C1 - SENSE AND SENSITIVITY: THE ROLE OF SALIVA IN BLOOD SUCKING BY ARTHROPODS, OR WHY THEY SPIT ON US?

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We know for more than 80 years that saliva from blood sucking arthropods contain anti-clotting substances [1]. However their role in feeding was not clear. Indeed, in 1949 De Meillon [2] claimed that saliva of mosquitoes could be a vestigial organ from plant feeding ancestors, because the allergic reactions it provoked in the host were thought to be disruptive to blood feeding. Supporting this line of reasoning, Rossignol and Spielman [3] concluded that mosquito saliva played no role in blood feeding. However, other studies on salivary duct ablation in mosquitoes, or removal of the salivary glands of kissing bugs, indicated that saliva of blood sucking arthropods could play a positive role in blood feeding, more specifically by shortening the probing phase, where the skin is being cut and suitable vessels are being searched for a meal [4-7].

In the past 20 years we have learned a lot from those little creatures that most of us want extinct for their role in both annoying us and transmitting some of the most devastating diseases to humans and animals. In addition to anti-clotting agents, anti-platelet and vasodilatory substances were discovered in the saliva or salivary glands of these invertebrates [8,9]. Host hemostasis is a very complex and redundant physiological process. Blood sucking arthropods have found that against a redundant target it is best to fight using a magic potion, not magic bullets. Accordingly, for each insect studied in some detail, an incredibly varied combination of pharmacological reagents was discovered. For example, the bug *Rhodnius prolixus* has a novel anti-clotting agent that prevents activation of the clotting Xase complex [10,11], a salivary apyrase to prevent platelet aggregation, [12], and nitric oxide as vasodilator [13,14]. The highly reactive and unstable gas nitric oxide was stored and stabilized within the salivary glands by a novel class of hemeproteins, the nitrophorins, which have been cloned [15], and crystalyzed [16,17]. Additionally, a number of other anti-platelet compounds derived from the lipocalin gene family have been found in *Triatoma* [18], and in *Rhodnius* (Champagne, Anderson and Ribeiro, unpublished). Similarly, black flies have a sophisticated cocktail of anticlotting reagents, inhibiting thrombin, activated factor X and factor V [19-21], apyrase activity [22], and a novel vasodilatory peptide [23], (Cupp et al. 1998, in press). The bed bug *Cimex lectularius*' salivary cocktail includes a novel factor X inhibitor [24], an apyrase that belongs to a novel class of this enzyme (Valenzuela et al. *J Biol Chem*, accepted for publication), and nitric oxide [25] carried by a nitrophorin that has no relationship to *Rhodnius* nitrophorin. The mosquito *Aedes aegypti*, on the other hand, has an apyrase that belongs to the 5'-nucleotidase gene family [26], an anti Xa belonging to the serpin gene family [27] (Stark & James 1998 *J Biol Chem*, in press), and vasodilatory tachykinins [28]. The mosquito *An. albimanus*, who shared a common ancestor with *Aedes* about 150 million years ago (to place things in perspective, we shared a common ancestor with whales 110 million years ago…), has a salivary peroxidase as a vasodilator [29] which shares homology to human myeloperoxidase (Ribeiro & Valenzuela, unpublished). *Anopheles albimanus* salivary anticlotting bears no relationship to *Aedes* salivary serpin, being an anti-thrombin of novel primary sequence (Valenzuela, Francischetti & Ribeiro, unpublished). I hope these examples will help us to realize that we should not condemn these insects to extinction, at least not before listening carefully to what they know about us.

If these arthropods are so smart, why in their pharmacological wisdom they allow us to develop skin reactions, both of immediate and delayed type hypersensitivity, that we believe is bad for us and also bad for the insect? Perhaps our beliefs are wrong and the insects may benefit from our skin reactions. We are presently studying the induction of delayed type hypersensitivity by the sand fly *Phlebotomus papatasi*, and we find that flies feeding at DTH sites feed twice as fast as flies feeding in normal skin sites. Using laser doppler fluxometry, we found, not surprisingly, that human DTH sites have 3-5 times more blood flow than normal skin sites. Flies that feed, at least on average, on the same host within a few days' interval, may 'purposefully' induce a skin DTH response on their hosts in order to have a fast food service. Indeed, when saliva is HPLC fractionated, we observe DTH inducing activity in only one fraction, indicating it to be a specific inducer of such reaction (Belkaid, Valenzuela, Rolton, Sacks & Ribeiro, unpublished). We cloncude it may be a very sensible thing for some insects to induce hypersensitive reactions on their hosts.

In addition to interfering with our hemostatic system, blood sucking arthropods also play with our immune system to their advantage, and to the advantage of the parasites they transmit. Evidence is accumulating for a role of saliva in the transmission of vector borne pathogens. Since our initial work on the subject [30], vector salivary components have being implicated to be of fundamental importance to transmission of viruses by ticks [31, 32], and by mosquitoes [33] . Using a mouse model of transmission of *Leishmania major* and *P. papatasi*, we observed that saliva had a significant enhancement of the infection in naïve mice. However, an aborpted infection followed co-injection of *Leishmania* parasites with salivary homogenate when mice were previously exposed to salivary homogenates or by uninfected sand fly bites. This aborpted infection was of less severity than infection caused by injection of *Leishmania* alone (Belkaid, Valenzuela, Ribeiro and Sacks, J. Exp. Med., in press). Immunity to sand fly saliva may be an important component of the epidemiology and natural hystory of leishmaniasis. Search for

immunemodulators in arthropod saliva, particularly saliva of ticks (which feed on their hosts for several days) may yield an array of substances that will be even more impressive than the anti-hemostatic components.

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C2 - NATURAL MODELS OF CUTANEOUS LEISHMANIASIS: EFFECTS OF LOW DOSE, SAND FLY SALIVA, AND SALIVA PRE-EXPOSURE ON *LEISHMANIA MAJOR* INFECTION IN THE MOUSE EAR DERMIS

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In attempts to mimic the biology of natural transmission, we have established a model of cutaneous leishmaniasis in the mouse that includes inoculation of low numbers of *Leishmania major* metacyclic promastigotes plus salivary gland lysates from a natural vector, *Phlebotomus. papatasi*, into the mouse ear dermis. Blood sucking arthropods, including infected sand flies capable of egesting *Leishmania*, will salivate into the skin in order to locate blood and maintain its flow during ingestion (1). The importance of taking into account this aspect of natural transmission was first addressed by Titus and Ribeiro (2), who showed that in addition to its powerful anti-hemostatic activities, sand fly saliva will enhance the infectivity of *Leishmania* in a sub-cutaneous site of infection, the mouse footpad. The rationale for extending these observations to the skin is that in addition to being the tissue environment in which these encounters normally occur, the skin is comprised of specialized cells (DETC, Langerhans cells, keratinocytes) that may be better adapted to respond to these exposures in a host protective rather disease promoting manner. We found, on the contrary, that the ability of *P. papatasi* saliva to exacerbate *L. major* infection is especially pronounced in the skin. The lesions progressed faster, to a larger size, and were associated with far more tissue destruction. The effect in C57Bl/6 mice was especially dramatic in that a large proportion of the dermal lesions failed to heal for up to eight months after infection. Thus the presence of saliva in a dermal site of infection converted B/6 mice into a non-healing phenotype, something that was not observed when infections were initiated in the footpad.

By using recently developed methods that permit recovery of cells from the inflammatory ear dermis (3), and by using for the first time intracellular staining and flow cytometry to analyze cytokine production by epidermal cells at the single cell level, a consistent pattern of host response to sand fly saliva has emerged. Flow cytometric analyses of epidermal cells stained for intracellular cytokines in response to the tissue injury associated with needle injection of PBS revealed a significant increase in the frequency of cells producing IL-3, IL-5, TNFa IFNd, and MCP-1. The extremely high frequency of cells staining for TNFa is consistent with the ability of keratinocytes to produce this cytokine in response to different epidermal exposures, including barrier disruption, burns, UV irradiation, and TNFa itself (4). The inclusion of saliva in the low dose inoculum elicited a consistent increase in the frequency of IL-4, IL-5, and in the case of BALB/c mice, GM-CSF producing cells. The kinetics of the type 2 cytokine response in the epidermis (6 hr) suggests that components in sand fly saliva are able to activate innate responses by dendritic epidermal T cells, which in the mouse skin bear primarily the a form of the antigen receptor (5). Direct evidence that IL-4 elicited by saliva was at least partially responsible for the enhancement of infection was provided by the studies in IL-4 deficient BALB/c and B/6 mice, in which saliva failed in each case to significantly alter the severity of the dermal lesions.

We have used this model of natural infection to introduce an additional variable into the interplay between vector, parasite, and host. Since in endemic areas it is likely that individuals at risk of exposure to infected sand flies will have prior exposure to the bites of uninfected flies, we investigated whether pre-exposure to vector saliva via intradermal inoculation of saliva could modify the course of *L. major* infection in mice challenged with parasites plus saliva. In both BALB/c and C57Bl/6 mice, pre-exposure to salivary lysate completely abrogated the exacerbative effect of saliva on the development of the dermal lesions. Two intradermal injections of salivary gland sonicate elicited both anti-saliva antibodies and DTH responses in these mice. We were able to formally demonstrate a role for the anti-saliva antibodies in the reversal of the salivary effect since pre-incubation of saliva with serum IgG from sensitized animals completely neutralized its ability to enhance infection in both mouse strains. In addition, the anti-saliva antibodies neutralized the ability of saliva to elicit early type 2 responses in the epidermis.

Despite the fact that *Leishmania* parasites are transmitted exclusively by the bite of an infected sand fly, there are few reports of successful transmission to experimental animals by bite, and virtually none have explored the host inflammatory and immune response to sand fly initiated infections. A reproducible murine ear model based on the transmission of *L. major* by bite of *P. papatasi* was established. The model was used to investigate the effect of exposure to uninfected sand fly bites on the course of infection in both BALB/c and C57Bl/6 mice. The progression and associated pathology of the ear lesions, measured by lesion diameter,thickness, and ulceration, was dramatically faster and more severe in naive mice. The data clearly demonstrates an increased resistance to *Leishmania* as a result of host pre-exposure to uninfected sand fly bites in both genetically susceptible and resistant mouse strains.

These results are the first to suggest that for individuals at risk of vector borne infections, their exposure history to vector saliva might influence the outcome of their exposure to transmitted parasites, and they validate the prior suggestion (6) that salivary antigens might be effective components of an anti-*Leishmania* vaccine.

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C3 - THE FUSION MACHINERY IN EXOCYTOSIS IN *PARAMECIUM*: A PARADIGM

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Paramecium tetraurelia is an excellent eukaryotic system in which to study the process of regulated exocytosis since exo- mutants are available and the cell is structurally well defined and biochemically easy to handle. We have focused on a particular protein, parafusin (PFUS), a phosphoglycosylated component that is part of the fusion machinery in this cell. Early *in vivo* 32Pi labeling studies had shown that in wild type cells a Ca2+-dependent dephosphorylation of this protein took place concomitantly with exocytosis. This dephosphorylation and exocytosis were both blocked in wild type in the presence of extracellular Mg2+ and in the presence of extracellular Ca2+ in the exo- temperature sensitive mutant, nd9, at the non-permissive temperature (Gilligan & Satir 1982). In addition, another covalent modification was described for this protein; it was shown to have a-phosphoglucose (Glc-1-P), bound to a short chain of mannoses O-linked to a serine residue of the protein (Satir et al. 1990).

A series of *in vitro* experiments later suggested that it was this modification, Glc-1-P that was removed upon exocytosis by a Ca2+-dependent phosphodiesterase, which was defective in the exo- mutant nd9. It was further hypothesized that PFUS might represent a new type of signal transduction molecule involving carbohydrate cycling (Subramanian & Satir 1992).

PFUS has been cloned and sequenced and found to have 51% identity to rabbit muscle phosphoglucomutase (PGM), a glycolytic enzyme, although PFUS showed no or minor PGM activity (Andersen et al. 1994, Levin et al. 1998). Compared to PGM, PFUS has several specific insertions and deletions in the primary sequence (Subramanian et al. 1994). These regions have been shown to be located in surface loops of the molecule, thus conserving the overall three-dimensional structure of PGM.

The association of this protein with exocytosis was further corroborated using a peptide specific antibody to PFUS and laser scanning confocal microscopy. These experiments showed that PFUS was localized to the surface of the secretory vesicle (trichocyst) membranes and to their docking sites below the cell membrane. In addition, upon stimulation of exocytosis, PFUS dissociates from these sites, only to re-associate with the new secretory vesicles in the cytoplasm; this dissociation does not take place in the exo- mutant in which both *in vivo* dephosphorylation and exocytosis are blocked, which therefore suggests that the membrane bound form of PFUS is the phosphoglucosylated form (Zhao & Satir 1998).

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C4 - CYSTEINE PROTEINASES AS VIRULENCE FACTORES OF *TRICHOMONAS VAGINALIS*

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Trichomonas vaginalis a flagellatte protozoan is responsible for trichomonosis, one of the most common sexually transmitted disease in humans aurond the world. This disease is now been recognized as being life threatening for infected women, it is associated with adverse pregnancy outcomes (5) enhanced predisposition to HIV infection (9), and possibly cevical neoplasia. The mechanism of trichomonal cytopathogenicity is a multifactorial process where cytoadherence, cytotoxicity, phagocytosis, haemolysis, nutrient acquisition systems and immune evasion among other are contributing as virulence properties. Trichomonads have many proteinases, including numerous cysteine proteinases (11) and several metalloproteinases (4). At least 23 distinct CP activities with relative molecular masses between 23,000 daltons (23-kDA) and 110-kDA have been characterized by two dimendional substrata gel electrophoresis. Loately parasite proteinases have received attention for their possible role as virulence factors involved in haemolysis (6), immunoglobulin degradion, complement resistance (1), cytosqueleton disruption (8), and may as well be responsible for the adverse pregnancy outcome which occurs by membrane rupture. Recently, we have demonstrated that cysteine proteinanse activity of *T. vaginalis* is required for cytoadherence and cytotocity (2,3). Two cysteine proteinases (CPs) with relative molecular masses (Mr) of 65-kDa (CP65) and 30-kDa (CP30) avidly bound to both HeLa and vaginal epithelial cell surfaces. Assays with cysteine proteinase inhibitors such N-a-p-tosyl-L-lysine chloromethyl Ketone (TLCK) and leupeptin, inhibited cytoadherence and cytotoxicity at different levels. Leupeptin only affected CP30 proteinase activities and reduced cytoadherence up to 80%, TLCK inhibited both proteinases and completely protected HeLa cell monolayers from parasite destruction. These data suggested that CP30 is related with cytoadherence and CP65 with cytotoxicity. Also, analysis of about 50 different trichomonad isolates showed that isolates with low levels of cytoadherence also hand, isolates capacle of attachment but with little or no cytotoxicity toward HeLa cell had no detectable host cell-bound CP65 proteinase (3). The same relationship between levels of cytoadherence or cytotoxicity and the activity of the two proteinases was observed on nine independent agar-clones from a single isolate (7). Lately, specific polyclonal antibodies to each one of the CPs demonstrated their participation in the corresponding function previously assessed by inhibiting

cytoadherence to or destruction of HeLa cell monolayers up to 50%. These specific antibodies recognized the corresponding proteinaase by immunoblot or immunoprecipitacion assays and localized them on the trichomonad surface. Both proteinases are active at body temperature, at the pH range of the vaginal environment of patients with trichomonosis and use as natural substrates extracellular matrix proteins i.e., collagen aand fibronectin as haemoglobin. Both CP30 and CP65 were detected in vagina; secretions of patients and antibody to them were found in patient sera (2,3), showing the *in vivo* relevance of these CPs.

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C5 - MOLECULAR GENETIC APPROACHES TO THE *LEISHMANIA* INFECTIOUS CYCLE

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In the last 10 years, tools for the manipulation of the *Leishmania* genome have emerged and advanced to the point where many difficult procedures are now considered routine. Here we briefly review the present status of tools available to the prospective *Leishmania* molecular geneticist, identify some of our present challenges, and discuss possible future directions.

Considerations arising from the Leishmania genome and its evasive sexual cycle - The *Leishmania* genome is conveniently small, with estimates ranging from 35 to 50 Mb of DNA organized into more than 30 different chromosomes, depending on the species. Current data suggest that the genome is predominantly diploid, although several studies have suggested that portions of the genome can be aneuploid, and tetraploids have been obtained as a by-product in some gene knockout studies. Whether aneuploidy occurs in nature, or arises from pressures or freedoms posed by cultivation in the laboratory, remains to be determined.

Diploidy poses a problem to geneticists, as this requires that two copies must be altered in order to obtain mutants by traditional or gene replacement methods. With normal mutation rates, the frequency of homozygous loss-of-function mutants will be low, requiring the use of powerful selections to recover mutants, which typically occur at a frequency of less than 10^{-7} even after mutagenesis. In gene targeting experiments, removing both copies of the gene sequentially solves this problem. This can be accomplished by targeting with two constructs containing independent selectable markers in succession (1), or by one targeting event followed by generation of homozygotes through a process involving loss of heterozygosity (2).

Sexual crosses are powerful tools that can be exploited in many ways, for equalizing the background of mutant lines or to establish the genetic dominance of mutant or other phenotypes. To date, no successful genetic cross has been reported in *Leishmania*, despite many attempts (including unpublished negative results). Provocative data concerning the existence of *Leishmania* hybrids has emerged, although whether these constitute evidence for productive sexual exchange or represent sterile cellular fusions is unknown. In the laboratory, cell fusions would be highly useful in assessing the dominance of many mutant phenotypes. However, the rate of hybrid generation in nature is unknown; if hybrids arise only on an evolutionary time scale, it would preclude their use in most genetic tests!

Standard Leishmania genetics: expression vectors and gene knockouts - This technology allows the investigator to remove or express any known gene in *Leishmania*. Gene knockouts and expression vectors have been used to establish the function now of a variety of *Leishmania* genes; these include genes encoding drug targets (*DHFR-TS*, *PTR1*, trypanothione reductase), drug resistance genes (*PGPA*) and virulence factors (gp63, cysteine proteases, *HSP100*),

amongst others. When attenuated, knockout parasites offer good potential for use as live vaccination lines (3).

One concern in interpreting the results of gene knockout experiments is that, like other pathogens, *Leishmania* undergoes spontaneous alterations in virulence and other phenotypes during culture *in vitro*, and DNA transformation itself is known to be mutagenic. Thus, steps must be taken to ensure that the mutant phenotype in fact arises from the planned alteration. Since a sexual back-cross is impossible, this may be accomplished by re-expressing the wild-type gene in the mutant line. Notably, it is the comparison between the 'add-back' line and the mutant that establishes the role of the gene of interest. This test is often referred to as one of Koch's 'molecular postulates', as set forth by Stanley Falkow (4).

In trypanosomes, it has proven possible to generate inducible expression systems, enabling the comparison above to be performed in knockout parasite lines in which the gene is now selectively induced. Such isogenic comparisons would be ideal, and several groups are working on analogous systems in *Leishmania*. Another application of inducible expression system is in the study of essential or toxic genes.

Functional genetic rescue of loss-of-function mutations - *Leishmania* episomal vectors can carry at least 40 kb of DNA, and genomic DNA segments carried on such vectors appear to function more or less autonomously. This suggested that transfection of genomic libraries into mutants could be used to identify relevant genes. This was first shown by functional rescue of lipophosphoglycan (LPG) mutants isolated in Turco's laboratory, and several LPG biosynthetic genes have now been identified (5). Recently this approach has been extended to other mutants, such as ones affecting glycosomal protein import (6) and pteridine and nucleoside uptake (7), as well as to other LPG phenotypes. Thus, if appropriate mutants and selective protocols are available, functional genetic rescue works well in *Leishmania*. At present, the only limitation to widespread application of this powerful methodology is the availability of appropriate mutants.

Dominant genetics: over-expression mediated by multi-copy suppression - Prior to the advent of DNA transfection, a common method for isolating *Leishmania* drug resistance genes relied upon gene amplification. Since *Leishmania* episomal cosmids exist in multiple copies following transfection, genes carried on these can be viewed as pre-engineered amplifications. The advantage is that their frequency in cosmid transfected parasite libraries is approximately 10^{-3}, which is much higher than that of gene amplifications or parasite mutants.

Drug selections of cosmid transfected libraries has been used to recover a number of drug resistance loci in *Leishmania*, including ones implicated in resistance to antifolates, toxic nucleoside and sterol synthesis inhibitors. In theory, any quantifiable phenotype would be amenable to this approach; for example, in mammalian cells, FACS sorting based upon the abundance of cell surface molecules has been used.

Genetic suppression mediated by over-expression is a well-known mode of genetic rescue in many organisms. The advantage here is that one expands the range of genes that are recoverable by functional rescue experiments, beyond the defective gene anticipated through strict genetic complementation. In studies seeking to rescue the defect of an avirulent line of *L. major*, we found that every active locus obtained functioned through suppression, rather than complementation (unpublished data).

Dominant genetics: gene fusions and transposable elements - A common method for gene identification and analysis in both prokaryotes and eukaryotes has been the generation and screening of gene fusion libraries. From these, genes can be recovered constitutively or selectively, such as those showing differential regulation or encoding proteins showing differential protein targeting. In *Leishmania*, we created small gene fusion libraries by simply ligating genomic DNA to appropriately constructed episomal vectors. Following transfection of these, we have identified a number of *Leishmania* proteins fused to the Green Fluorescent Protein, which show targeting to specific cellular compartments such as the mitochondrion, kinetoplast, nucleus, or Golgi apparatus (unpublished data).

To date, integration into the trypanosomatid genome occurs exclusively through homologous recombination, and in this respect, *Leishmania* resembles the yeast *Saccharomyces cerevisiae*. Since the fidelity of episomal gene regulation in *Leishmania* is not yet established, there is considerable motivation to score gene expression in a chromosomal context. To achieve this, some method of random chromosomal integration is required.

We showed that the *Drosophila* element *mariner* was able to transpose when properly introduced and expressed in *Leishmania*. We were able to inactivate a hemizygous gene, as well as to design a vector for trapping active genes (8). Significantly, insertion of selectable modified *mariner* constructs resulted in little change in mRNA processing and abundance, and some of the 'trapped' genes have since appeared in EST databases. Recently, we have been able to purify the *mariner* transposase and demonstrate transposition *in vitro*. This system has several advantages: it can be used in "primer-island" DNA sequencing, in functionally identifying genes on cosmids, in testing transposons for use *in vivo*, and in shuttle mutagenesis applications.

Diploidy can be an advantage - While diploidy is an obstacle in making mutants and/or gene knockouts, it is an advantage in making gene fusion libraries by transposon insertion methods. This is because the disrupted gene remains 'covered' by a wild-type allele, which is usually sufficient to maintain a normal function. Thus, insertions giving rise to reporter gene fusions cause relatively little problem to the host cell, and can be scored independently of their phenotypic effect. This permits us to contemplate the generation of large reporter or selectable gene fusion libraries in the readily cultivatable promastigote stages of *Leishmania*, which can then be scored for differential expression during the metacyclic promastigote or amastigote stage.

An interesting comparison is the challenges faced by *Leishmania* geneticists with those studying obligate intracellular Apicomplexan parasites. While mutants and or insertions are readily induced in these haploid parasites, their phenotypic consequences are also immediately exposed. The ideal parasite from a genetic point of view would resemble yeast, which can undergo genetic exchange and be cultivated at the experimenter's need as haploids or

diploids. While it is unlikely that *Leishmania* genetics will completely become as amenable as that of yeast, there is every possibility that workable alternatives may be found which allow us to meet most of our genetic needs.

Prospects for the future: functional genomics - In the future, the nascent *Leishmania* genome project offers a fresh perspective and resource for many questions. Not only will this provide a seemingly limitless supply of new genes for functional testing by established knockout approaches, but it provides a powerful new resource for rapidly characterizing the fruits of functional genetic rescues of genetic mutants and screens of gene fusion libraries. The combination of genetic tools with genomic insights promises to make our next decade of parasite genetics a particularly fruitful one.

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C-6 - STRUCTURE AND FUNCTION OF PROTOZOAN CILIA

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Cilia and flagella are important organelles of motility, sensory response and behavior among many protistan groups. Considerable information has been obtained on the structure and function of cilia especially from three organisms: *Chlamydomonas*, *Paramecium* and *Tetrahymena*, reviewed by Satir and Barkalow (1996). The basis of motility and motor response lies in the universal construction of the 9+2 axoneme, while the ciliary membrane contains specific receptors and channels that regulate the environment around the axoneme, allowing the cilium to change its beat parameters to cause the cell to swim in a novel manner in response to a stimulus or a concentration gradient. Among the responses of protistan cilia are changes in beat frequency, which cause an increase or decrease in swimming speed, changes in beat form, which can cause the cell to turn or to reverse its swimming direction and complete arrest. Since ciliary motility in protozoa usually is a low Reynolds number hydrodynamic system, complete ciliary arrest results in virtually instantaneous cessation of cell movement. Turning and reversal responses are the basis of complex behaviors, such as phototaxis. Over the past decade or so, we have begun to dissect out the molecular mechanisms that govern these responses.

Two small signalling molecules, Ca^{2+} and cAMP, act on the axoneme directly to initiate the responses. Ca^{2+} normally enters the space around the axoneme through voltage-gated Ca^{2+} channels in the ciliary membrane. Upon stimulation, axonemal internal free Ca^{2+} changes from less than 100 nM to more than 1 mM. As originally shown by Naitoh and Kaneko (1972) for *Paramecium*, the effect of this Ca^{2+} increase can be mimicked by removing the ciliary membrane by detergent treatment and adding appropriate Ca^{2+} buffers to a reactivation solution containing Mg^{2+}-ATP. At 100 nM Ca^{2+}, the reactivated cells swim normally forward much like their living counterparts, but as Ca^{2+} concentration increases systematically they slow, stop and finally, at concentrations above 1 mM, they swim backwards. Although the phenomenology is clear, we know much less about the Ca^{2+} sensor. Two well-known Ca^{2+} binding proteins are permanently found as part of axonemal structures: calmodulin (CaM) and centrin. Centrin is a constituent of certain inner dynein arms, but the exact localization of CaM within the axoneme has not been determined. However, with high Ca^{2+} in the presence of CaM antagonists, arrest and reversal responses are abolished, which suggests that CaM is a likely candidate for the Ca^{2+} sensor. Ca^{2+} might act directly by binding to a CaM or centrin switch or indirectly, if Ca^{2+}-centrin or Ca^{2+}-CaM activates an axonemal kinase or phosphatase to change the phosphorylation status of a switch protein, possibly associated with the spoke-dynein regulatory complex at the base of the inner dynein arms (IDAs).

A protein kinase permanently built into the axoneme acts as the cAMP sensor. This PKA is activated by increased cAMP concentration after cell hyperpolarization via membrane K^+ channels that are tightly coupled to adenylyl cyclase. Phosphorylation of inner and outer dynein arm components occurs, which can be predicted to produce a faster, shallower ciliary beat. Detergent-permeabilized *Paramecium* reactivated in the presence of 10-100 mM cAMP swim faster than controls. Faster swimming depends primarily on increased beat frequency. It is

abolished by the addition of 10^{-4}M Ca^{2+}, but not by removal of cAMP, suggesting that a Ca^{2+}-sensitive cAMP-dependent protein phosphorylation is responsible for the beat frequency change. In the absence of a change in bend amplitude, beat frequency is directly proportional to doublet microtubule sliding velocity produced by outer dynein arms (ODAs). In *Paramecium* isolated ODAs are three-headed bouquet-like molecules sedimenting at 22S, where each head is a specific dynein isoform. A regulatory light chain, p29, of the a ODA isoform is phosphorylated in a cAMP-dependent and Ca^{2+} sensitive manner, corresponding to the beat frequency responses. The conclusion that p29 phosphorylation is responsible for the change in beat frequency response is supported by *in vitro* experiments where 22S dynein with experimentally phosphorylated p29 produces faster microtubule sliding than 22S with unphosphorylated p29 (Barkalow et al. 1994). Hamasaki et al. (1995) conclude that p29 phosphorylation alters 22S dynein mechanochemistry in such a way as to account for frequency changes over most of the beat frequency range.

In contrast to the beat frequency dependence on ODA activity, changes in beat form depend on the IDA. The structure of the IDAs, now effectively reconstructed by computer modelling, is more complex than that of the ODAs, and different isoforms may subserve different functions in controlling bend parameters. At least one function is dependent on phosphorylation of a p138 subunit associated with the I1 isoform. Phosphorylation of p138 slows microtubule sliding velocity of IDAs in *Chlamydomonas* mutants (Habermacher & Sale 1996), as the end result of the spoke signalling pathway.

Although the integration and switching of ODA and IDA activity that produces the final beat is not completely understood, the mechanisms presented here provide the beginnings of an understanding of the conversion of molecular events into ciliary and flagellar movement in protista.

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C7 - ABSTRACT NOT RECEIVED

C8 - SUSCEPTIBILITY OF BALB/C MICE TO THE INTRACELLULAR PARASITE *LEISHMANIA MAJOR* DEPENDS ON T CELL PRIMING BY ENVIRONMENTAL ANTIGENS

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Susceptibility of BALB/c mice to the intracellular parasite *Leishmania major* correlates with the development of a strong Th2 response to parasite antigens. In agreement with a critical role of IL-4 for the differentiation of Th2 cells, an early burst of IL-4 mRNA is observed in the draining lymph nodes of BALB/c mice as early as 16 hr after infection (Launois et al. 1995). Moreover, early neutralization of IL-4 at the time of infection results in the development of protective Th1 effector lymphocytes and in the healing of the lesions. A similar healing phenotype is observed in BALB/c mice with a disruption of the IL-4 gene. We and others have previously shown that the early accumulation of IL-4 which is induced by the parasite in BALB/c mice occurs within a subset of CD4+ T cells which react to the previously identified LACK antigen (Mougneau et al. 1995). Indeed, BALB/c mice which have been made tolerant to LACK by transgenic expression of this antigen in the thymus, or by deletion of the LACK-reactive T cells using a superantigen, do not exhibit an early burst of IL-4 and develop a default Th1 response and a healing phenotype (Julia et al. 1996, Launois et al. 1997). Here, we show that LACK-specific T cells are stimulated prior to infection by mimicry peptides derived from microbial antigens from the intestinal flora. The early production of IL-4 is prevented in germ free (GF) mice, and splenocytes from these mice are unable to adoptively transfer susceptibility when inoculated into immunodeficient recipients. Thus, molecular mimicry between parasite and microbial antigens is critical for the early production of IL-4 and for the development of protective immunity in susceptible BALB/c mice. These results may have important implications for the design of vaccination strategies.

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C9 - USING INDUCIBLE GENE EXPRESSION TO STUDY GENE FUNCTION IN *TRYPANOSOMA BRUCEI*

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From the point of view of chemotherapy, the most interesting genes in kinetoplastids are the essential ones. But it is precisely these genes whose function cannot be studied by classical knockout technologies. If a gene is essential attempts to delete it from the genome must fail. We have therefore used inducible gene expression to find out (a) the phenotype of cells lacking essential gene products; (b) how much of an essential gene product is needed to allow cell survival and cell growth (c) the phenotype of cells over-expressing toxic products. The proteins studied so far include a glycosomal membrane protein, and enzymes involved in glycolysis and thiol metabolism.

Details of the experimental approaches

A. Inducible expression - When we start studying a gene, we usually want to find out the effects of both over-expression and the absence of the gene product. We first make cells which can inducibly over-express the gene in question. (a) We start with trypanosomes that express the procaryotic *tet* repressor. These cells contain a plasmid that bears the *tet* repressor gene, linked to the phleomycin resistance (*BLE*) gene, integrated in the tubulin locus. Very often, these cells serve as "wild-type" controls in our subsequent experiments. (b) The gene we want to over-express is cloned into an inducible expression vector. The vector bears a *PARP* promoter and a binding site (operator) for the tet repressor, upstream of the gene of interest. Expression is turned on by adding tetracycline and is turned off in the absence of tetracycline [1]. Downstream of the inducible gene is a well-characterised 3'-untranslated region, chosen to enable optimal expression in the life cycle stages that interest us. For example, if we want high expression in bloodstream forms, we use the untranslated region of a variant surface glycoprotein (*VSG*) gene. After that comes a second promoter (a *VSG* promoter) that drives expression of the hygromycin resistance marker *HYG* [2]. The *PARP* and *VSG* promoters are recognised by RNA polymerase I, and unfortunately do not work in other kinetoplastid species. (c) The inducible construct is transfected into the trypanosomes and we select appropriate clones by adding hygromycin. We then test the clones by adding tetracycline to the medium. We measure expression of the RNA using Northern blots, or protein either using antibodies (Western blots, immunofluorescence) or enzyme assays. At this stage we always look at both the morphology and growth rate of the over-expressing cells, to see if over-expression is toxic.

B. Preliminary knockouts - While these over-expression experiments are in progress, we generate contructs to knock out the gene. (a) It is important to design the constructs such that they target the endogenous gene copies - and not an inducible copy we have added! For that reason, our inducible construct contains the complete coding region of the gene (generated by PCR), but absolutely none of the 3'- or 5'-untranslated regions. The knockout contructs contain 3'- and 5- downstream and upstream regions - but no coding sequence. We normally place the G418 (*NEO*) resistance marker between the 5'- and 3'- ends. (b) The *NEO* construct is transfected into trypanosomes to knock out the first copy of the gene. We check the knockout by Southern analysis and then by measuring the level of protein product. So far we have always found that the heterozygous knockouts express roughly half the normal level of RNA or protein, but there may well be cases where internal regulation mechanisms serve to restore 100% levels of the preotein even if one of the genes has been removed. (c) Next, we want to find out if the gene is essential. We therefore make a second knockout construct, usually bearing the *HYG* marker, and transfect it into the cells. The transfection mixture is split in half: one half is selected with hygromycin and G418, and the other half is selcted with hygromycin alone. If the gene is not essential, either the gene itself or the NEO gene should be replaced with equal frequency, so we should get clones from both selections. If, however, the gene is essential, we get clones by selecting wtih hygromycin alone: in these, the *NEO* gene has been eliminated but the endogenous gene is retained. When we select with both drugs, we can only obtain clones under two circumstances: either integration occurs in an unexpected fashion, so that the targeted gene is retained, or the chromosome concerned becomes triploid [3]. These are rare events and can readily be detected by Southern analysis.

C. Generating cells with an "inducible knockout" - Supposing the gene we are interested is essential, we return to our cells with the inducible gene. We very carefully check several clones in order to find the cells which have the lowest expression in the absence of tetracycline (i.e. no detectable "leakage" of the inducible system). We knock out the first endogenous gene copy using the *NEO* construct. We check that the cells over-express the gene in the presence of tetracycline and have 50% expression in the absence of tetracycline.

So far the experiments have been technically straightforward, if time-consuming. Now things can get very difficult indeed We need to knock out the second endogenous copy of the gene. There are two major problems associated with this: (a) The cells are already resistance to phleomycin, hygromycin, and G418. Several additional selectable markers, such as puromycin resistance (*PAC*) are available for procyclic trypanosomes, but we have so

far found no satisfactory additional marker for bloodstream forms. To obtain the double knockout, our only option is to gradually increase the level of G418 in the medium to select for cells with more than one copy of the *NEO* gene. Usually such cells have undergone "loss of heterozygosity" [4], so that the second copy of our target gene has been replaced by *NEO*. (b) In order to knock out the endogenous gene, we must provide sufficient gene product from our inducible system to replace all the essential function. That means we must put tetracycline in the medium. If we have too little tetracycline, the cells will die. But often, over-expression of a gene product inhibits cell growth. So if we put in too much tetracycline, the cells will also die! This difficulty is compounded by the short half-life of tetracycline in the medium, which means that some tetracycline must be added every 2-3 days.

In practice, it usually takes several months of trial-and error, with a multitude of different combinations of tetracycline and G418 concentration, to get a double knockout in bloodstream forms. The results we obtain with such cells are however so useful - and often so unexpected - that so far the effort has always proved worthwhile.

The role of glycolytic compartmentation

One of the first really surprising results we obtained using our inducible expression did not involve a knockout. *Trypanosoma brucei* has two dividing life-cycle stages: the bloodstream form and the procyclic (tsetse fly) form. It also has two prinipal isoforms of phosphoglycerate kinase. One is found in the cytosol, and is expressed in procyclics, and the other is located in the glycosome, and is expressed in bloodstream forms. To our surprise, we discovered that she could not get any clones expressing the cytosolic phosphoglycerate kinase in bloodstream forms. Using the inducible expression system, she was able to show that the presence of phosphoglycerate kinase enzyme activity in the cytosol is toxic to bloodstream forms - even if the normal amount of enzyme is present in the glycosome [5]. This demonstrates that the compartmentation of glycolysis is very important in trypanosome metabolism. We suspect that the extra cytosolic enzyme upsets the balance between ATP and ADP in the glycosome.

The function of glycosomal membrane proteins

In another approach to studying the role of the glycosome, we cloned the genes encoding the two most abundant integral glycosomal membrane proteins. These have molecular weights of 24kD and 26 kD and have a predominance of basic and hydrophobic amino acids. Over-expression of either protein is toxic to bloodstream trypanosomes. We have so far done most work with the 24kDa protein gene. The 24kDa protein has two predicted transmembrane domains. It is targeted to peroxisomes when expressed in mammalian cells and yeast. The protein is a functional homologue of Pex11p from *Saccharomyces cerevisiae*: *pex11*D mutants, which are defective in peroxisome proliferation, can be complemented by the trypanosome gene. Sequence conservation is significant in the N-terminal and C-terminal domains of all putative Pex11p homologues known, from trypanosomes, yeasts and mammals. Several lines of evidence indicate that these domains are oriented towards the cytosol. TbPex11p can form homodimers, like its yeast counterpart. The *TbPEX11* gene is essential in trypanosomes. Inducible overexpression of the protein in *T. brucei* bloodstream forms causes growth arrest, the globular glycosomes being transformed to clusters of long tubules filling significant proportions of the cytoplasm. Inducible under-expression (10% of normal amounts of protein) results in trypanosomes with fewer, but larger, organelles [6].

The role of trypanothione reductase: verification of a possible drug target

In trypanosomes and in *Leishmania*, trypanothione and trypanothione reductase are thought to provide an intracellular reducing environment, substituting for the glutathione and glutathione reductase found in mammalian hosts. Several investigators have already studied the role of trypanothione reductase in *Leishmania* and *T. cruzi*. The cells studied had either a hemizygous knockout, or they contained an episome that constitutively expressed either antisense RNA or a dominant-negative mutant enzyme [7, 8]. In either case, of course, the cells could grow well on normal media - otherwise the clones would not have been obtained. Experiments did show, however, that the parasites had a restricted ability to infect activated macrophages. These results suggest that trypanothione reductase plays a role in resistance to oxidative stress. However the cells obtained still expressed so much enzyme (at least 15% of normal levels) that it was not possible to see any increase in susceptibility to agents causing oxidative stress directly, such as peroxide.

To investigate the physiological role of trypanothione reductase in *T. brucei*, we constructed cells containing a single trypanothione reductase gene under control of a tetracycline-inducible promoter; the endogenous genes were knocked out as described above. In normal growth medium (which contains reducing agents), trypanosomes containing less than 10% of wild-type enzyme activity arrested in the G2/M phase of the cell cycle, but contained normal levels of small thiols and showed normal sensitivity to arsenical drugs. These cells survived for several days before gradually dying. In non-protective media, hypersensitivity towards hydrogen peroxide (k_i=3μM) was found, finally confirming the role of trypanothione reductase in protecting against oxidative stress. Careful titration of the levels of tetracycline yielded cells with a wide range of trypanothione reductase levels. By observing the behaviour of these parasites we concluded that a drug that inhibited trypanothione reductase by 90-95% should be completely cytostatic.

Most exciting were the results in mice. We infected mice with the trypanosomes containing the inducible gene. Mice given normal water did not become infected, but mice given tetracycline in their water did become infected. Thus infectivity of the trypanosomes was dependent on expression of trypanothione reductase.

Conclusion

Our results so far have shown that the inducible expression system is extremely useful in testing the role of different proteins in trypanosome metabolism, growth and survival, and in verifiying potential drug targets. In many cases - as for the *PEX11* gene - our results with *T. brucei* define the role of the gen in all trypanosomatids. But *T. brucei* has no intracellular phase, so that to study many functions that are required for intracellular survival it will be necessary to develop similar genetic toools for *T. cruzi* and *Leishmania*.

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C10 - *LEISHMANIA* AND *MYCOBACTERIUM*: CONTRASTING STYLES OF INTRACELLULAR PARASITISM

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Both *Leishmania* and *Mycobacterium* parasitize the phagocytes of their vertebrate hosts and both pathogens go on to induce chronic, enduring infections that only show clinical symptoms in a subsection of the infected population at any given time. The cryptic nature of the infections provides graphic demonstration of the balanced nature of the interaction between both pathogens and their host cell. Macrophages are known to possess highly effective antimicrobial properties ranging from innate defences such as lysosomal pH and hydrolases, and the superoxide burst, to immune-mediated modulators such as the inducible nitric oxide synthase. Nonetheless, both *Leishmania* spp. and pathogenic *Mycobacterium* are capable of residing and persisting in these cells. This lecture will deal with the strategies employed by both pathogens to ensure the continuation of their infection.

Leishmania

The parasitophorous vacuole of *Leishmania* spp. is a hydrolytically-competent compartment that exhibits most of the properties of a late endosome or lysosome. It has a pH of 4.7-5.2, contains active cathepsins B, L, and D and is freely accessible to cargo trafficking through the host cell's endosomal system. Indeed, parasites of the Mexicana complex actually increase the fusigenicity of their vacuole to the point where phagosomes containing large, particulate material fuse freely with the parasitophorous vacuoles.

In addition to access the endosomal network parasitophorous vacuoles of *L. mexicana* also acquire material from the host cell cytoplasm via two independent mechanisms. (i) Small anionic molecules such as lucifer yellow are rapidly transported into the parasitophorous vacuoles by active transport. This transport can also be demonstrated in isolated parasitophorous vacuoles and is sensitive to specific inhibitors indicating that it is a product of the host cell's organic anion tranporter. (ii) Larger molecules such as fluorescent dextrans introduced into the host cell cytosol are also delivered to parasitophorous vacuoles. This transport is considerably slower and is sensitive to modulators of autophagy. Although the rate of autophagous transfer was comparable in infected and uninfected cells, infected cells retained hydrolyzed substrate to a greater degree. Acquisition of host material via these routes have possible significance for both nutrition and immune evasion.

***Mycobacterium* spp.**

Pathogenic *Mycobacterium* also reside in vacuoles inside the macrophage, however, the compartments harboring these pathogens show marked differences from those of *Leishmania*. Vacuoles inhabited by *M. avium* and *M. tuberculosis* show limited acidification (pH 6.2-6.3), and restricted hydrolytic capacity. However, despite the fact

that these vacuoles do not fuse with host lysosomes, they are highly dynamic, fusing readily with certain plasmlemma-derived constituents. Analysis of transferrin trafficking in infected macrophages reveals that the bacilli secure their vacuole within the sorting/recycling endosomal system of their host cell.

Interestingly, activation of infected macrophages induces acidification and maturation of the mycobacterial vacuoles prior to the drop in viability of the infecting organisms. This suggests that macrophage activation induces a change in endosomal/lysosomal fusion, and that this change may play a role in the subsequent killing of these bacteria.

The interplay between both pathogens and their host cell as finely balanced, a state which may be tilted ineither direction by appropriate or inappropriate immune responses. In addition to detailing the characteristics of the compartments inhabited by both pathogens, this lecture will discuss strategies employed by the pathogens to avoid the induction or effects of stimulating a productive immune response.

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C11 - WHAT IS *ENTAMOEBA DISPAR*, AND WHY IS IT IMPORTANT?

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In the last few years the name *Entamoeba dispar* has reappeared in the literature after a long absence. The species was described by Emile Brumpt in 1925 but it was regarded as identical to *E. histolytica* by almost everyone at that time and, until recently, by almost everyone working in the field of amebiasis research since then. We now know that Brumpt was correct and that a separate species does exist. Because the change in our view is still relatively recent and not widely known, this presentation will review the history of *E. dispar* and contrast what we know about its biology with the better known species *E. histolytica*.

History

When Brumpt described *E. dispar,* he considered it to be morphologically identical to *E. histolytica* but non-pathogenic. He based this on both observation of an infected individual over a period of years and on experimental animal infections none of which developed amebic disease. By this time it was known that in many countries there were significant rates of infection with what appeared to be *E. histolytica* but no invasive disease, and Brumpt concluded that most of these infections were due to *E. dispar*. The main reason for Brumpt's interpretation being rejected was that it was known from experimental human infections that not everyone infected with an ameba capable of causing disease develops symptoms. Therefore Brumpt's observations could be explained as asymptomatic carriers of *E. histolytica* without needing to describe a new species.

The evidence for *E. dispar* being a distinct species did not really start to be reexamined until 1978, when Sargeaunt and Williams showed that all *E. histolytica* could be divided into two groups using isoenzymes, one of which ('pathogenic *E. histolytica*') contained all isolates from cases of invasive disease. The second group ('non-pathogenic *E. histolytica*') contained most of the isolates from asymptomatic carriers. This distinction held true for several thousand isolates they examined. By 1990 the evidence had been expanded to include antigen and DNA markers in addition to more isoenzymes, all of which showed the same two groups of amebae. The data were such that in 1993 Dr. Louis Diamond and I decided that there was sufficient evidence for the existence of two species, *E. histolytica* and Brumpt's *E. dispar*, and so we formally redescribed *E. histolytica* to separate the two. All the data since then have only reinforced the distinction between the two species. The two species names have been almost universally accepted in the field of amebiasis research and were incorporated into the latest WHO recommendations.

How does *E. dispar* differ from *E. histolytica*?

Most differences between the two species are not absolute. *E. histolytica* produces more protease, has a thicker glycocalyx, a lower surface charge, binds more strongly to target cells, and is more cytotoxic. However, the similarities are more obvious than the differences - both use a galactose lectin to bind to target cells and disrupt their membranes with pore-forming proteins. The only major difference found so far is that *E. dispar* lacks two of the six cysteine proteinase genes found in *E. histolytica*. How important this may be in the relative ability to cause disease will require the construction and testing of transgenic *E. dispar.*

Every gene sequence that has been compared between *E. histolytica* and *E. dispar* so far has been different. Although it is clear that the two species are quite closely related, using ribosomal RNA and cysteine proteinase gene sequences for comparison the two are as different as humans and mice. In addition to the genetic differences and their disease-causing potential there are a number of other biological characteristics that separate them. The most significant observation, however, is that no forms with a mixture of genetic characteristics have ever been detected.

The importance of *E. dispar*

One objection raised to Brumpt's original proposal was that the fact that because the two species are indistinguishable under the light microscope diagnosis would be very difficult. This is still true. Even where invasive disease is widespread *E. dispar* is still easily the more common of the two species. A reliable, simple and inexpensive method for distinguishing the two species is urgently needed otherwise there will be a very large number of unnecessary courses of antiamebic drug therapy, with the associated side effects and the potential for developing drug resistance. The recognition of *E. dispar* as a distinct species represents one of the most significant changes in medical parasitology in recent years, but it also means that we need to start collecting prevalence data all over again, this time distinguishing the two species. At this point in time we have no idea of the true prevalence of *E. histolytica.*

C12 - IMMUNOLOGICAL CONTROL MECHANISMS OF *GIARDIA LAMBLIA* INFECTIONS AND THE BIOLOGICAL ROLE OF ANTIGENIC VARIATION

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The adult mouse model of *Giardia lamblia* was used to study how the infection is controlled in mice and the biological role of antigenic variation. In immunocompetent mice the number of intestinal trophozoites peaks at about one week post inoculation (PI) and then declines precipitously until at three weeks PI only rare organisms can be detected by culture. This chronic infection continues from weeks to months. In nude, scid or ab T cell deficient mice high levels of infection are maintained for at least one month. In contrast mice lacking IgA, B cells or gd T cells are able to control acute infections similar to immunocompetent mice. Anti-CD4 treated B cell deficient mice were unable to control infections compared to control treated B cell deficient mice confirming that the control of *G. lamblia* infections is primarily T cell mediated and not B cell mediated. In previous studies anti-IgM treated mice experimentally infected with *G. muris* were not able to self-cure suggesting an important role for antibody in the control of this infection. In contrast B cell deficient mice were able to self-cure and control *G. muris* infections. Therefore, B cell mediated mechanisms are not important in the control of either acute *G. lamblia* or *G. muris* infections in adult mice but could still play a role in the formation of chronic *G. lamblia* infections after three weeks PI.

We as well as others have shown that the ability to make specific anti-VSP antibodies is essential to select for variant specific surface proteins (VSPs) but as indicated above these antibodies are not essential in the control of acute infections. If the role of antigenic variation is not immune evasion what is its biological function? In immunocompetent mice inoculated with our standard clone expressing a particular VSP, the initial VSP is maintained until two weeks PI when it is replaced by a large number of other VSPs. Different VSP expressing clones derived from one isolate and MAbs to the VSPs expressed by these clones were produced and used to study infections and VSP expression in scid mice. Since these mice are unable to produce antibodies, changes in VSP expression cannot be antibody mediated and must be due to other mechanism(s). A majority of clones continue to express the original VSP but some clones could not maintain expression of the original VSP and express a mixture of VSPs. Over the same time in vitro, VSP expression was very heterogeneous and only a few per cent of the initial VSP was expressed. Therefore, *in vivo* some VSPs are favored in mice while others are not favored and the selective pressures for VSPs differ in vivo compared to culture *in vitro*. This suggests that VSPs differ biologically and may allow infection of a wider range of animals or survival in different intestinal environments. The ability to survive in vivo is dependent on both biological and immunoselection mechanisms.

C13 - THE CELL CYCLE AND ORGANELLE MORPHOGENESIS IN *TRYPANOSOMA BRUCEI*

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The trypanosome cell has a precise shape and form which is modulatied through the cell cycle with a clearly recognisable structural precision. Moreover, since the cell contains single copy organelles such as the nucleus, the kinetoplast, and the flagellum each of which must be replicated and distributed to daughter cells these events provide a rather unique set of morphological landmarks for cell cycle position and progression [1, 2].

The early interphase procyclic trypanosome possesses a single flagellum, basal body and probasal body, a single nucleus and a single kinetoplast. The earliest recognisable event signifying entry into a new cell cycle is the elongation of the probasal body to form a mature basal body. This is followed by a sequence of events which include: the appearance of a new flagellum on the mature posterior basal body, formation of two new probasal bodies adjacent to each of the existing basal bodies, elongation and emergence of the new flagellum out of the flagellar pocket, initiation and extension of the paraflagellar rod, segregation of kinetoplast: basal body complexes, mitosis, and cytokinesis.

These events require extensive remodelling of the cytoplasmic array of sub-pellicular microtubules and the formation of a new flagellum pocket. Studies of the distribution of gamma - tubulin in the region of the basal bodies and the three dimensional positioning of the basal bodies themselves suggest that there is extensive remodelling of this area of the cell in the G1 / S period of the cell cycle.

Obviously, with respect to genomes we have to consider the trypanosome cell as possessing TWO UNIT genomes [nuclear and kinetoplast] that need to be replicated and segregated in the cell cycle. Bromodeoxyuridine incorporation into the replicating DNA of the nucleus and kinetoplast reveals that both S phases are periodic occupying, respectively, 0.18 and 0.12 of the unit cell cycle. The initiation points of both S phases are almost in register. Estimates of the cell cycle timings with respect to both the kinetoplast and nucleus have been obtained [2] and show a significant nuclear G1 phase length [0.4 of the unit cell cycle] and that although mitosis is relatively short [0.08] there is a significant period spent in the cytokinesis phase [0.12]. With a doubling time of 8.65 hours for the cultured procyclic trypanosomes this translates to more than an hour spent in the post-mitotic, cytokinesis phase of the cell cycle. Presumably this reflects the complexity of the late division process.

Mitosis occurs via an intranuclear mitotic spindle [3]. Kinetoplast segregation, however, is orchestrated by other structural events in the cell cycle. Structural reorganisation of the kinetoplast can be seen during S phase when observations reveal an antipodal pattern of replicated DNA [4]. Subsequent segregation of the replicated kinetoplast is mediated by attachment to the basal bodies of the flagella [5]. Attachment of the basal body to the kinetoplast is through a direct connection *via* sets of filaments and a differentiated zone of the mitochondrial membranes: a structure that we have termed the tripartite attachment complex [TAC]. This structure appears to be central to both positioning and segregation of the kinetoplast.

Drug treatment experiments have been informative in revealing control points in the trypanosome cell cycle. Low concentrations of the antimicrotubule drug Rhizoxin inhibit mitosis yet cytokinesis proceeds. This cleavage produces a nucleated flagellated cell and a flagellated, anucleate cytoplast. We term these cytoplasts ‘zoids’ and have shown that they are born from the posterior of the cell and contain the new flagellum and associated basal body/kinetoplast complex [6]. Progression through cytokinesis [zoid formation] whilst mitosis is compromised suggests that the usual mitosis to cytokinesis checkpoints do not operate in these ancient eukaryotes. Trypanosomes may possess novel cell cycle control features that are a consequence of its possession of two unit genomes and the need for coordinated replication and segregation of both in the cell cycle. Similarly, experiments with DNA synthesis inhibitors and the application of our markers of cell cycle position have revealed interesting phenotypes that are indicative of unusual cell cycle controls [checkpoints?] in the trypanosomes. Moreover, such experiments give insight into cell cycle events leading to the formation of dyskinetic strains of the parasite.

The life cycle of the trypanosome and control of its pathogenicity is intimately linked to control of the cell cycle. Efficient transfer between insect vector and mammalian host; control of parasite population and pathogenicity in the bloodstream; efficient differentiation from bloodstream to procyclic [insect midgut forms] and maybe even its ability to undergo a sexual division are all influenced by the parasite’s ability to excercise control at specific points in its cell division cycle [7, 8, 9]. Thus, an understanding of the basic cell cycle is providing insight into these more complex processes that are at the heart of the organism’s success as a parasite.

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C14 - *ENTAMOEBA HISTOLYTICA* CELL SIGNALING, MOTILITY, AND INVASION

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The tenacity of parasitic diseases may be attributed to the fact that most parasites adapt rapidly to vastly different environments. The trigger to adaptation lies within the intracellular changes that occur in the parasitic cells, elicited in response to signals from the extracellular milieu. Cells respond to external stimuli, activating several metabolic pathways that, in turn, trigger a cascade of internal signals to regulate processes such as movement, cell shape, endocytosis, secretion, proliferation, and differential gene expression. Cell signaling is then an important regulator of cellular activity.

In parasites, that have to respond to the interaction with the host and the host's environment, the understanding of signal transduction mechanisms has become a fundamental topic. We approached the study of the human parasite *Entamoeba histolytica* signaling pathways, using an experimental model designed for the interaction of trophozoites with extracellular matrix, in particular, fibronectin (FN). The idea for this model was based on our pioneering observations of extracellular matrix binding and local degradation of the substrate by the parasites. Binding also elicited drastic modifications in the organization of the cytoskeleton, consisting in the formation of actin-rich structures similar to the adhesion plaques described in other eukaryotic cells at the sites of contact with extracellular matrix proteins, and known to function as signal transduction organelles.

Having characterized a 37 KDa FN-binding protein, located in the trophozoite surface in close association with plasma membrane proteins, the stimulus by FN was used as a tool to identify receptor-coupled signal transduction pathways in the amebas. We hypothesized that activation of signaling pathways could result in the formation of the actin plates to facilitate adhesion and local secretion of proteases, and as a further step, dissemination of the parasite within the host tissues.

Evidence has been obtained for the presence and function of phospholipase C, inositoltriphosphate, diacylglycerol, Ca2+-dependent kinases and Ca2+ associated proteins, as well as rapid Ca2+ influx, all of them stimulated by the interaction of trophozoites with FN. The observed increment in phosphorylated proteins was correlated with increased adhesion to FN, increased protease secretion, and shifts of the soluble actin to its polymerized form. We also obtained evidence for the stimulation of actin mRNA synthesis after activation of the protein kinase C pathway.

Adenylyl cyclase activation in the throphozites has also been studied as well as the effect of augmented cytoplasmic cAMP levels on actin gene expression and actin organization. It is now clear that *E. histolytica* responds to activation of Gs and Gi -like proteins and therefore, more than one FN receptor must operate in the trophozoites. As support for this, the presence of an integrin b1-like protein has been recently described which binds FN with high affinity. There are also strong indications for the presence, in at least one actin gene, of regulatory elements in the actin promoter sequence that respond to increases in the levels of both cAMP and diacylglycerol, up-regulating the transcription of actin mRNA. The interaction of protein kinase C and protein kinase A phosphorylated proteins with these cre and sre-like elements could modulate the organization and function of the actin cytoskeleton, by regulating the levels of actin mRNA and the protein. As motility functions, that depend on actin organization, are directly related with the invasive behavior of the parasite, we could suggest that interaction of trophozoites with extracellular matrix proteins triggers processes conducive to facilitate invasion.

To further explore this idea, we tested the induction of chemokinesis and chemotaxis in trophozoites by FN fragments generated by digestion with cathepsin B. Some of the proteinases identified in trophozoites and partially released to the external medium are similar to cathepsins. It was found that two fragments of the FN molecule, one corresponding to the central domain containing the cell binding GRD sequence, and one corresponding to the NH2-terminal end, previously identified to be the binding site for the 37 KDa FN-binding protein, were capable to induce significant chemokinesis and chemotaxis of the trophozoites, while the complete FN molecule or other fragments of the FN failed to do so.

In the proposed model, binding to FN followed by release of FN fragments, generated by substrate degradation as result of induction of signal transduction processes, will guide directed motility and locomotion of the trophozoites. At the same time, the generated FN fragments will attract other cells that contribute to the inflammatory processes observed at the sites of tissue damage.

C15 - ULTRASTRUCTURAI. ASPECI-S OF SOME LIFE CYCLE STAGBS OF PROTOCTIST PARASITES OF DIFFERENT HOSTS

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Protozoology, Protistology, and Protoctisology are terms usually applied to different descriptions of several kinds of microorganisms effectively considered, as "first animals ".

Recently, it was erected the kingdom Protocfista that includes seven phyla (Sarcomastigophora, Labyrinthulomycota, Apicomplexa, Microspora, Myxozoa, Ascetospora and Ciliophora), most of them containing different species of parasites some of which are pathogenic.

This exposition was organized around the two partners, or antagonists, involved in a biotic disease, agent and host. The disease agent (pathogen) benefits flom its host, another different organism, which tends to be negatively affected by the presence of the agent.

Since the number of recognived infection agents and facts of known diseases am continuously increasing, it is necessary to update current knowledge. Thus, both published and new accounts of viruses, bacteria, proloctista, fungi, and infectious diseases of unknown aetiology in commercially exploited shellfish (molluscs, mhinoderms, crustaceans) and fishes are summatised.

In this explanation we try, therefore, to present the life cycle of some species of the phyla Apicomplexa, Microspora, Myxozoa and Ascetospora, which are associated with host cell degradation during the developmental life cycle stages. On the other hand, we try to take advantage of our own experience in these phyla, and the results and of the results obtained by transmission electron microscopic studies (1, 2, 3, 4).

Phylum Apicomplexa - This phylum is by far one of the largest of protoctistan parasites, estimated in about 5,000 described species. They are characterized by the presence of an apical complex found in the infective stages of life cycles, composed by the conoid, polar ring, micronemes, rhoptries and subpellicular microtubules.

Among these, protoctista, the best known species of the class Pcrkinsanida is *Perkinsus marinus*, that causes extensive and serious mortality rates in the American oyster, *Crassostrea virginica* (11). *Perkinsus atlanticus* is another pathogen found in European clams that causes high mortalities in this region. The merogony is represent by numerous spherical trophozoits found mainly in the gill tissues. These successive binary fissions occurred within a prezoosporangium, each giving rise to some hundred prezoospores. Cell multiplication occurs as a result of the different life cycles of one karyokinesis followed by cytokinesis. This process is called zoosporulation producing numerous zoospores within a zoosporinginm During the zoosporulation the cells became, elongate and a typical apical complex as differentiated. The zoospores are biffagellated and uninucleatt cells, constituting the cellular agent of direct of infection transmitted from clam to clam.

Phylum Microspora - This phylum is characterized by a considerable diversity of ultrastructures of spores: extrusion apparatus with a well-devoloped polar filament and polaroplaste- unicellular spore without aperture in the wall.

Recently, it was compiled a final list of 118 available generic names of the microsporidan species (13), which presents some thousand species. For explanation of the general ultrastructurat study of the spores and life cycle, we may use the the species *Abelspora portucalensis*, parasite of a commercially important crab from Portugal (2). Generally in the same host it was possible to observe all life cycle stages. The schizonts are uninucleate. Later, centriolar plaques begin their differentiation around the nucleus. The nuclear division is followed by cytokinesis, giving rise to two sporonts. The latter were, ultrastructurally characterized by the appearance of an incomplete coat of amorphous electron-dense material. During this please several vesicles are in close contact with the plasmalemma. Uninucleate sporont begins its nuclear division as signalled by the appearance of mitotic figures. A microtubular manchette is diffrerentiated in the nucleoplasm. Each sporont gives rise to two sporoblasts within a sporophorous vacuole. By a complex differentiation the sporoblasts developed directly into spores.

Phylum Myxozoa - This plylum, also known by tht name Myxosporida, contains more than one thousand of parasitized species, mostly in fishes. These parasites are represented in host tissues by numerous spores, that are multiccllular, with one or more polar capsules and sporoplasm. One to three, delimiting wall valves are, formed around the internal cells.

In this explanation we described *Henneguva amazonica*, recently erected in honour of the Amazon river, where the parasite was found, parasitizing an Amazonan fish (12). Sporogenesis begins by one uninucleate cell envolving another uninucleate cell. These two cells are, known as sperogonic cells that were engulfed by an enveloping cell or periryte. Together they are considered to be, a young sporoblast. The sporogonic cell starts successive bipartitioning, while the pericyte enlarges, within each periryte vacuoic are formed five cells which then diffetentiate by a complex proccss into a spore. For each spore two sporogonic cells become valvogenic cells, two become capsulogenic cells, while one undergoes karyokine,sis and becomes a binucleate sporoplasm. During this phase each of the valvogenic cells differentiate and tapes as a caudal projection, forming a long tail.

Phylum Ascetospora - This phylum consists of about 30 described species, most of which belong to the genera *Haplosporidium* and *Minchinia*. Both belong to a group of parasitic protoctist that form spores with only one,

sporoplasm contained in a spore wall that has an orifice covered by an opercular sistem. The uninucleate sporoplasm contains one spherulosome and several haplosporosome that are typical structures in this group. The ornaments (filaments or tails) attached to the spore wall or the absence of them are used to distinguish the genera *Haplosporidium* and *Minchina*.

For the general description of the haplosporidian species we use the species *Haplosporidium Iusitanicum* parasite of a Portuguese mollusc that was described some years ago (1). This parasite is represented in all life cycle stages of the host including mature spores. After excystment, the endosporoplasm becomes an amoebae that develops into plasmodium by enlargement and nuclear multiplication as evidenced by mitotic figures, each consisting of a bundle of microtubules attached to spindle polar bodies. This cell becomes successively bi-tetrad-octanucleated, etc... forming a multinucleate sporont containing several hundred nuclei. By complex diferentiation, each nucleus with its cytoplasm and surrounding plasmmalema, becomes a sporoblast; among the soporoblasts a light space of variable size is formed as the sporoblast becomes more spherical in shape. The sporoblasts are surrounded by a very irregular wall and this assemblage is collectively called a sporocyst. Each uninucleate sporoblat then forms a single spore, a process in which the protoplast is separated into separate uninucleate and anucleate units, the latter almost completely encasing the former except where the future spore orifice is located. Spore wall AL'AIXL;Al @.yllth@ in the epispore cytoplasm against the membrane facing the sporoplasn plasmalemma. The wall ornaments (filaments, or tails), are synthesized in the epispore cytoplasm in vacuoles and are then fused with the outer layer of the wall.

Finally, I hope that protoctistological topics of different species will be subject to review and discussion.

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C16 - EVIDENCE IN SUPPORT OF A CARRIER-MEDIATED CHLOROQUINE UPTAKE MECHANISM IN *PLASMODIUM FALCIPARUM*

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Chloroquine was the first choice antimalarial drug for more than three decades until the emergence and spread of chloroquine resistant *Plasmodium falciparum* strains rendered its application ineffective in many parts of the world. Over recent years the incidence of malaria has soared to approximately 500 million clinical cases per year as other available antimalarial drugs are not as effective, safe nor affordable as chloroquine. Understanding the molecular mechanism of chloroquine resistance and action may aid in the rational design of novel antimalarial drugs with improved pharmacological properties. Chloroquine targets the intra-erythrocytic stages of *P. falciparum* (1, 2), which are responsible for the high morbidity and mortality associated with tropical malaria. These stages feed on the host erythrocyte's hemoglobin which is digested within the acidic vacuole of the parasite. Heme released during this process is capable of amaging biological membranes and inhibiting the activity of several enzymes (3). By polymerizing free heme into an insoluble hemozoin the parasite has contrived a novel mechanism to detoxify heme (2). Chloroquine, accumulating to high concentrations in the acidic vacuole, exerts its specific antimalarial effect in the inhibition of heme polymerization (4, 5, 6). Chloroquine resistance does not seem to involve changes in hemoglobin degradation, heme detoxification or drug metabolism, but appears to be associated with the reduction of the intracellular chloroquine concentration (7). While chloroquine sensitive (CQS *P. falciparum* clones accumulate the drug to high concentrations, chloroquine resistant (CQR) parasite clones do not, suggesting that CQR parasites are capable of keeping their chloroquine concentration below toxic levels. This conclusion had previously led to the hypothesis that CQR parasites have either lost a chloroquine import mechanism (8) or, alternatively, have acquired a rapid chloroquine efflux mechanism (9). While alteration in chloroquine transport supplied an attractive model for

the CQR phenotype, the existence of a chloroquine transport mechanism had never been conclusively substantiated in *P. falciparum*.We have investigated the kinetics of chloroquine uptake by *P. falciparum* infected erythrocyte to ascertain whether chloroquine enters the parasite by passive diffusion or a carrier-mediated process. The finding that the chloroquine uptake by *P. falciparum* s temperature-dependent, saturable and inhibitable has provided strong evidence in favor of a carrier that mediates chloroquine uptake in *P. falciparum* (10). In comparison, uptake of chloroquine by uninfected erythrocytes was found to be a diffusion-controlled process (10). We were further able to show that, while facilitated chloroquine uptake exists in both CQS and CQR parasite clones, the kinetics differ. CQR parasites consistently have a chloroquine importer with a reduced transport efficiency (10). The genetic linkage oberved between changes in the chloroquine import kinetics and the CQR phenotype has provided strong evidence that a reduction in chloroquine uptake is a minimal and necessary event in the generation of the CQR phenotype. Several lines of evidence exist to suggest that the chloroquine importer is the *P. falciparum* NHE, a plasma membrane protein involved in cytoplasmic pH and cell volume regulation (11). Firstly, chloroquine uptake is competitively inhibited by a broad rang of NHE inhibitors. The apparent constants of inhibition observed vary amongst the NHE inhibitors examined, defining a potency scale, which, significantly, directly correlates with the ability of these NHE inhibitors to block NHE activity. Secondly, chloroquine uptake coincides with changes in the cytoplasmic pH and sodium ion concentration, both of which are indicative of NHE activity (11). On the addition of chloroquine, protons move from the parasite cytoplasm into the host erythrocyte, resulting in an alkalization in the parasite and an acidification of the host erythrocyte. At the same time, the parasite's cytoplasmic sodium ion concentration rises. As chloroquine uptake, proton efflux and sodium ion influx all take place at the same time and, significantly, are all inhibited by EIPA, this would suggest a common basis for these events, i.e. NHE activity. On the basis of these data we propose that the *P. falciparum* NHE takes up chloroquine during a sodium/proton exchange reaction. A quantification of this reaction revealed that this is not a simple exchange reaction. We estimate that for each chloroquine molecule taken up approximately 500 protons are extruded. It was this disparity that let us to investigate the effect of chloroquine on *P. falciparum* NHE activity, thereby providing the third line of evidence linking chloroquine uptake with NHE activity. It was found that chloroquine activates the NHE of the CQS parasite clone HB3, resulting in an increase in the pH-dependent activity and working range (11). The activation of an NHE is facilitated by the sodium ion gradient across the plasma membrane, as shown in other systems. Thus, activation of the *P. falciparum* NHE appears to be required for chloroquine uptake and accumulation, suggesting that it provides the energy, stored in the sodium ion gradient across the parasite plasma membrane, to concentrate chloroquine against its gradient into the parasite. Once the NHE has reached its activated steady-state in the presence of chloroqui , the surge in ion exchange abates and no more chloroquine is taken up. Chloroquine uptake therefore appears to be a secondary active transport mechanism in *P. falciparum*. CQR parasite clones appear to have preempted most of the stimulatory effect caused by chloroquine, as chloroquine has no significant effect on the activity of their NHE (11). Their NHE already have an increased pH-dependent activity and working range in the absence of chloroquine, which suggests that the NHEs of CQR parasite clones are constitutively activated. As there is no further activation of the NHE of CQR parasites by chloroquine, there is no release in energy; no transient surge of sodium/hydrogen ion exchange occurs and, hence, no chloroquine is concentrated into the parasite. Based on these data we propose that the inability of chloroquine to effectively stimulate its own uptake through the constitutively activated NHEs of CQR parasites constitutes a minimal and necessary event in the generation of the CQR phenotype. The molecular basis for the constitutively activated NHE of CQR parasites remains to be determined. It is also not yet clear how these o 2, recently found to be genetically linked with the CQR phenotype.

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