Attachment and invasion of *Toxoplasma gondii* and *Neospora caninum* to epithelial and fibroblast cell lines *in vitro*

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SUMMARY

Attachment and invasion of *Toxoplasma gondii* and *Neospora caninum* to a cat and a dog fibroblast cell line and 2 epithelial cell lines (a cat kidney and Vero) were compared *in vitro* using fluorescence antibody methodology. In addition, trypsin treatment of tachyzoites was used to determine whether protein molecules were essential to the process of invasion. The results show that both *T. gondii* and *N. caninum* invaded all 4 cell lines, and that pre-treatment of *T. gondii* tachyzoites with trypsin caused an increase in the ability of the parasite to invade these host cells. Furthermore *T. gondii*, in comparison to *N. caninum*, invaded all 4 cell lines at greater levels. The results here support the conclusion that both *T. gondii* and *N. caninum* have the ability to invade a variety of cell types including both dog and cat cells, and questions the utility of Vero cells as an appropriate host cell for *in vitro* studies on the biology of these taxa.

Key words: *Neospora caninum*, *Toxoplasma gondii*, attachment, invasion, host cell, immunofluorescence.

INTRODUCTION

Toxoplasma gondii and *Neospora caninum* are two closely related cyst-forming coccidia with similar cellular morphology and structure and closely related molecular and antigenic characterisitics. They both can invade a broad range of intermediate hosts. Although these two apicomplexans appear similar in so many aspects they differ in their definitive hosts and predilection for host cell types (Dubey, 1994; Dubey and Lindsay, 1996; McAllister *et al*. 1998). For example, in most intermediate hosts, *N. caninum* is found predominantly in the central neural system, whereas *T. gondii* can infect almost all host cell types (Frenkel, 1988; Dobrowolski and Sibley, 1996; Hemphill, 1999). Attachment to, and invasion of a host cell is a crucial step in the establishment of infection and subsequent survival and proliferation of a protozoan. In this respect, many aspects of the cell biology of *T. gondii* have been studied and reviewed Ortega-Barria and Boothroyd, 1999; (Dowse and Soldati, 2004; Ngo, Yang and Joiner, 2004; Sibley, 2004).

Using a modified fluorescent antibody method, we have compared the ability of 4 different cell lines to support attachment and invasion by tachyzoites of either *T. gondii* or *N. caninum*. Attachment and invasion was measured 60 min post-infection (p.i.). The cells used were dog and cat fibroblast lines. At the same time, experiments with Vero and a cat epithelial cell line were performed in parallel in order to determine whether any of the differences observed were cell-line specific. Vero was included because it is a commonly used cell line for the propagation of both *T. gondii* and *N. caninum*. The results obtained show that all 4 cell lines supported the attachment and invasion by both *T. gondii* and *N. caninum*. In general, *T. gondii* attached to and invaded all 4 cell types at higher levels compared to *N. caninum*. A novel observation was made in that removal of tachyzoite surface proteins by trypsin, resulted in a significant increase in invasion by *T. gondii* but not by *N. caninum*. These results suggest that the underlying mechanisms of invasion differ among the two parasite species. In addition, results are presented which question the utility of Vero as a host cell line for studies on the cell biology of these two taxa.

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MATERIALS AND METHODS

Toxoplasma gondii *and* N. caninum *strains*

The RH strain of *T. gondii*, and NC-1 strain of *N. caninum* were used in this study. Tachyzoites of the parasites were maintained in Vero cell (African green monkey kidney cell) monolayers at 37 °C in a 5% CO_2 atmosphere in tissue-culture medium (RPMI, GIBCO, USA) containing 2 mM glutamine, 50 U/ml penicillin/50 μg/ml streptomycin (ICN Biochemicals, USA) supplemented with 2% newborn calf serum (NBS; CSL, Australia). Parasites were harvested from cultures when about 70–80% of the Vero cells were lysed. The lysed cell preparation (containing tachyzoites and host cell debris) was

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passed through a 26-gauge needle 3 times to release tachyzoites from host cells and then washed in cold phosphate-buffered saline (PBS) by centrifugation at 4 °C. The pellet was resuspended in 5 ml of cold PBS and passed through a 3 μm sterile filter unit to separate tachyzoites from host cells. The eluted, purified parasites were centrifuged at 4 °C and were resuspended in cold PBS. Purified tachyzoites were counted with a haemocytometer, and the percentage of viable cells was determined by trypan blue exclusion (0·25% in PBS). Preparations containing at least 95% or more viable parasites were immediately used for attachment and adhesion assays.

Host cells

Dog fibroblast cells 1542 (ATCC, CRL-1542), cat epithelial cells (CRFK) and Vero cells were cultured in RPMI supplemented with 2% NBS, 2 mM L-glutamine and 50 U/ml penicillin/50 μg/ml streptomycin at 37 °C in a 5% CO_2 atmosphere. A cat fibroblast cell line (ATCC, CRL-176) was cultured in RPMI supplemented with 10% NBS, 2 mM L-glutamine and 50 U/ml penicillin/50 μg/ml streptomycin at 37 °C in a 5% CO_2 atmosphere. All cell cultures were free of *Mycoplasma* infection as verified by commercial PCR testing. For the immunofluorescence method described below, host cells were grown overnight to a monolayer in 8-well chamber slides with removable gaskets (Lab-Tek, USA) before use.

Antibodies

The antibodies used in this study are to tachyzoites of *T. gondii* and *N. caninum* and were DG 52 (kindly provided by Professor Boothroyd, Stanford University School of Medicine, USA) and NcmAb-4 (a generous present from Dr Björkmann, Swedish University of Agriculture Sciences, Sweden) respectively. They are species-specific, tachyzoite-specific monoclonal antibodies. The secondary antibodies used were FITC conjugated anti-mouse Fab fragment (Sigma) and Texas red conjugated anti-mouse IgG (Amersham, Australia).

Parasites/host cell pre-treatment with trypsin and neuraminidase

Freshly purified tachyzoites of RH and NC-1 were incubated in PBS containing trypsin protease type I (CSL, Australia) at 0·25 mg/ml at 37 °C for 20 min or neuraminidase at 0·05–0·1 IU/ml at pH 5·5 for 30 min at 37 °C. After the incubation, the tachyzoites were put on ice and the reaction with trypsin was terminated by addition of fetal bovine serum (FBS) in PBS, to a final concentration of 20%. Tachyzoites from both treatment groups were then washed twice in a vast excess of PBS prior to exposure to host cells.

As a control, host cell monolayers were produced in an overnight incubation, and then washed with PBS and incubated in PBS containing trypsin at 37 °C for 15 min. The reaction was terminated by addition of FBS as described above. The host cells were washed twice with an excess of PBS prior to parasite infection.

Attachment and invasion assays

For each species, 5×10^5 freshly purified tachyzoites were added to each of 3 wells of a chamber slide, 2 species per slide. Slides were incubated at 37 °C for 5, 30 and 90 min. At the end of each incubation period, the culture medium was removed, and the wells were rinsed 3 times in PBS to remove extracellular and unattached tachyzoites. Slides were then placed into fixation buffer (3% paraformaldehyde and 50 mM glycine in PBS) for 15 min at room temperature, after which they were rinsed 3 times in PBS and subsequently incubated in blocking buffer (2% bovine serum albumin (BSA) and 50 mM glycine in PBS) for 30 min. The first antibody was applied at a dilution of 1 : 500 (DG52) or 1 : 200 (NcmAb-4) in blocking buffer for 45 min at room temperature followed by 3 rinses in wash buffer. Slides were then incubated in the Texas Red conjugated secondary antibody at a 1 : 100 dilution in blocking buffer, for 45 min. Slides were then rinsed in PBS 3 times and were placed into pre-cooled methanol and acetone (−20 °C) for 5 min each. This step fixed the cells, the parasites, and the antibodies, and also permeabilized the monolayers. After rehydration 3 times in PBS, the slides were placed into blocking buffer again for 30 min, and intracellular parasites were labelled with the same primary antibody diluted as before. The secondary antibody used for the detection of intracellular parasites was a goat anti-rabbit antibody conjugated to FITC at 1 : 200 dilution. Slides were then rinsed in PBS 5 times and subsequently stained with the DNA-specific dye Hoechst 33258 (2·5 μg/ml in PBS) for 2 min. Finally, the preparations were mounted in anti-fading reagent (Calbiochem, Germany) and a cover-slip applied.

Results were obtained by inspection of 15 randomly chosen fields at 40× magnification on an Olympus BX5 reflected epifluorescence microscope with a PM-30 automatic photomicrographic system. Counting of the Hoechst 33258-stained host cell nuclei gave the number of host cells within a given field. The overall numbers of tachyzoites were determined by counting the numbers of FITC-labelled parasites within the same field. The number of external parasites, which attached to host cells was determined by counting the Texas red immunolabelled tachyzoites. Finally, the number of parasites invaded into host cells was obtained by taking away external parasites (Texas

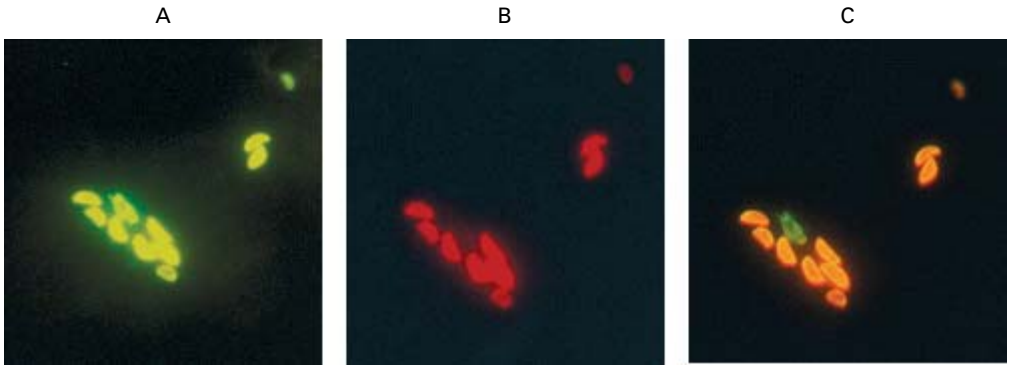

Fig. 1. Immunofluorescence assay carried out to identify intra- and extracellular tachyzoites of *Toxoplasma gondii* and *Neospora caninum*. (A) Total tachyzoites (FITC-labelling). (B) Extracellular tachyzoites (Texas red-labelling). (C) Double staining showing both external and internal parasites.

red-labelled) from the total parasite number (FITC-stained).

DTAF labelling and SDS-PAGE of tachyzoite surface proteins

Freshly purified RH tachyzoites were incubated with a fluorescent dye 5-(4, 6-dichlorotriaziny) aminofluorescein (DTAF) at 0·5 mg/ml (stock solution 7·5 mg/ml in sodium borate, pH 9·0) in Hank's solution (pH 8·0) in total darkness for 15 min at 4 °C. The cells were then washed once in PBS containing 20% FBS and incubated in the same buffer for 5 min at 4 °C. After washing with PBS (Henriques and De Souza, 2000) again, the tachyzoites were incubated with trypsin at several different concentrations.

After the trypsin treatment, the tachyzoites were washed twice in PBS and resuspended in PBS and subjected to SDS-PAGE. Equal amounts of protein from each experimental group were added to sample buffer and proteins were then separated on 12% SDS-PAGE gels in Tris-SDS-Glycine running buffer (Laemmli, 1970). Molecular weight markers (Bio-Rad) were used for gel calibration. A reducing sample buffer was used (62 mM Tris-HCI, pH 6·8, 10% glycerol, 2·3% SDS, 0·5% bromophenol blue) and β-mercaptoethanol was added to a final concentration of 5%. Immediately following electrophoresis, the gels were removed from their moulds and DTAF labelled proteins were detected with a UV transilluminator (Transilluminator TM 40; Ultra-Violet Products Ltd) with a surface light energy of approx 7000 μW/cm^2 (>320 nm). For comparison, after taking the fluorescent pictures, the same gels were stained with Coomassie Blue using standard procedures. Images were taken using a Kodak imaging system.

Evaluation of results

In this assay, results are presented as attachment or invasion indices (Hemphill, Gottstein and Kaufmann, 1996). The invasion index was calculated as the number of intracellular parasites (FITC-labelled parasites minus Texas red labelled parasites) in a certain field divided by the host cell number in the same field. Attachment index was calculated as the total number of parasites (FITC labelled) in a certain field divided by the host cell number in the same field. The data were obtained by using a double-blind method for examining, randomly, at least 500 host cells. All samples were analysed in triplicate in each experiment, and each experiment was performed 3 times on 3 independent dates. The data presented show the mean counts from 3 experiments.

RESULTS

Attachment versus invasion

Like other apicomplexan parasites, *T. gondii* and *N. caninum* invade host cells in 2 main steps: through attachment and active penetration. In order to study, and to be able to distinguish between these two processes, we modified a previously described immunofluorescence staining *in vitro* attachment/invasion assay (Hemphill *et al.* 1996), in which purified tachyzoites of *T. gondii* and *N. caninum* were allowed to interact with host cell monolayers grown on chamber slides. In order to detect attached, non-invaded tachyzoites, chamber slides containing cultured tachyzoites and host cells were fixed with 3% paraformaldehyde and surface labelled with monoclonal antibodies (DG52 or NcmAb-4) directed against tachyzoites of *T. gondii* or *N. caninum* first, and then incubated with a Texas red-conjugated secondary antibody. After this, the monolayers were permeabilized with methanol and acetone and incubated again with the same monoclonal antibodies (DG52 or NcmAb-4) and a secondary antibody conjugated to FITC, thus distinguishing between intra-and extracellular parasites (Fig. 1). Tachyzoites on the outside of the cell were labelled with Texas red, while those internal were labelled with FITC.

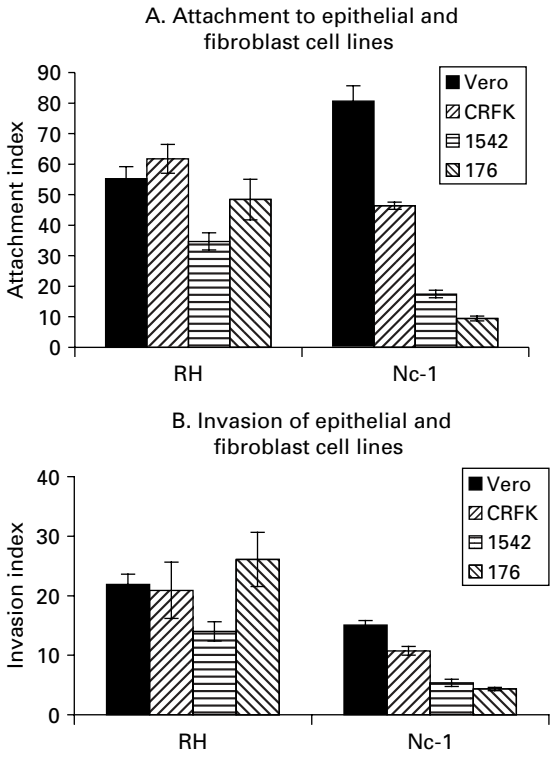

Fig. 2. Quantitative analysis of attachment (A) and invasion (B) by *Toxoplasma gondii* (RH) and *Neospora caninum* (NC-1) tachyzoites to 4 host cell lines. The cell lines used were Vero cells, dog fibroblast (1542) cells, cat fibroblast (176) cells and cat epithelial (CRFK) cells. Invasion and attachment indices are defined in the Materials and Methods section. Values are derived from 3 independent experiments, and the error bars represent standard error.

Preliminary investigations of the time-course of events relating to attachment and invasion of dog fibroblasts, indicated that a time-point between 30 and 90 min was an appropriate time to assay attachment and invasion, because the invasion index reached a plateau. Hence, 60 min p.i. was chosen as the time of study and hence the results presented here relate to experiments assayed at this time-point.

Both RH and NC-1 attached and invaded all 4 cell lines. While RH attached to 3 of the 4 host cells at similar levels at 60 min p.i. compared to NC-1, NC-1 bound at higher levels to Vero cells (compared to *T. gondii*), than to cat and dog fibroblast cells ($P<0{\cdot}05$) (Fig. 2). For all cell lines attachment indices of both species are much higher than their invasion indices ($P<0{\cdot}05$). This indicates that although attachment is necessary for successful invasion, not every adherent parasite infects a host cell.

Attachment and invasion to Vero cells of RH and NC-1

Attachment and invasion of *T. gondii* and *N. caninum* to a commonly used and well-described cell line (Vero) was compared. Attachment to Vero cells by RH and NC-1 is significantly different ($P<0{\cdot}005$) with NC-1 possessing a much higher attachment index than RH at 60 min p.i. (Fig. 2). However, more RH invaded into Vero than NC-1 ($P<0{\cdot}005$).

Attachment and invasion to fibroblasts of RH and NC-1

Attachment and invasion to cat and dog fibroblast cell lines were compared. Overall more RH attached to, and invaded, both of the fibroblast cell lines than NC-1 ($P<0{\cdot}005$, Fig. 2). In comparing the 2 fibroblast cell lines more RH attached to cat fibroblasts than to dog fibroblasts ($P<0{\cdot}05$). Although more NC-1 attached to dog fibroblast cells ($P<0{\cdot}05$), it invaded both fibroblasts with a similar efficiency ($P>0{\cdot}05$, Fig. 2).

Attachment and invasion of fibroblasts and epithelial cells

Epithelial and fibroblast cell lines were compared. While RH attached and invaded all four cell lines similarly ($P>0{\cdot}05$, Fig. 2), more NC-1 attached and invaded into epithelial cells than fibroblast cells ($P<0{\cdot}05$, Fig. 2).

Effects of enzymatic treatments

In order to investigate if a specific recognition process was mediated by parasite surface proteins during the interaction of *T. gondii* or *N. caninum* and their host cells, tachyzoites were pre-treated with either trypsin, a protease that can alter the parasites surface protein composition, or neuraminidase, an enzyme which cleaves terminal sialic acid residues from carbohydrate moieties on the surfaces of parasites. The results obtained showed that pre-treatment of tachyzoites with neuraminidase prior to host cell interaction did not affect tachyzoite invasion for both *T. gondii* and *N. caninum*. Trypsin pre-treatment, however, resulted in a significant increase in invasion by RH ($P<0{\cdot}01$), but it did not affect the ability of NC-1 to invade (Fig. 3). These results were consistent in all 4 cell lines used.

To confirm the effects of trypsin treatment on tachyzoite surface membrane proteins, RH tachyzoites were labelled with DTAF, and then the labelled RH tachyzoites were treated with trypsin. As expected after treatment with different concentrations of trypsin for different time-periods, surface proteins were removed from the RH tachyzoite surface as revealed by fluorescence microscopy and SDS-PAGE gels viewed for fluorescence (not shown).

To determine whether the enhancing effect on *T. gondii* RH was due to a sole effect on the parasite,

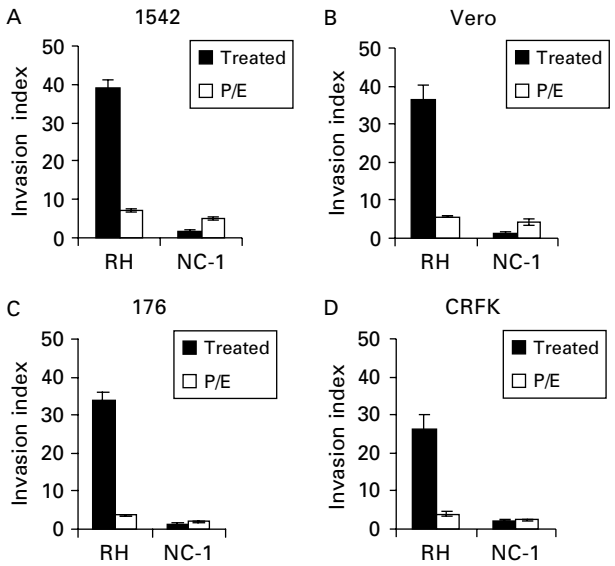

Fig. 3. Effects of trypsin pre-treatment of tachyzoites on invasion indices of *Toxoplasma gondii* (RH) and *Neospora caninum* (NC-1). The cells used were (A) dog fibroblast (1542) cells, (B) Vero cells, (C) cat fibroblast (176) cells and (D) cat epithelial (CRFK) cells. The control group was treated with P/E (PBS/EDTA). The results shown are the means of 3 independent experiments, and the error bars represent standard error.

host cell monolayers were treated with trypsin before being infected with untreated tachyzoites of *T. gondii*. While pre-treatment of dog or cat fibroblasts and cat epithelial cells with trypsin had no discernible effect on attachment and invasion ($P > 0{\cdot}05$), treatment of Vero cells with trypsin caused a significant decrease in both attachment and invasion by both *T. gondii* and *N. caninum* tachyzoites. Pre-treatment of Vero cells resulted in a 42% decrease in the *T. gondii* attachment index and a 30% invasion index compared to untreated controls, whereas the treatment only decreased *N. caninum* attachment and invasion indices by 30% and 12% respectively (not shown).

DISCUSSION

In this study, the attachment and invasion of *T. gondii* and *N. caninum* tachyzoites to 2 fibroblast and 2 epithelial cell lines were investigated. To demonstrate and quantitate tachyzoite attachment and invasion, an assay described by Schenkman, Diaz and Nussenzweig (1991) and Hemphill, Gottstein and Kaufmann (1996) was modified in order to clearly distinguish between intracellular and extracellular tachyzoites by fluorescence light microscopy.

Under the conditions used in these experiments, both attachment and invasion took place within a relatively short time. Preliminary experiments not presented here showed that a 5 min incubation of the tachyzoites with host cells was sufficient to allow attachment and invasion. At 60 min p.i. it was found that attachment to, and invasion of all cell lines by *T. gondii* and *N. caninum* occurred in a non-homogeneous manner. Some host cells would contain several parasites, while others contained none. Similar patterns of heterogeneous host cell attachment and invasion have been previously reported (Schenkman *et al*. 1991; Mineo and Kasper, 1994; Hemphill *et al*. 1996).

The initial time-course experiments showed that during incubation of tachyzoites with dog fibroblast cells, the invasion index increased and reached a plateau at 60 min p.i., although there were still additional tachyzoites attached onto the host cell surface, and there were still a considerable number of uninfected host cells present. This suggested that attachment onto the host cell membrane did not automatically lead to invasion of host cells and, that attachment and invasion are 2 distinct steps. These assumptions were confirmed by the fact that a disproportional attachment rate and invasion rates were found within experiments, and although attachment rates were similar between RH and NC-1, the corresponding invasion indices of NC-1 were much smaller than RH. In all cases *T. gondii* invaded host cells more efficiently than *N. caninum*.

In contrast to our data, Sundermann and Estridge (1999) compared invasion by RH and NC-1 isolates to human foreskin fibroblasts and found that 2 to 3-fold more NC-1 tachyzoites were intracellular compared with RH at 1 or 3 h p.i. One possible explanation for the difference between our results and theirs, is that *T. gondii* can display different growth dynamics depending on the type of cultured cell used (Hughes, Hudson and Fleck, 1986). Furthermore, the assay used here is not the same as the one used by Sundermann and Estridge (1999), who used an absolute parasite number (tachyzoites/well), which were not necessarily intracellular, and host cell number was not taken into consideration at the same time. We applied a double staining immunofluorescence protocol that clearly distinguished intracellular parasites from extracellular ones and at the same time we took host cell number into consideration when defining rates of attachment and invasion. We therefore argue that our method of assay and analysis is considerably more robust and clearly distinguishes attachment and invasion. Furthermore, it recognizes the fact that invaded parasites must also have attached to the cell prior to invasion.

With respect to attachment and invasion of different host cells, the RH strain showed no difference among the four cell lines whereas NC-1 attached to, and invaded epithelial cells significantly more efficiently than fibroblast cells. Of interest here is the fact that *T. gondii* is known to be able to invade almost all nucleated cell lines examined (Hartley, 1966; Miller, Frenkel and Dubey, 1972; Canfield, Hartley and Dubey, 1990; Inskeep *et al*. 1990;

Hartley and Dubey, 1991; Dubey, Lindsay and Speer, 1998; Hall, Ryan and Buxton, 2001). Although *in vitro* studies may have no correlation to *in vivo* studies, *N. caninum* is known from pathological evidence, to prefer certain tissues such as brain, placenta and foetal tissues (Dubey *et al*. 1988; Buxton *et al*. 1998; Naguleswaran *et al*. 2002).

Naguleswaran *et al*. (2002) showed that *T. gondii* and *N. caninum* tachyzoites exhibit differential adhesive properties to host cell surface glycosaminoglycans, which suggested that the interaction of *N. caninum* with a host cell differs from that for *T. gondii*. They reported that *N. caninum* showed a high affinity to chondroitin sulphates, which may reflect the preference of *N. caninum* for certain tissues such as brain, placenta and foetal tissues observed upon natural and experimental infections (Dubey *et al*. 1988; Buxton *et al*. 1998). In addition, while adhesion of *N. caninum* tachyzoites to Vero cell monolayers was not markedly affected by treatments with heparinases, a 30% reduction was observed in attachment of *T. gondii* RH strain tachyzoites, which confirmed a previous study which demonstrated the affinity of *T. gondii* tachyzoites to heparan sulphate glycosaminoglycans (Carruthers *et al*. 2000). Finally, binding of *N. caninum* tachyzoites to Vero cells treated with ABCase at 1 unit/ml demonstrated a 43% reduction in the number of adherent parasites, while *Toxoplasma* did not show any reduction in bound tachyzoites. All these results provide convincing evidence that *T. gondii* and *N. caninum* differ in their mechanisms of invasion of host cells.

Enzymatic treatment of RH or NC-1 tachyzoites with neuraminidase and trypsin prior to their interaction with host cells provided further information on the nature of the molecules on the tachyzoite surface and their involvement in attachment and invasion. For both species, neuraminidase pre-treatment did not affect host invasion by tachyzoites. These findings are in keeping with a recent study demonstrating that pre-treatment of tachyzoites of *T. gondii* and *N. caninum* with 0·05 I.U. of neuraminidase/ml prior to host cell interaction resulted in no alteration to tachyzoite invasion rates (Vonlaufen *et al*. 2004). This implies that sialic acid plays no role as a ligand for the parasites. Interestingly, when bradyzoites of *N. caninum* were pre-treated with neuraminidase, invasion rates were more than doubled. This result also indicates that the two stages employ different receptor-ligand interactions for invading host cells (Vonlaufen *et al*. 2004).

For RH tachyzoites, trypsin pre-treatment resulted in a marked increase in invasion of host cells. In contrast, trypsin pre-treatment of NC-1 tachyzoites had little or no effect. These results suggest that the plasmalemma, and the proteins it contains, probably differ between *T. gondii* and *N. caninum* in composition and function. Indeed the results suggest that in *T. gondii*, surface membrane proteins mask the real determinants that are involved in host cell attachment and invasion.

The effect of trypsin treatment could be attributed to the effect on the parasite, and not the host cells, since the phenomena occurred only when tachyzoites were treated with trypsin. Pre-treatment of 3 of the 4 host cells with trypsin had no effect on parasite invasion rates. Linch *et al*. (2001) recently demonstrated that the tachyzoite stage of *T. gondii* is resistant to trypsin at concentrations higher than the physiological concentrations found in the intestinal fluid and described the presence of a trypsin inhibitor associated with the surface of *T. gondii*.

Previous studies have demonstrated that SAG1, the most abundant and immunogenic surface protein, is important for the success of invasion (Grimwood and Smith, 1992, 1996). Neutralizing this protein with a monoclonal antibody caused a significant drop in invasion of host cells (Mineo *et al*. 1993; Mineo and Kasper, 1994). Our findings that trypsin pre-treatment of tachyzoites, which removes surface proteins including SAG1, markedly increased invasion of *T. gondii* contradicts these described phenomena. Our results are, however, in keeping with more recent studies which showed (1) that pre-incubation of SAG-1 in polyclonal sera from chronically infected mice failed to block binding (Robinson, Smith and Miller, 2004); (2) parasites engineered to lack the major surface protein, SAG1, appear to invade fibroblasts with faster kinetics than wild-type parasites (Lekutis *et al*. 2001). This challenges the role that anti-SAG1 antibodies have in blocking parasite attachment through the masking of SAG1 host cell binding domains.

Hemphill *et al*. (1996) reported that pre-treatment of NC-1 tachyzoites with trypsin decreased its invasion into bovine endothelial cells. We did not detect a decrease using this methodology, however, we did not include bovine cells in this study.

Four cell lines were used in this study including a well-described and widely used one (Vero). While both species attached to and invaded both the cat and dog fibroblasts and the cat epithelial cells in a similar way, Vero cells were the only host cell to which *N. caninum* showed a higher attachment index compared to *T. gondii*. Invasion of Vero cells by *T. gondii* and *N. caninum* was also affected by trypsin treatment of the Vero cells. This suggested that unlike the other three cell types, the surface membrane of Vero cells may play a role in the attachment and invasion process. This raises the question of the suitability of Vero as a host cell for *in vitro* studies using these two parasite taxa. However, given that both RH and NC-1 are routinely propagated in these labs in Vero, it is highly likely that selection for compatible parasite and host cells is occurring. This

may also have an impact on the biological properties of the two cell populations.

In summary, this study represents an analysis of interactions between *T. gondii* and *N. caninum* with four different host cells. These findings may have important implications for our understanding of the mechanism of entry of *T. gondii* and *N. caninum* into vertebrate cells. For example, the results suggest that the cell surface properties of *T. gondii* and *N. caninum* exhibit functional differences with respect to their role in attachment and invasion of host cells. Further studies on this topic are underway. In addition, further study using both dog and cat intestinal cell lines are required before any suggestions can be made regarding why these two closely related Apicomplexas have different definitive hosts and why this is so.

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