C-001

DOUBLY INFECTED CELLS: TOOLS TO ASSESS THE DOMINANCE OF SIGNALS THAT CONTROL THE FUSION OF VACUOLES THAT ENCLOSE INTRACELLULAR PATHOGENS

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Numerous intracellular pathogens enter cells by mechanisms akin to classic phagocytosis but markedly distort post-engulfment events (Moulder JW, Microbiol Rev 49, 298, 1985; Falkow S, et al Ann Rev Cell Biol 8, 333, 1992). Thus, *L. monocytogenes*- and *T. cruzi*-secreted lysins allow the pathogens to leave their entry vesicles and find solace in the cytosol. *Chlamydia* or *Mycobacteria* remain in unacidified vacuoles that seem arrested in their maturation; fusion of these vacuoles with other vesicles belonging to the endocytic of phagocytic pathways is often restricted. Lastly, *Leishmania sp.*, or the rickettsia *Coxiella burnetii,* thrive within acidified, hydrolase-rich, fusion prone, phagolysosome-like vacuoles (Russell DG, Trends Cell Biol 5,125, 1995; Baca, OG et al, Trends Microbiol 2, 476, 1994).

Little is known of the signals and mechanisms by which pathogens control the fusion of their vacuoles with other host cell membrane-bound organelles. Fusion competence of phagocytic vacuoles obviously depends both on the nature of the particles and on the "cytosolic" software required for vacuolar fusion. Most studies have dealt with fusion inhibition, for which two general, non-exclusive, mechanisms can be proposed: a) Inhibition could be mediated by systemic effects of pathogen-associated- or -derived molecules. As a corollary, the maturation and fusion of bystander phagosomes with lysosomes might be also inhibited in infected cells; such "spreading" inhibition was not found in *Chlamydia* infected cells (Eissenberg LG, Wyrick PB, Inf & Immun 32, 889, 1981). b) A second hypothesis is that inhibition of fusion is strictly localized to pathogen-containing vacuoles; again, it could be mediated by molecules expressed on the surface of the pathogens, and/or secreted into the lumen of the phagosomes. Fusion *inhibition* is easier to define and to detect than fusion-*enhancement*. The latter could signify increased efficiency of fusion and/or changes in particle selectivity. At this time it is only possible to rank different vacuoles as to their selectivity and efficiency of fusion with other vacuoles.

Uses of dual infections. Cells coinfected with different viruses, virus strains or mutants have often been used to examine viral recombination, selection or interference. Cells were also coinfected with a virus (HIV, CMV...) and a bacterium or protozoan. Only rarely were mammalian cells doubly infected with bacteria and/or protozoa. In two instances, cells were coinfected with *Toxoplasma* and with either *T. cruzi* (Meirelles, MN, de Souza, W, J Submicr Cytol 15, 889, 1983) or *M. tuberculosis* (Black, CM et al, J Exp Med 172, 977, 1990). Colocalization of the two organisms in the same vacuoles was only rarely found.

We chose to study coinfection models in which at least one of the pathogens is sheltered in vacuoles capable of fusion. These experiments were prompted by earlier studies of the vectorial transfer of zymosan particles, phagocytosed by infected macrophages, to the lumen of *L. amazonensis* parasitophorous vacuoles (PVs) (Veras, PST et al, J Exp Med 176, 639, 1992). Fusion was inferred from the colocalization of amastigotes and particles in the same vacuoles. It should be noted that, at this time, detection and measurement of fusion of phagocytic vacuoles require some form of microscopy. The *L. amazonensis* model proved useful in studies of the pharmacological control of particle transfer to the PVs. Time-lapse cinemicrography revealed that transfer events were often clustered in time. We also compared the fusion competence of *L. amazonensis* and *C. burnetii* (phase II) vacuoles in CHO and Vero cells. We found that *Coxiella* vacuoles were both more fusogenic and more "promiscuous" than the particle-selective *L. amazonensis* PVs (Veras, PST et al J Cell Sci 107, 3065, 1994).

Fusion between Leishmania vacuoles. Our first coinfection studies were performed in 1991-92 with Dominique Germain. The large *L. amazonensis* PVs were used as recipient vacuoles to detect fusion

with donor vacuoles that contained homologous or heterologous parasites. Macrophages infected with "cold" or with 3H-thymidine labeled *L. amazonensis* amastigotes, were superinfected, respectively, with hot or cold parasites. Autoradiography was used to detect labeled- from unlabeled-amastigotes in the same PVs. In heterologous fusion, cells infected with *L. amazonensis* were superinfected with promastigotes of *L. braziliensis*; colocalization was ascertained by phase microscopy or confocal laser fluorescence microscopy. Examples of homologous and heterologous fusion were documented, but quantitative studies could not be reproducibly performed.

Fusion between Coxiella and *Leishmania vacuoles*. Vacuoles containing both *Coxiella* and *L. amazonensis* rapidly formed when *C. burnetii*-infected cells were superinfected with *Leishmania*. Amastigotes increased in numbers within the mixed vacuoles and some transformed into promastigotes when the cultures were shifted to 25°C (Veras, PST et al, Inf & Imm, in press). *Fusion between T. cruzi and Coxiella vacuoles*. *T. cruzi* invade non-professional phagocytes by non-conventional phagocytosis and enter host cell lysosomes that migrate to the cell periphery (Andrews NW, Trends Cell Biol 5, 133, 1995). When *C. burnetii* infected cells were challenged with *T. cruzi*, trypomastigotes entered *Coxiella* vacuoles and differentiated into amastigotes without exiting to the cytosol. The impressive movement of the parasites within the vacuoles was documented in real time video with J.M. Panaud and O. Touchard (Pasteur). Amastigotes did not enter *Coxiella* vacuoles when cells were infected first with *T. cruzi* and one day later with *Coxiella* (Veras PST et al, experiments in progress).

In the previous experiments, fusion probably took place between phagolysosome-like partner vacuoles as, in single infections, *C. burnetii*, *L. amazonensis* or, transiently, *T. cruzi* all live within phagolysosomes. These experiments not only tested for the fusion between pathogen-containing vacuoles, but also for the survival of the parasites in the "hybrid" vacuoles. In the next experiments, one of the partners, *Mycobacterium avium*, normally resides in a fusion restricted vacuole. Would fusion- inhibitory or fusion-enhancing signals predominate ?

Fusion between M. avium and C. burnetii vacuoles. *M. avium* vacuoles most likely fuse with early endosomes but not with secondary lysosomes or phagosomes (Frehel C et al, Inf & Immun 52, 252, 1986; Xu, S et et al, J Immunol 153, 2568, 1994; de Chastellier C et al, Eur J Cell Biol in press). Mouse macrophages, infected with *M. avium* for 8 days, were challenged with *C. burnetii* for 6 to 48 h, fixed and examined by transmission electron microscopy. Twenty four h after superinfection 90% of the *M. avium* colocalized with *Coxiella* in the same vacuoles. Whether in single or in mixed vacuoles, *M. avium* were well preserved ultrastructurally. In parallel experiments, transfer of *M. avium* to *L.amazonensis* PVs was far less efficient, as only 15% of the *Mycobacteria* were found within the PVs 2 days after superinfection (de Chastellier, Thibon and Rabinovitch, in preparation). It will be important to determine if *M. avium* can survive and perhaps multiply within *C. burnetii* vacuoles and if the transferred *Mycobacteria* modify the pH, hydrolase content or membrane markers of the hybrid vacuoles.

C. burnetii vacuoles, novel fusion-meters ? It is likely that vacuoles will be found that resist fusion with *Coxiella* vacuoles. However, the high efficiency of fusion of *Coxiella* vacuoles with other phagocytic vacuoles, and the finding that *Coxiella* can open up *M. avium* vacuoles, suggest the search for vacuole fusion-enhancing factors associated with or secreted by *C. burnetii*.

C-002

NEW SIGNAL TRANSDUCTION PATHWAY IN *TRYPANOSOMA CRUZI.*

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In mammalian cells L-arginine is metabolized to yield nitric oxide (NO), also known as endothelium-derived relaxing factor. Within neural or endothelial cells, where it originates or in neighboring cells, NO activates heme-containing soluble guanylyl cyclase , acting therefore as either an intracellular

or an intercellular signaling molecule. Consequently, NO formation is associated with a rise in cyclic GMP levels. NO synthases are the enzymes responsible for the conversion of L-arginine to NO. These synthases exist as two groups of isoforms: constitutive and inducible. NO synthases of the first group require Ca2+ and calmodulin and are found in endothelium and the central nervous system. These constitutive NO synthases also require NADPH, have binding sites for tetrahydrobiopterin, flavin adenine dinucleotide, flavin adenine mononucleotide, calmodulin, and heme and can be phosphorylated by a variety of protein kinases . NO synthases of the second group are found in macrophages, vascular smooth muscle cells, fibroblasts and hepatocytes. In the central nervous system, excitatory synaptic transmission is mediated by L-glutamate. This ligand interacts with at least three functional receptor groups: a-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)-kainate, N-methyl-D-aspartate (NMDA) and metabotropic receptors .

From an evolutionary viewpoint, evidence indicates that the NO transduction signaling pathway is operative only in higher eukaryotic organisms. Our work provide the first demonstration that this pathway is also present in the lower eukaryotic organism *Trypanosoma cruzi.*

A nitric oxide synthase was partially purified from *T.cruzi* epimastigote soluble extracts. The conversion of L-arginine to citrulline by this enzyme activity requires NADPH, it was activated by Ca2+, calmodulin, tetrahydrobiopterin and FAD and inhibited by N-methyl-L-arginine. L-glutamate and N-methyl-D-aspartate (NMDA) stimulated *in vivo* conversion of L-arginine to citrulline by epimastigote cells. These stimulations were blocked by EDTA, MK-801 and ketamine and enhanced by glycine. A sodium nitroprusside-activable guanylyl cyclase activity was detected in cell-free, soluble, preparations from *Trypanosoma cruzi* epimastigotes. L-glutamate, NMDA and sodium nitroprusside increased cyclic GMP levels in *T. cruzi* epimastigotes. This evidence shows that in *Trypanosoma cruzi* epimastigotes, L-glutamate controls cyclic GMP levels through a pathway mediated by nitric oxide.

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C-003

QUANTITATIVE PREDICTION OF MHC-BINDING SEQUENCES WITHIN PROTEIN ANTIGENS

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The identification and analysis of MHC binding sequences within protein antigens and ultimately the ability to predict them is central to immunology. Recent advances reveal increasingly complex MHC binding motifs and allow prediction of sequences that bind to MHC molecules. The systematic characterisation of binding motifs for ail human MHC alleles is now possible and will facilitate the design of peptides for therapeutic intervention.

The rules for peptide-MHC class II interaction

Recently, the motifs of various human class II (DR) molecules have been identified by the characterisation of large, DR-bound peptide pools selected from M13 peptide display libraries [1-3]. These motifs consist of several peptide positions where amino acids with similar side chains occur with increased frequencies (anchor positions).

The anchor position closest to the N-terminus (anchor position 1) is essential for a high affinity interaction between peptides and all DR molecules tested so far [4-5]. The position 1 anchor accepts only aromatic or large aliphatic peptide side chains that interact with a deep pocket in the DR binding groove, built by both the invariant DR alpha-chain and a fairly conserved part of the DR beta-chain [6-7]. Other major, but less essential, anchor positions are found at peptide positions 4, 6 and 9 [3-4]. The peptide side chains in these positions interact with shallow pockets shaped by clusters of polymorphic residues of the DR beta-chain and determine allele specific peptide binding.

Of similar importance for peptide-class II interaction is the presence or absence of peptide side chains which can interfere with peptide binding (inhibitory residues). Studies on designer peptide libraries and natural peptide sequences have indicated both position- and allele-specific properties of inhibi-

tory residues [3-4]. Interestingly, inhibitory residues are found more frequently at anchor positions, since these are the major contact sites for the peptide side chains with the class II molecule. The capacity of a given peptide to bind a certain MHC class II molecule therefore, is the result of attracting and repelling forces between peptide side chains and residues lining the MHC binding site.

Quantitative MHC binding motifs

The growing evidence that peptide side chain effects (anchor, inhibitory or neutral effects) depend on the position within a particular peptide-frame rather than on neighbouring amino acids, has led to the approximation that each amino acid in a peptide sequence contributes independently of one another to the affinity of the peptide [2-3]. The increasingly available multiple-peptide synthesis technology, together with new high-flux in vitro binding assays allowed this approximation to be tested. The important consequence is the possibility to quantitatively predict the affinity of MHC binding sequences, once the effects of each amino acid at all positions have been.

We have used the DRB1*0401 molecule to design a strategy for identifying quantitative motifs, that is widely applicable to many other human class II DR molecules. These studies confirm (i) the working hypothesis of independent binding of side chains as a useful first approximation, and (ii) that quantitative motifs can have a remarkable predictive power. Algorithms based on these quantitative motifs ranked high affinity binding peptides and T cell epitopes in the top 2-4% of all possible peptide-frames of given antigens. The immunodominant T cell epitopes of the human myelin basic protein or the influenza haemagglutinin, for example, were placed at the top of all possible frames [2].

Vaccine development

The identification of the rules for peptide binding to MHC molecules makes it possible to predict antigenic peptides within proteins of pathogens with known sequences. This facilitates the development of subunit vaccines. To be effective and applicable to the outbred population, subunit vaccines have to contain either a cocktail of peptides or a single promiscuous T-cell determinant that would protect individuals with a variety of MHC combinations. In theory, promiscuous peptides should either contain overlapping MHC binding motifs or, when only one binding frame is used, they should use anchors that are conserved among MHC molecules and should lack allele-specific contact sites that prevent binding to other MHC molecules. The latter is likely to occur for peptides binding to HLA-DR alleles, which account for approximately 90% of all human class II molecules expressed on the surface of antigen presenting cells. Unlike the polymorphic beta-chain, the alpha chain is invariant in all DR alleles and forms half of the binding cleft [6], thus providing the structural basis for promiscuous peptide binding. More specifically, the DR alpha-chain together with a fairly conserved part of the beta-chain, creates a deep pocket in the binding groove that interacts with a conserved anchor position 1 (p1) in class II binding peptides [1, 7]. The p1 anchor is essential for high affinity interaction with most DR alleles, and can even be sufficient in peptides which otherwise contain no inhibitory residues [8]. Substitution experiments and high stringency screenings of phage libraries defined a second conserved anchor position at p2, and a third, but less conserved one at p4 [4]. Altogether, they increase the chance of peptides binding in a promiscuous mode. The prediction of promiscuous T helper cell epitopes may, therefore, be rather simple, once quantitative motifs for most HLA-DR molecules have been determined. The quantitative values of each allele could then be combined into a "supermotif" that is able to predict epitopes effective in most individuals of an outbred population.

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C-004

"BASIC RESEARCH IN CHAGAS' DISEASE: SCENARIOS FOR THE FUTURE"

Carlos Médicis Morel

MC-001

RNA EDITING IN KINETOPLASTID MITOCHONDRIA AND THE GUIDE RNA PARADIGM: SEVERAL CONUMDRUMS AND THEIR SOLUTIONS

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RNA editing is the post-transcriptional modification of the sequence of RNA molecules. Both substitution editing and insertion/deletion editing, have been described in a variety of eukaryotic organisms. The uridine addition/deletion type of RNA editing that occurs in the kinetoplast-mitochondrion of the kinetoplastid protozoa is one of the most well-studied of these phenomena. Uridines (U's) are inserted or (more rarely) deleted from multiple specific sites mainly within coding regions of mRNA transcripts of the mitochondrial maxicircle DNA. The guide RNA (gRNA) paradigm states that the sequence information for the insertion and deletion of uridines in mitochondrial DNA transcripts resides in gRNAs, which are small 3'-oligo-uridylated RNAs that can form duplex anchors with preedited mRNAs downstream of specific editing blocks and are in addition complementary to cognate blocks of mature edited mRNA sequence. The evidence for the involvement of gRNAs in the editing of maxicircle transcripts is compelling, but the precise mechanism for the transfer of U's to the editing site is still an open question. Two models are under investigation, the first involving a specific cleavage, a U addition, and a ligation, and the second involving two successive transesterifications transferring U's from the 3' tail of the gRNA to the editing site.

Recently, several challenges to the gRNA paradigm have arisen which are addressed in this presentation. First, the C1 laboratory strain of *Crithidia fasciculata* was reported to contain a single homogeneous minicircle class, which would be insufficient to encode the variety of required gRNAs for the known editing of maxicircle transcripts. Secondly, the minicircle DNA molecules from *Trypanosoma cruzi* are extremely heterogeneous in sequence within a strain and do not even cross-hybridize between different strains or stocks, thereby raising the question of the existence of a homologous set of encoded gRNA genes in such a diverse population of minicircle molecules. Finally, the cryptobiid kinetoplastid, *Trypanoplasma borreli*, does not even contain minicircular DNA in its mitochondrion, but does undergo editing and even pan-editing of some mRNA transcripts. This raises the question of the genomic localization of the gRNA genes in this species.

These conundrums have been solved and the solutions firmly establish the robustness of the gRNA paradigm.

MC-002

THE ENZYMOLOGY OF GPI BIOSYNTHESIS IN TRYPANOSOMES

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Protein-linked glycosyl-phosphatidylinositol (GPI) anchors and GPI-related glycolipids, such as the lipophosphoglycans and glycoinositol-phospholipids of the *Leishmania*, are particularly abundant in the protozoa (McConville and Ferguson, 1993). The African trypanosomes are able to survive in the mammalian bloodstream by virtue of their dense cell-surface variant surface glycoprotein (VSG) coat. This coat consists of 10 million 55 kDa GPI-anchored VSG molecules (Cross, 1990). The relative abundance of the VSG protein in *Trypanosoma brucei* has made this organism extremely useful for the study of GPI anchor biosynthesis. The structure of the VSG GPI anchor is known (Ferguson *et al.*, 1988) and principal features of the GPI biosynthetic pathway in trypanosomes were elucidated using a cell-free system based on washed trypanosome membranes (Masterson *et al.*, 1989, 1990; Menon *et al.*, 1990b). The first step in the pathway involves the transfer of GlcNAc from UDP-GlcNAc to endogenous phosphatidylinositol (PI), via a sulphydryl-dependent GlcNAc-transferase (Milne *et al.*, 1992), to form GlcNAc-PI which is rapidly de-N-acetylated (Doering *et al.*, 1989) to form glucosaminyl-PI (GlcN-PI). Three a-mannose residues are sequentially transferred onto GlcN-PI from dolichol-phosphate-mannose (DPM) (Menon *et al.*, 1990a) to form the intermediate Man3GlcN-PI. At least this much of the pathway is believed to occur on the cytoplasmic face of the endoplasmic reticulum (Vidugiriene and Menon, 1994). Ethanolamine phosphate (EtNP) is then transferred from phosphatidylethanolamine (Menon *et al.*, 1993) to the terminal mannose residue to form EtNP-Man3GlcN-PI (known as glycolipid A'). This species then undergoes a series of fatty acid remodelling reactions (Masterson *et al.*, 1990) as follows: The *sn*-2-fatty acid of glycolipid A' is removed to form a *lyso*-species called glycolipid q. Glycolipid q is myristoylated to form glycolipid A'' (which contains *sn*-1-stearoyl-2-myristoylglycerol). The sn-1-stearoyl group is removed from glycolipid A'' and replaced by myristic acid to form glycolipid A. The donor molecule for the two myristoyl-transferase steps is most likely myristoyl-CoA. Concomitant with the formation of glycolipid A is the formation of glycolipid C (the inositol-acylated version of glycolipid A). The structures of glycolipids A and C are: NH2-(CH2)2-PO4-6Mana1-2Mana1-6Mana1-4GlcNa1-6*myo*-inositol-1-PO4-*sn*-1,2-dimyristoylglycerol (EtNP-Man3GlcN-PI) and NH2-(CH2)2-PO4-6Mana1-2Mana1-6Mana1-4GlcNa1-6(acyl)*myo*-inositol-1-PO4-*sn*-1,2-dimyristoylglycerol (EtNP-Man3GlcN-(acyl)PI), respectively (Mayor *et al.*, 1990a,b). The acyl group in hydroxy-ester linkage to the inositol ring in glycolipid C is believed to be predominantly palmitate (Mayor *et al.*, 1990b) and is attached to the 2-position of the *myo*-inositol ring (Ferguson, 1992). Both glycolipid A and glycolipid C have been shown to be competent for transfer to VSG polypeptide when added exogenously to a trypanosome cell-free system (Mayor *et al.*, 1991), although there is no evidence for the transfer of glycolipid C *in vivo*. The role of glycolipid C is unclear. However, recent data have shown that it is not an obligatory intermediate on the pathway to glycolipid A and suggest that it is in dynamic equilibrium with glycolipid A, via the action of a phenylmethylsulphonyl fluoride (PMSF)-sensitive inositol-acyltransferase (AC) and a diisopropylfluoro phosphate (DFP)-sensitive inositol-deacylase (CA) (Güther *et al.*, 1994b; Güther and Ferguson, 1995). It is possible that glycolipid C can act as a reservoir for excess glycolipid A.

Recent data on the substrate specificities of the second and third enzymes of the GPI biosynthetic pathway (i.e., the GlcNAc-PI de-N-acetylase and the GDP-Man:GlcN-PI 1-4 mannosyltransferase) will be presented, as well as a description of the roles of inositol-acylation and inositol-deacylation.

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MC-003

CAN IMMUNITY TO TRANS-SIALIDASE AND SIALIC ACID ACCEPTORS CONTROL PARASITISM OF CHAGAS' DISEASE?

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Trypanosoma cruzi expresses an enzyme, trans-sialidase (TS), which removes sialic acid from host derived glycoconjugates and attaches it to mucin-like glycoprotein acceptors on the parasite surface (4). The exact role of this process is unknown, but it has been postulated that expression of high levels of TS and the sialylation of the parasite might be important for the infection and development of Chagas' disease. We have previously shown that *T .cruzi* infection induces antibodies to the catalytic domain of TS that inhibit TS enzymatic activity (2). The catalytic domain of the TS is located at the N-terminus of the protein, and it is sufficient for activity in the absence of C-terminus region which contain repeats of 12 amino acids (3). On the other hand, antibodies to the sialic acid acceptor mucins were shown to neutralize *in vitro* infection and neutralize parasites injected into mice (5). In human Chagasic patients, anti-a-galactose antibodies, which also recognize epitopes present in the mucin molecules, were shown to cause lysis of infective forms in absence of complement (1). In this presentation, we will show our recent experiments intending to test whether the TS or the sialic acid acceptor-mucins induce immunoprotection to *T. cruzi* in the murine model.

To study the immunogenic characteristics of TS we have cloned a PCR derived fragment of TS gene in the plasmid pQE-60 (Qiagen) and expressed its amino-terminal domain in *Escherichia coli.* Recombinant protein was obtained by growing bacteria containing the recombinant plasmid (PTS-cat) in the presence of IPTG. The bacterial lysate was incubated with a NiNTa-agarose and bound proteins were eluted with imidazole. TS was further purified by a MonoQ-FPLC ion exchange chromatography. The recombinant enzyme eluted with a NaCl gradient and appears as a single band of 70 kD on SDS-PAGE. Several parameters of the enzymatic activity of the recombinant TS were shown to be similar to those found for the native TS purified from trypomastigotes supernatant (native TS) (3).

To investigate whether antibodies to TS participate in the control of *T. cruzi* infection in mice, the recombinant TS, as well as the native TS, heat denatured recombinant TS and a synthetic peptide based on the repeats of the carboxyl terminus of TS conjugated to hemocyanin were used for immunizations. A/Sn or BALB/c mice received 3-4 doses of 5 to 20mg of each protein and adjuvant (Alum) in intervals of 10 to 15 days. Sera from mice immunized with either the native TS and the recombinant TS (active form) strongly inhibited TS enzymatic activity as measured by the transfer of sialic acid from sialyllactose

to D-glucose-1-[14C]lactose. In contrast, sera from mice immunized with denatured recombinant TS (inactive), or the synthetic peptides to the repeats had no inhibitory activity, but displayed high antibody titers when assayed by ELISA with the native TS. Upon challenge with a dose of 500-103 blood forms trypomastigotes ip, the parasitemia of mice immunized with native TS, recombinant (native or denatured) or even the synthetic peptide was lower than control mice. However, there was no significant difference in the parasitemia among mice challenged with 105 trypomastigotes. To confirm whether the antibodies generated with the native forms of TS were inhibiting the parasite sialylation *in vivo,* trypomastigotes were collected from the blood of mice, fixed to glass slides and incubated with a biotinylated monoclonal antibody specific for sialic acid (mAb 3C9) and FITC-streptavidin. The extent of sialylation was much lower in parasites colletect from mice immunized with active TS than with the controls. Therefore, inhibition of TS *in vivo* is insufficient to induce strong protection, and other epitopes of TS not to the active site can generate a partial protective response. To determine whether the inhibitory antibodies could have an impact on the tissue distribution of parasites, heart, striated muscle, liver and spleen were obtained from mice immunized with recombinant TS and the parasite DNA measured 4 days after a challenge with 2 X 106 culture trypomastigotes iv. The tissue DNA was extracted and parasite DNA estimated by hybridization with *T. cruzi*-specific DNA probe. The distribution of parasites were similar in both groups, demonstrating that inhibition of TS does not have an important role in the tissue distribution of parasites.

Next, we asked if antibodies to the sialic acid acceptors could have protective role. For this purpose, we investigated whether a passive transfer of a sialic acid-specific monoclonal antibody (mAb 3C9) could modify acute blood and tissue parasitism. Mice received 0.5 mg of purified mAb 3C9, subclass IgG1, iv, before infection with tissue culture derived trypomastigotes. The mAb 3C9 decreased significantly the parasitemia even when we used for challenge a dose as high as 2 x 106 parasites iv. The effect was dose dependent and specific. Adoptive transfer of the same amount of mAb 39, which recognizes the carboxyl-terminal region of trans-sialidase, had no inhibitory activity. To determine whether mAb 3C9 also reduced tissue parasitism, we estimated the amount of parasite DNA in the different organs. Mice passively transferred with mAb 3C9 had a significant lower number of parasites in the heart, skeletal muscle, liver, spleen as well as in the blood. These results were further confirmed by light microscopy analysis of hystological slides made from different organs of the same animals. In conclusion, the immunity to TS participate in the control of *T. cruzi* infection and the sialic acid acceptors are important targets for the immune system.

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MC-004

IDENTIFICATION OF *LEISHMANIA* GENES BY FUNCTIONAL COMPLEMENTATION: APPLICATION TO DRUG RESISTANCE AND VIRULENCE

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There are many strategies for identifying parasite genes important to the infectious cycle. For ex-

ample, one may seek genes that are developmentally regulated, or proteins targetted to the parasite surface, or genes homologous to ones known to be important in other organisms. Another approach is to use genetic methods, in a way that permits the parasite itself to functionally identify the gene of interest. In fact, functional genetic complementation of avirulent mutants has revolutionized our understanding of the virulence factors of many prokaryotic pathogens. Our laboratory has recently developed such methods in *Leishmania* and here I discuss some of the parameters important in establishing this system, as well as its application to the identification of *Leishmania* genes involved in virulence and drug resistance.

Functional complementation begins with the ability to sample the complete genome by DNA transfection. We developed a shuttle vector, cLHYG, which can replicate in both *E. coli* and *Leishmania*, and contains drug resistance markers that can be selected for in either organism (Ryan *et al* 1993a). cLHYG is a cosmid vector and as such, has a capacity to carry 40 kb of passenger *Leishmania* genomic DNA. Since the *Leishmania* genome contains about 50 x 106 bp, only 1250 cosmids are minimally required to encompass the entire genome when inserted into cLHYG. Since this number of transfectants can be obtained on a single plate (Kapler *et al* 1990), it is possible to obtain 30,000 independent *Leishmania* transfectants with cLHYG libraries without much difficulty. It should be emphasized that the use of plating technology is a key factor, as this helps ensure that all cosmid transfectants are equally represented (this is well known to molecular biologists screening libraries in *E. coli*).

Another requirement is the mutant line destined to receive the library for complementation. Since *Leishmania* are predominantly diploid, even after heavy mutagenesis loss-of-function mutants are recovered at a low frequency, typically 10-6 or less. This makes brute force screening for avirulent mutants unfeasible, and selective methods are required, which poses a severe problem for the recovery of avirulent mutants. However, in special cases this limitation can be overcome. For example, Sam Turco's lab has shown that mutations affecting the synthesis of lipophosphoglycan (LPG), a key virulence determinant, can be recovered by selection for cells unable to be agglutinated by ricin (King & Turco, 1988). This approach could in principle be applied to other parasite surface molecules, with appropriate selective agglutinins. Our knowledge of mutagenesis is not well advanced in *Leishmania*, and basic studies here could prove invaluable in our efforts to further develop genetics in this parasite.

Complementation of *L. donovani* LPG mutants. In collaboration with Turco's lab, we have succeeded in recovering cosmids which rescue all five different *L. donovani lpg*- mutants attempted thus far. The mutants R2D2 and YODA are defective in the synthesis of the LPG glycan core, while C3P0, OB1 and JEDI are defective in the synthesis of the LPG phosphodisaccharide repeats. We also have a large panel of new independent mutants currently under investigation. In this manner, we are following the classic model established in the analysis of microbial operons.

As expected, a number of genes encode the biosynthetic enzymes catalyzing the synthesis of LPG. For example, the gene which rescues R2D2, *LPG1*, predicts a glycosyltransferase responsible for addition of a Gal*f* residue within the LPG core (Ryan et al. 1993b). Since Gal*f* is a sugar common to many pathogens but absent in mammals, inhibition of this pathway could be an attractive target for rational chemotherapeutic attack.

Another class of LPG genes functions in assembly or cellular compartmentalization. *LPG2* is related to the yeast *VAN2/VRG4* gene, which is involved in general Golgi function, and *LPG3* is related to the HSP90 family (Descoteaux *et al* 1995). However, the C3P0 and OB1 mutants which are rescued by these genes show only defects in addition of the disaccharide phosphate repeating units, to both LPG and the secreted acid phosphatase. Remarkably, C3P0 extracts can make LPG in vitro, suggesting that its defect involves compartmentalization of a key precursor or enzyme. Otherwise, OB1 and C3P0 are normal with respect to Golgi function, and secretory or surface protein expression. These data suggest that *Leishmania* may have enlisted proteins of the general secretory pathway, to the specialized LPG pathway.

Studies of the LPG1 protein tagged with the Green Fluorescent Protein (GFP), and LPG2 tagged with the flu hemagglutinin epitope, suggest that both are localized in the Golgi apparatus (Ha *et al.* 1995; Descoteaux *et al.* 1995). Like many parasite surface proteins, LPG is anchored by a GPI anchor, however the enzymes responsible for protein GPI anchor biosynthesis are thought to be associated with the endoplasmic reticulum. The cell biology of LPG biosynthesis thus promises to yield a number of interesting surprises in the future.

Complementation of avirulent *L. major*. Although it is difficult to select for avirulent mutants, it

is well known that after lengthy periods of growth *in vitro* many *Leishmania* species lose the ability to cause animal infections. For use in complementation these lines have some potential drawbacks, such as the possibility of multiple mutations, but they are readily available. We explored their use by transfecting the avirulent *L. major* A1 line with a cLHYG library made from the parental virulent *L. major* V1 Friedlin strain DNA. After infection of mice with the transfected *Leishmania* library, a number of them showed infections, and the parasites and their cosmids were recovered. Retransfection of these showed that 10 were able to give infections. However, the phenotype of the cosmid transfectants was not identical to V1, as they do not give rise to metacyclic parasites and showed at best only a slight improvement in their ability to infect macrophages. Moreover, after infection the transfected parasites show a prolonged interval before the appearance of lesions. Amastigotes recovered from these lesions were much more virulent. However, if allowed to differentiate back to promastigotes before infection, they again showed a prolonged delay in lesion appearance. Our current model is that A1 possesses multiple defects, in both metacyclogenesis and growth as amastigotes, and that the genes recovered thus far in our screens primarily affect the latter process.

Molecular characterizations show that the 10 cosmids represent different loci, suggesting the possibility of multiple defects or the occurrence of genetic suppression. Dissection of the first cosmid shows the presence of number of interesting candidate genes, including a protein kinase and a protein phosphatase. Studies are underway to determine the responsible locus or loci therein, and the biochemical and genetic mechanism(s) by which they influence virulence.

Drug Resistance. One common mechanism of drug resistance is gene amplification, and in *Leishmania* this has been a favored method for the isolation of new genes (Beverley, 1991). Since cLHYG exists in multiple copies, genes included therein are effectively amplified. Following a protocol well-established in other microbes, one can identify cosmids mediating resistance by directly plating cosmid-transfected *Leishmania* libraries on various drugs. Our experience is that this is much faster and directed than traditional gene amplification-based strategies. Thus far we have applied this strategy to a number of different inhibitors, including nucleotide analogs and inhibitors of sterol biosynthesis. In total, more than two dozen loci have been identified and confirmed by re-transfection. One example is the identification of the *L. major* homolog of squalene synthase.

Summary. It is clear that under the right circumstances, functional genetic complementation can be profitably applied to the recovery genes relevant to several areas of *Leishmania* biology. Currently, the major limitations are 1) the ability to generate mutants, 2) the formulation of a feasible screen for the desired phenotypes, and 3) the rate at which the genes within a functional cosmid can be mapped and sequenced.

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MC-005

LEISHMANIA AND *MYCOBACTERIUM*: STOWAWAYS WITHIN THE ENDOSOMAL NETWORK.

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Much of the literature discussing intramacrophage pathogens is fixated on the apparent anomoly of why pathogens "choose" to parasitize a cell as hostile as the macrophage. However, the macrophage is actually the most likely cell to be exploited by microbes because of its very ability to phagocytose particles. Intramacrophage pathogens do not need a specific entry strategy, all they require is a suitable ligand on their surface, frequently provided by the third component C3 of the host's complement system. Once bound to an appropriate receptor, or receptors, they are phagocytosed. The frequency of the macrophage/microbe encounter throughout the evolution of both host and pathogen will have placed strong selective pressure on microbes to develop defences that prevent their subsequent degradation within the lysosomes of the macrophage. This presentation discusses the diametrically-opposite strategies employed by the intramacrophage pathogens *Leishmania* and *Mycobacterium* to ensure their survival within the endosomal/lysosomal system of their host cell.

The *Leishmania* amastigote resides in a membrane-bound compartment inside the macrophage of its vertebrate host. This interaction with the macrophage is an extremely stable one which appears to inflict minimal damage on the host cell. Indeed, protective immunity to leishmanial infection is mediated through activation of the host macrophage by interferon-g and TNF-b, and the macrophage "absorbs" the parasites and recovers its normal appearance and function.

This presentation will summarize much of our current knowledge about the biology of the parasitophorous vacuole inhabited by the *Leishmania mexicana* complex, which characteristically induce large, fluid-filled vacuoles that contain multiple parasites. The ability of this vacuole to mix with material in the endocytic pathway was first described by Alexander and Vickerman1. More recently Antoine and colleagues, and my own laboratory, have conducted a more extensive analysis of this compartment. Both laboratories demonstrated that the vacuole was acidic, Antoine's experiments on established *L. amazonensis*-containing vacuoles estimated the pH to be between pH 4.7 and 5.22, and our analysis on the acidification kinetics of *L. mexicana*-containing phagosomes demonstrated that they equilibrated to pH 5.3 within 30 minutes of internalization3. The vacuole contains lysosomal hydrolases4,5, and class II MHC in an *in vivo* infection or macrophages activated *in vitro*.5,6 The vacuole is positive for lysosomal membrane proteins such as LAMP/lgp glycoproteins2,5. It also accumulates the lysosomal-trafficking protein, the cation-independent mannose 6-phosphate receptor as the vacuole matures beyond 48 hrs5. Much of this label is associated with the lumen of the vacuole, as reported previously in autophagic compartments. The vacuole is readily accessible to fluid-phase endocytic markers and intersects with the trafficking of certain ligands internalized via receptor-dependent mechanisms5.

Mycobacterium species also infect macrophages but each bacillus tends to occupy an individual parasitophorous vacuole. These PVs appear to replicate with the bacilli. PVs that contain *Mycobacterium* have quite different properties to those that contain *Leishmania*. *Mycobacterium*-containing PVs are relatively inaccessible to the endocytic pathway of the host cell and their internal pH remains close to neutral. Consistent with this more neutral pH, the PVs containing *Mycobacterium* do not seem to have the proton-ATPase that generates an acidic pH within endosomes and lysosomes, suggesting that they do not fuse with lysosomes3. Nevertheless, the *Mycobacterium*-containing PVs contain a lysosomal membrane protein, LAMP 13,7.

There are at least two possible explanations for these apparently paradoxical findings: either lysosomes fuse with the PV, but the proton-ATPase is then selectively removed from the PV membrane, or the LAMP1 in the PVs is delivered not from lysosomes but as newly synthesised protein from the *trans* Golgi network in vesicles that do not carry the proton-ATPase. Either possibility would imply that the PVs containing *Mycobacterium* are not static, but communicate with certain elements of the cellular membrane systems. This is also indicated by the observation that a bacterial cell wall constituent, lipoarabinomannan, can be observed in vesicles that do not contain bacteria7, and by the theoretical

consideration that the total surface area of the PVs must increase as the bacteria divide and new PVs form. The components for this membrane growth most probably originate from other biosynthetic compartments such as the endoplasmic reticulum. Recently we have shown that despite the mycobacterial vacuole's reluctance to fuse with lysosomes, it is directly accessible to glycosphingolipids derived from the host macrophage plasmalemma providing direct evidence that the vacuole is in a dynamic state8.

Like macrophages infected with *Leishmania*, *Mycobacterium*-infected cells can be activated by IFN-g and TNF-a to promote killing of the bacilli. Remarkably, under these circumstances the PVs coalesce and are then able to acidify, presumably by fusion with lysosomes.

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MC-006

MOLECULAR ASPECTS OF INVASION OF MAMMALIAN CELLS BY METACYCLIC TRYPOMASTIGOTES OF *TRYPANOSOMA CRUZI*

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Infection of mammalian hosts by *Trypanosoma cruzi* is initiated when host cells are invaded by metacyclic trypomastigote forms released in the feces of triatomine vectors during their bloodmeal. This event, upon which depends the developments of *T. cruzi*, is probably a multi-step process involving a number of molecules on both the parasite and the host cell membranes. We have identified two surface molecules of metacyclic forms that bind to nonphagocytic mammalian cells and mediate parasite invasion (1,2): gp82, a metacyclic stage-specific 82 kDa glycoprotein (1,3) and gp35/50, a mucin-like glycoprotein of 35/50 kDa (4) expressed by the developmental stages found in the insect vector (5). Either one of these molecules can bind to target cells in a dose-dependent and saturable manner, and significantly inhibit cell invasion by metacyclic trypomastigotes.

To determine whether attachment of gp82 or gp35/50 to host cells could trigger Ca2+ signal, which has been reported to be a requisite for invasion of tissue culture trypomastigotes (6,7,8), experiments of Ca2+ mobilization were carried out by exposing HeLa cells preloaded with fura-2 to either of these molecules. Positive signals were obtained when 1.25 to 10 μg of purified gp82 or gp35/50 were added. At 10 μg, the rise in [Ca2+]i was lower than at 1.25-5 μg range. More intriguing was the finding that the Ca2+-signaling activity was further reduced or even abolished when HeLa cells were exposed to 25μg or higher amounts of gp35/50 or gp82. Why higher concentrations of these glycoproteins impair Ca2+-signaling activity is not clear. Our results also indicate that mammalian stage and insect stage trypomastigotes use different molecules to induce Ca2+ signal. For tissue culture trypomastigotes, it has been suggested that a Ca2+ signaling membrane factor, which remains to be defined, is generated by proteolysis of a precursor molecule by an alkaline peptidase (9). In the case of metacyclic forms, a sonicated extract containing a mix of protease inhibitors, including leupeptin that completely abolishes

Ca2+ signal by sonicated tissue culture trypomastigotes (9), induced a rise in HeLa cell [Ca2+]i of 100% or more above the basal level, the signal in the presence of protease inhibitors being always comparable to that detected in their absence. Furthermore, it should be noted that one of the metacyclic trypomastigote Ca2+ signaling molecules, gp35/50, is a highly glycosylated molecule (4) resistant to proteolysis (10), and the other, gp82, is generated from a 70 kDa precursor molecule by N-glycosylation (1).

Provided that Ca2+ signaling is a requisite for *T. cruzi* entry into host cells (8), factors that interfere with calcium mobilization would impair the rate of infection by metacyclic forms. To ascertain that this was the case, we performed a series of experiments by incubating HeLa cells with metacyclic trypomastigotes at 37oC for 3 h under different conditions. Treatment of HeLa cells with 1 μM thapsigargin, a global inhibitor of intracellular Ca2+ transport ATPases (11), reduced the Ca2+ signal to a value below the threshold level and significantly decreased the rate of cell invasion, in PBS containing 1% BSA plus 1mM Ca2+ Absence of calcium from the assay buffer had a moderate inhibitory effect (20-30%), but in Ca2+-free medium contaning 10μM calcium ionophore A23187 invasion of Hela cells was drastically reduced. These data suggest that release of Ca2+ from intracellular compartments, more than calcium influx from extracellular medium, may be regulating the process of host cell invasion by metacyclic forms.

In addition to these data, observations that gp35/50 and gp82 are differentially used by *T. cruzi* strains with different invasive capacity, and the possible role of another metacyclic stage-specific surface glycoprotein, gp90, as a negative modulator of invasion, will be discussed.

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MC-007

THE ROLE OF LPG IN LEISHMANIA-SANDFLY INTERACTIONS

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While there is much information on the biology of *Leishmania* within their phlebotomine vectors, this information is especially lacking in molecular detail. Understanding the basis of molecular interactions at the sandfly-*Leishmania* interface is fundamental to any study of vector competence and disease transmission.

The life-cycle of *Leishmania* parasites within the sandfly vector includes the development of extra-

cellular promastigotes from a non-infective, procyclic stage into an infective, metacyclic stage which is uniquely adapted for transmission by the sandfly and survival in the vertebrate host (1). These adaptations were explored in the context of the structure and function of the abundant surface LPG of *Leishmania* promastigotes. Structurally, LPG is a lipid containing polysaccharide composed of several distinct regions (2,3). One of the regions is an unconventional lyso-alkyl phosphatidylinositol anchor attached to a glycan core. The core consists of 3 Gal, 2 Man, an unacetylated glucosamine and a Glc-P residue. The salient feature of LPG is a repeating oligosaccharide-phosphate unit. In the case of *L. donovani*, it is a Gal(b1,4)Man unit linked together by phosphodiester bridges. LPG is capped by one of several small oligosaccharide-P units. The LPGs from all species of *Leishmania* have an identical lipid anchor and glycan core. The Gal-Man-P backbone is also conserved, but the LPGs from other species of *Leishmania* can have additional sugars branching off the Gal residue of the backbone repeat units. The structure of the cap can also vary depending on the species and the virulence of the parasite. Thus, the *Leishmania* LPG express both stage- and species-specific polymorphisms that are defined by variations in the type and number of phosphorylated oligosaccharide repeats.

During metacyclogenesis, the LPG from *L. major* undergoes two modifications (2,3): (*i*) a 2-3-fold increase in size due to an increase in the number of repeat units expressed, and (*ii*) a change in the composition of the terminal sugars of some these units. Specifically, there is a pronounced decrease in the abundance of repeat units with side-chains of bGal or Gal(b1,3)Gal(b1)-, and a corresponding increase in repeat units with either no side-chains or with side-chains of Ara(a1,2)Gal(b1)-. This structural modification appears to have a consequence in controlling the stage-specific adherence of promastigotes to midgut epithelial cells in *Phlebotomine papatasi* (4).

Relative to developmental polymorphisms of the *L. major* LPG, there are similarities and some important distinctions that occur with the *L. donovani* LPG during metacyclogenesis: (*i*), the size of the molecule is also substantially increased due to an approximate 2-3-fold increase in the number of phosphorylated disaccharide repeat units expressed, and (*ii*) there is a down regulation of terminally exposed capping sugars on metacyclic LPG, resulting in the loss of lectin binding sites for both peanut agglutinin and concanavalin A, as well as sugar substrate for galactose oxidase. Refined structure analysis revealed that despite the loss of terminal sugars available for binding, metacyclic LPG continues to express several neutral capping oligosaccharides which terminate in either b-linked galactose or a-linked mannose. The masking of these terminal sugars is attributed to a change in conformational structure associated with folding and clustering of the extended phosphoglycan chains, which form densely distributed particulate structures visible on fracture-flip preparations of the metacyclic surface. The exposure and subsequent masking of the terminal capping sugars were shown to control the stage-specificity of promastigote attachment and release from the midgut of *P. agentipes* (5,6).

In summary of *Leishmania*-sandfly interactions, it appears that LPG is involved in promoting survival of the parasite in the sandfly midgut, in regulating the development and behavior of infective metacyclic promastigotes, and in determining species-specific vectorial competence.

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