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Toxoplasma gondii virulence is predictable in cultured human cells

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In the absence of valid serological and molecular markers to identify virulent strains of T. gondii, injection of the putatively contaminated clinical sample into mice and* monitoring *the survival of mice, is still used as the biological test of virulence. The inoculation of several thousands of mice annually for this purpose represents an ethical problem. In project No. 107-07, Dr Sushila D'Souza as principal investigator and PhD student Dr Vijay Morampudi investigated an in-vitro cell culture method to determine T. gondii virulence. Cell cultures of intestinal epithelial cells, were established to be appropriate hosts. The early activation pattern of host genes was seen to be predictive of T. gondii virulence.*

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In-vitro propagation of *T. gondii* parasites

Propagation of *T. gondii* isolates by inoculation into mice is a commonly used method. In order to reduce the use of mice, we evaluated the use of human foreskin fibroblast cells (HFF-1) for *T. gondii* propagation. Three strains with high (Type I), intermediate (Type II) and low virulence (Type III) were successfully grown in fibroblasts and did not alter their distinct virulence compared to that seen in mice. Propagation of *T. gondii* isolates in cell culture is therefore a suitable alternative to the *in vivo* approach with mice (Fig. 1).

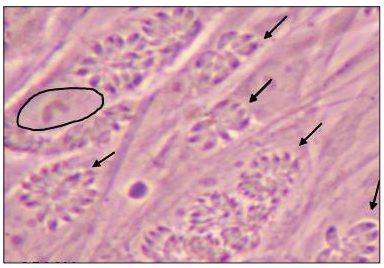

Fig. 1: Tachyzoites (arrows) derived from Type I parasites replicating as rosettes in parasitophorous vacuoles in human fibroblasts (nucleus of one cell is encircled).

Intestinal epithelium as primary target

After ingestion of foods contaminated with *T. gondii*, the parasite (cyst or oocyst derived bradyzoites or tachyzoites) can infect the gut mucosa by direct invasion of small intestinal epithelial cells (IEC) (1). Therefore, IEC respond directly to *T. gondii* infection and initiate early local mucosal responses. Within 8 days of *T. gondii* invasion into IEC, a severe form of intestinal inflammation is observed in mice characterized by recruitment of pro-inflammatory cytokine producing T cells (2). Although infection in the majority of healthy individuals remains asymptomatic, there are studies showing that this parasite can induce severe inflammation of the intestine in mice, rats, pigs and in certain species of nonhuman primates.

Suitable cellular hosts *in-vitro*

An immortalized small intestinal human epithelial cell line (HCT-8) was used as *in vitro* target for comparative studies of the three *T. gondii* strains which differ in their virulence. Cells were cultured to a monolayer and infected with an inoculum of the parasites. Intracellular replication in HCT-8 cells, even at a very low multiplicity of infection, was observed of both the highly virulent Type I parasites and the low virulent Type II and Type III parasites. Type I parasites had a significantly higher rate of replication than Type II and III which correlated with a deleterious effect on host cells, as indicated by the subsequent release of lactate dehydrogenase, a marker of host cell cytotoxicity (Figs. 2A, B). Further, foci of cell destruction in the monolayer were rapidly formed and were more abundant after infection with Type I strains compared with Type II strains. These *in-vitro* findings correspond with the virulence of Type I strains observed in mice, and served as a basis to further explore the early molecular mechanisms that can be induced by *T. gondii* parasites in cultured cells.

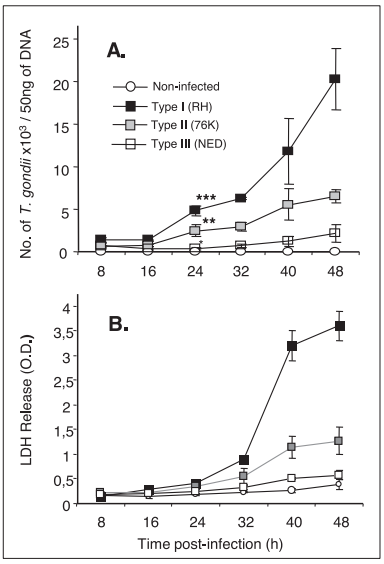

Fig. 2: Replication (A) of high and low virulent *T. gondii* is highly correlated to cytotoxicity (B) of human intestinal epithelial cells (IEC).

Transcription factor and cytokine production

We compared the activation of NF-κB (a key transcription factor required for the activation of host genes), in HCT-8 cells by the three *T. gondii* genotype strains. Type II parasites, that displayed intermediate replication, induced higher NF-κB activity in HCT-8 cells than

Type I and III parasites, confirming previous observations in human macrophages (3). We further examined whether the distinct levels of NF-κB activity could be correlated to downstream cytokine production. In accordance with the high NF-κB activity observed, Type II parasites induced host cells to secrete significantly higher levels of IL-8 and IL-6 than Type I and Type III strains until 48h post-infection.

Expression of human b-defensin 2

Human b-defensins (HBD) are effector molecules that play an important role in early intestinal innate immune defense by their dual function primarily as antimicrobial factors to defend against pathogens and secondarily as chemotactic factors to recruit cells for adaptive immune responses (4). Thus, the differences observed in the replication capacity between the three *T. gondii* types could reflect differences to stimulate IEC for the secretion of antimicrobial immune effectors such as b-defensins. To investigate this possibility, we examined the early expression of antimicrobial peptide genes HBD1, 2 and 3 in IEC upon infection by the three *T. gondii* genotypes. As depicted in Figure 3, a clear increase of HBD2 mRNA levels occurred after 3h of infection

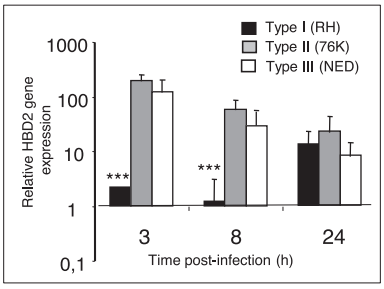

Fig.3: Type I *T. gondii* suppress HBD2 gene expression in IEC at early time points in comparison to less virulent strains.

with Type II and Type III *T. gondii* strains but not with Type I strains. On the contrary, no significant induction of constitutively expressed HBD1 mRNA levels was observed following infection by each of the three *T. gondii* genotypes. HBD3 gene expression on the other hand was down-regulated by all the three *T. gondii* genotype strains. Our findings support the notion that virulent *T. gondii* parasites (Type I), unlike the less virulent strains (Types II and III), do not activate HBD2 early after infection and use this as one of the mechanisms to evade early host antimicrobial effects (5).

A potential replacement method

An *in-vitro* test based on human cell cultures to detect the presence of *T. gondii* in clinical samples and to inform on its virulence would provide an alternative to current tests in mice and be more predictive of virulence in humans. Comparing the pattern of cellular response of human intestinal epithelial cells with the virulence known from mice studies, the high virulence of strain I can be distinguished from the ones with low virulence (Types II and III). The virulence is described by the combined analysis of *T. gondii* growth, cytotoxicity of target cells and the differential response of gene expression within 3-4 hours when there is no detectable replication of the parasite and therefore no cytopathic effect (Table 1).

T. gondii virulence	*T. gondii* replication	Epithelial cell damage	Foci formation	HBD2 in epithelial cells
Type I (high)	+++	+++	+++	+
Type II (intermediate)	++	++	++	+++
Type III (low)	+	+	+	+++

Table 1: Cellular host (IEC) correlates of *T. gondii* virulence

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* *Toxoplasma gondii* and its virulence

Congenital toxoplasmosis and toxoplasma encephalitis are serious human diseases caused by the protozoan parasite *Toxoplasma gondii*, with cats (felidae) as primary hosts in which the sexual part of the parasite's life cycle occurs. Uptake of oocysts derived from feces or cysts from undercooked meat enters the intestinal lining of warm-blooded vertebrates such as birds, and mammals including humans. In these secondary hosts, the parasite goes through the asexual part of its life cycle (bradyzoites to tachyzoites to encysted bradyzoites), resulting in disease symptoms which are usually self-limiting but can have serious or even fatal effects on immunocompromised humans, cats and on fetuses following primary maternal infection during pregnancy.

Molecular epidemiological studies on a wide collection of human and animal isolates of *T. gondii* obtained from Europe and North America have revealed the predominance of three major clonal lineages classified as highly virulent Type I and less virulent Type II and Type III parasites. To confirm the presence of *T. gondii* in clinical samples, currently laboratory mice are innoculated with the suspect samples and monitored for seroconversion and death. Each year several thousands of mice are used for this purpose. Recently, *T. gondii* organelles called rhoptries were shown to secrete kinases that dramatically influence host gene expression and are major parasite virulence factors (6). The data obtained from this study could finally serve as the experimental basis to develop an *in-vitro* test for the confirmation of *T. gondii* in clinical samples, thereby reducing or replacing the use of laboratory mice, and could go a step further by providing information on the virulence of the infecting strain.

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