane, for to pull on such molecules would only cause those molecules to move, not the parasite. The possibility that the key interaction between parasite and host molecules is occurring inside rather than on the surface of the host cell is an intriguing possibility to be tested.

3.13. Determine the trafficking signatures carried by secretory proteins that cause them to be delivered to the right compartment

Rhoptries, dense granules and micronemes each have distinct sets of proteins. What signature do these proteins have in common or is it just timing of expression? What causes some proteins to be targeted to discrete subcompartments like the rhoptry necks versus bulbs?
3.14. Identify the means by which rhoptry proteins are transferred across three membranes (rhoptry, parasite plasma membrane and host plasma membrane)

How do the rhoptry and dense granule proteins that enter the host cell get delivered? This is an intriguing topological problem and the solution may well involve a system as complex and elegant as those that appear to have evolved for this purpose in bacteria (e.g., the syringe like structure of type III secretion systems). Do different mechanisms operate for the proteins that are injected in soluble form (like ROP16 and PP2c-hn) versus ones that enter in association with vacuoles and end up associated with the parasitophorous vacuole?

3.15. Find the signals that cause differentiation from one developmental form to another

Many stimuli are known that will cause tachyzoites to differentiate to bradyzoites in vitro but which, if any, of these is a natural stimulus? And does reactivation occur spontaneously or can the parasite sense the time is right to return to the tachyzoite stage (and, if so, what useful purpose does such reactivation serve in immune-competent animals)?

3.16. Determine the nature of the cross-talk between intracellular stages and the host cell, especially the difficult-to-study bradyzoites

Much has recently been learned about the dialogue between tachyzoites and the infected host cell but clearly only the tip of the iceberg has been seen. And what of bradyzoites which must establish a far more subtle and balanced relationship with the host cell, assuming they are indeed intracellular? Is the parasite totally invisible to the host cell or does a state of détente exist?

3.17. Find the form of the parasite that disseminates through the body and, especially, that crosses the blood-brain barrier

Are the infectious forms of Toxoplasma that circulate through the body free, extracellular parasites or is the “Trojan horse” model correct, i.e., are the parasites (tachyzoites) transported inside cells that naturally migrate, including many immune cells that may be perversely commandeered for this role?

3.18. Understand the sexual cycle including how mating occurs in the feline intestine

Very little has been done concerning the sexual stages since they can only be studied in vivo in cats, at least currently. Major questions abound here: how and where does fertilization of the macrogamete by a microgamete occur? What launches the parasite into the developmental pathways leading to sexual differentiation? How and when does meiosis really occur? What drives Toxoplasma to the “2 × 4” mix of two sporocysts containing four sporozoites each (compared with the other permutations seen with many of its coccidian cousins)?

3.19. Develop methods for crossing strains in vitro or at least in animals other than cats

Classical genetics is still a powerful tool but requires the parasites to be “crossed” in cats. This is expensive, logistically involved, risky (the oocysts are highly infectious) and uses animals many prefer not to use for experiments. Finding a method to do this in vitro or perhaps in mice would be a huge benefit experimentally. It would simultaneously reveal much about what makes felines the exclusive definitive host (or are they really such for all strains of Toxoplasma)?

4. Conclusion

From the above, I believe it is clear that while the last 25 years have seen enormous advances in our understanding of Toxoplasma and its interaction with its host, very little of that knowledge has translated to improvements in the clinical care of patients and infected animals.
Continuing to discover the intricate biology of the host-pathogen interaction will undoubtedly bring many more exciting discoveries; the greater technical and intellectual challenge, however, may be how to take advantage of these advances to improve health and that will require a closer collaboration between physician and scientist than has traditionally been the case.

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