Effect of polyclonal antisera developed against dense granule-associated *Neospora caninum* proteins on cell invasion and development *in vitro* by *N. caninum* tachyzoites

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SUMMARY

The effects of polyclonal antisera to 2 recombinant dense granule (DG) antigens of *Neospora caninum* on invasion and development by *N. caninum* tachyzoites in baby hamster kidney cell cultures were examined. In immunofluorescent antibody tests, the anti-DG sera, at dilutions of 1:100 to 1:500, reacted intensely with individual intracellular tachyzoites and groups of tachyzoites enclosed in parasitophorous vacuoles at 2 and 52 h p.i., respectively. Tachyzoites suspended in diluted anti-DG sera and inoculated immediately into the cell cultures invaded cells in significantly fewer numbers (53–68% fewer by 2 h p.i.) than tachyzoites suspended in similarly diluted normal rabbit serum. In contrast, tachyzoites suspended in anti-*Eimeria tenella* sporozoite serum invaded cells as efficiently as those suspended in normal rabbit serum. Addition of anti-DG sera at the time of inoculation of tachyzoites, or to the cell cultures after the parasites had entered cells, had little effect on either the percentage or rate of their subsequent development.

Key words: *Neospora caninum*, dense granules, cell culture, invasion, development.

INTRODUCTION

Tachyzoites of *Neospora caninum* actively invade cells throughout the body of the host animal and cause cell death and a variety of clinical signs (Dubey & Lindsay, 1996). Tachyzoites also invade a number of well-established cell lines *in vitro* and undergo unlimited multiplication (Lindsay & Dubey, 1989). Although cellular invasion is a critical step in the establishment of infection both *in vivo* and *in vitro*, little is known concerning the mechanisms by which *N. caninum* tachyzoites bind to and penetrate the host cell. In one study, Hemphill, Gottstein & Kaufmann (1996) demonstrated that invasion of cultured cells by *N. caninum* was initiated by the attachment of tachyzoites to the plasma membrane of the host cell. Following attachment, the tachyzoites moved into an invagination in the host cell surface that formed at the point of parasite–host cell contact, and were enclosed by the host cell membrane. Elements of the process were judged to be similar, although not necessarily identical, to those that occur during cell invasion by other apicomplexans.

Dense granules (DG) have been implicated in cell invasion by sporozoites of several members of the Apicomplexa, but the primary activity of the DG, remodelling of the parasitophorous vacuole, would probably take place after the sporozoite enters the cell (Dubremetz *et al*. 1998). Recently, 2 cDNA clones that expressed recombinant DG antigens in *Escherichia coli*, were identified from *N. caninum* tachyzoites (Lally *et al*. 1997; Liddell *et al*. 1998). Polyclonal antisera, developed in rabbits to these antigens, recognized native antigens of tachyzoites with molecular weights of 33 kDa (NCDG1) and 37 kDa (NCDG2). In addition, intense extracellular labelling at one end of a few tachyzoites led the authors to hypothesize that the labelling represented DG protein that was discharged from tachyzoites of *N. caninum* and might be associated with host cell invasion (Lally *et al*. 1997). Polyclonal antiserum raised against a native antigen derived from fractionated *N. caninum* also reacted with DG of tachyzoites (Hemphill *et al*. 1998). The antibody, which was believed to be identical to that described by Lally *et al*. (1997), remained localized to the DG for at least 30 min after invasion and gradually became associated with the tachyzoite surface membrane, parasitophorous vacuole membrane, and intravacuolar network.

Antibodies reacting with other intracellular zoite proteins have been shown to inhibit parasite invasion and development *in vitro* (Jean *et al*. 1997; Matsuura & Kasper, 1997). Therefore, the aim of the present study was to determine if polyclonal anti-*Neospora*

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DG sera would have an inhibitory effect on invasion and subsequent development of *N. caninum* tachyzoites in cultured cells. Antisera developed to recombinant DG antigens (Lally *et al*. 1997; Liddell *et al*. 1998) were used.

MATERIALS AND METHODS

Cell cultures, tachyzoites and antisera

Equine dermal (ED) cell cultures were maintained in Medium 199 supplemented with 10% fetal bovine serum, 50 μg/ml streptomycin, and 50 U/ml penicillin (M199+10) at 37 °C in 5% CO_2 in 25- or 75-cm^2/tissue culture flasks. Baby hamster kidney (BHK) cell cultures were maintained in the same conditions and medium except that the serum concentration was reduced to 5% (M199+5). Both cell types were dispersed by trypsinization and replated at least weekly.

Neospora caninum (Nc-1 isolate; Dubey *et al*. 1988) was maintained *in vitro* by weekly transfer of tachyzoites from infected to uninfected ED cell cultures. To harvest tachyzoites for experiments, infected cultures containing 5 ml of fresh M199+5 were scraped from flasks and the scrapings were forced through a 26-gauge needle. Tachyzoites were separated from cell debris by running the centrifuge up to 2270 ***g*** and immediately turning it off; the supernatant fraction was collected and adjusted with M199+5 to contain the appropriate numbers of tachyzoites for the experiment. The adjusted tachyzoite suspension contained very few intact cells and little cell debris.

Polyclonal antisera were produced in rabbits from recombinant antigens expressed by 2 cDNA clones (NCDG1 and NCDG2) from *N. caninum* tachyzoites (anti-DG sera) (Lally *et al*. 1997; Liddell *et al*. 1998). Polyclonal antiserum was also produced in rabbits against *Eimeria tenella* sporozoites for use as an immunoglobulin (Ig) control (anti-*E. tenella* serum). By ELISA, the titres of the anti-DG sera exceeded 1:10000; by immunofluorescent antibody labelling, the titre of anti-*E. tenella* serum was 1:5000. Serum collected from rabbits before they were inoculated with antigens (normal rabbit serum; NRS) served as a negative control.

Invasion and development

Studies of *N. caninum* invasion (5 experiments) and development (3 experiments) were carried out in BHK cell cultures grown on 12 μm glass cover-slips in 24-well tissue culture plates. Purified tachyzoites were suspended in M199+5 containing 1:100 to 1:500 (v/v) of anti-DG sera or NRS and inoculated immediately onto BHK cell cultures; 1 ml of medium, containing from 1×10^5 to 3×10^5 tachyzoites, was inoculated onto each culture. Cultures were incubated at 37 °C for 2–52 h. Three to 5 cover-slips per treatment group per experiment were quantified. In 2 studies of invasion (Exps 4 and 5), tachyzoites were also suspended in M199+5 containing 1:300 (v/v) of anti-*E. tenella* serum and inoculated onto cultures. To quantify invasion, cultures were fixed in neutral buffered formalin at 2 h p.i. and stained with haematoxylin and eosin. The numbers of individual intracellular tachyzoites, enclosed in vacuoles, in 20 equally-spaced fields on each cover-slip were counted. Invasion was calculated as reported earlier for *Eimeria* sporozoites (Augustine & Jenkins, 1998). To quantify development, at 2 h p.i., cultures were washed twice in M199+5; fresh M199+5 containing the same concentrations of anti-NCDG serum or NRS used during the first 2 h incubation was reapplied to the cultures (the effect of anti-*E. tenella* serum on development of *N. caninum* was not examined). In 1 study of development, anti-DG sera were not added to the cultures until after invasion occurred (2 h p.i.). Two cover-slips from each treatment group were fixed at 2 h p.i. and examined for the presence of infected intact cells; infected cells in the inoculum could release additional tachyzoites onto the cultures and cause inaccurate estimates of later development. At 52 h p.i., single tachyzoites, stages containing 10 or fewer tachyzoites, and stages containing 10 or more tachyzoites were counted. All of the stages counted were enclosed in parasitophorous vacuoles. The overall percentage development in each treatment was calculated and the rate of development was estimated from the relative sizes of the developmental stages (numbers of tachyzoites/stage). Two experiments were run with 3–4 cover-slips per treatment group.

Differences in invasion and development among treatment groups were tested for significance by analysis of variance and Ducan's multiple range procedures. Probabilities at the 5% level were considered to be significant.

Immunofluorescent antibody labelling

BHK cell cultures, inoculated with *N. caninum* tachyzoites and incubated for 2 or 52 h in the absence of NRS or antiserum were fixed for 2 min in cold (4 °C) methanol and washed in cold PBS (pH 7·4). They were exposed to anti-DG sera, anti-*E. tenella* serum, or NRS (1:100 to 1:500 dilutions in Tris-buffered saline containing 1% BSA) for 1 h, washed for 10 min in 2 changes of PBS, and exposed to fluorescein-conjugated goat anti-rabbit serum for 1 h. Following 2×10 min washes in PBS, the cover-slips were mounted in buffered glycerol (pH 8·0). Specimens were examined with a Zeiss Ultraphot microscope equipped for epifluorescence microscopy.

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Table 1. Effect of polyclonal antisera developed against recombinant dense granule antigens of *Neospora caninum* or *Eimeria tenella* sporozoites on invasion of baby hamster kidney cell cultures by *N. caninum* tachyzoites (2 h p.i.)

Exp. no.	*n*	Serum dilution	Antiserum*	Culture conf. (%)†	Tachyzoites/mm² cells†	Change (%)
1	5	1:100	NRS	66 ± 1^{b}	45 ± 2^{a}	
	5	1:100	NCDG1	69 ± 0^{a}	17 ± 1^{b}	−62
	5	1:100	NCDG2	69 ± 1^{a}	21 ± 2^{b}	−53
		F value		3·68	50·80	
		P		0·0499	0·0001	
2	3	1:300	NRS	41 ± 2^{a}	241 ± 30^{a}	
	3	1:300	NCDG1	45 ± 3^{a}	82 ± 5^{b}	−66
	3	1:300	NCDG2	45 ± 3^{a}	106 ± 15^{b}	−56
		F value		0·44	56·53	
		P		0·4423	0·0001	
3	3	1:500	NRS	47 ± 3^{a}	269 ± 53^{a}	
	3	1:500	NCDG1	$45 \pm 1^{a,b}$	101 ± 21^{b}	−62
	3	1:500	NCDG2	41 ± 2^{b}	91 ± 10^{b}	−66
		F value		5·33	27·14	
		P		0·4670	0·0010	
4	3	1:300	NRS	52 ± 3^{b}	112 ± 16^{a}	—
	3	1:300	NCDG2	63 ± 2^{a}	48 ± 7^{b}	−57
	3	1:300	*tenella*	$57 \pm 2^{a,b}$	113 ± 2^{a}	+1
		F value		6·99	10·88	
		P		0·0357	0·0151	
5	3	1:300	NRS	30 ± 0^{a}	121 ± 13^{b}	—
	3	1:300	NCDG2	30 ± 0^{a}	39 ± 3^{c}	−68
	3	1:300	*tenella*	30 ± 0^{a}	208 ± 13^{a}	+72
		F value		> 99·9	9·27	
		P		N.A.	0·0146	

* NRS, Normal rabbit serum control; NCDG1 and NCDG2, polyclonal antisera raised in rabbits against recombinant *Neospora caninum* dense granule antigens; *tenella*, polyclonal antiserum raised in rabbits against *Eimeria tenella* sporozoites.
† Means ± S.E.M.
[a,b] Means followed by unlike superscripts differ significantly ($P \leqslant 0{\cdot}05$).

RESULTS

Invasion

No aggregation of tachyzoites was observed in medium containing anti-DG sera, anti-*E. tenella* serum, or normal serum at any of the concentrations that were used. Invasion of BHK cell cultures by tachyzoites of *N. caninum* in the presence of anti-DG sera was significantly ($P \leqslant 0{\cdot}05$) lower than invasion in the presence of NRS (Table 1, Exps 1–5). The decrease occurred with serum dilutions of 1:100 to 1:500 and remained between 53 and 68 % regardless of the size of the inoculum. In contrast, no inhibition of invasion occurred when tachyzoites were suspended in anti-*E. tenella* serum before inoculation into BHK cell cultures (Table 1, Exps 4–5). Counts of tachyzoites that adhered to the surface of the host cells but did not invade were not done. There were slight but inconsistent effects of the sera on the confluency of the cell cultures.

Development

There was little effect on parasite development at 52 h p.i. when anti-DG sera were added either before invasion (antisera included at the time of inoculation; Table 2) or after invasion (antisera added at 2 h p.i.; data not shown). The percentage development of both control and antiserum-treated cultures was in the range 82–94 % in Exp. 1 and 72–77 % in Exp. 2. No intact cells were seen in the inoculum for Exp. 1 and only 1 in each culture in Exp. 2.

Immunofluorescent antibody labelling

At 2 h p.i., both tachyzoites and BHK cells exposed to anti-DG sera and the fluorescein conjugant were labelled (Fig. 1 A), although the parasites were more intensely labelled than the cells. Most of the tachyzoites displayed an irregular surface reactivity. In addition, there were areas of intense fluorescence at one or both ends of approximately 20 % of the tachyzoites. Infected and non-infected BHK cells exposed to NRS were labelled to about the same degree as those exposed to anti-DG serum, but the tachyzoites were not labelled (Fig. 1 B). Tachyzoites and cells exposed to anti-*E. tenella* serum were not labelled.

At 52 h p.i., tachyzoites exposed to anti-DG sera were strongly reactive (Fig. 1 C) while those exposed

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Table 2. Effect of polyclonal antisera developed against recombinant dense granule antigens of *Neospora caninum* on development of *N. caninum* tachyzoites (TZ) in baby hamster kidney cell cultures (52 h p.i.)

Exp. no.	Serum dilution	Antiserum*	TZ†	Tachyzoites per developmental stage†		Development (%)‡
				<10	>10	
1	1:300	NRS	7 ± 5^{a}	15 ± 6^{a}	12 ± 6^{a}	82 ± 10^{a}
	1:300	NCDG1	2 ± 1^{a}	17 ± 6^{a}	18 ± 2^{a}	94 ± 2^{a}
	1:300	NCDG2	3 ± 1^{a}	19 ± 6^{a}	12 ± 2^{a}	92 ± 3^{a}
	F value		0·75	0·83	0·81	0·95
	P		0·5541	0·5137	0·5062	0·4604
2	1:500	NRS	42 ± 4^{a}	132 ± 9^{a}	11 ± 2^{a}	77 ± 2^{a}
	1:500	NCDG1	47 ± 10^{a}	123 ± 22^{a}	10 ± 1^{a}	74 ± 3^{a}
	1:500	NCDG2	50 ± 6^{a}	116 ± 15^{a}	13 ± 3^{a}	72 ± 4^{a}
	F value		0·4	0·24	0·63	0·93
	P		0·6848	0·7195	0·5650	0·4454

* NRS, Normal rabbit serum control; NCDG1 and NCDG2, polyclonal antisera raised in rabbits against recombinant *Neospora caninum* dense granule antigens.
† Means ± S.E.M.; means followed by like superscripts do not differ significantly ($P \leqslant 0{\cdot}05$).
‡ Percentage development = total number of parasites.

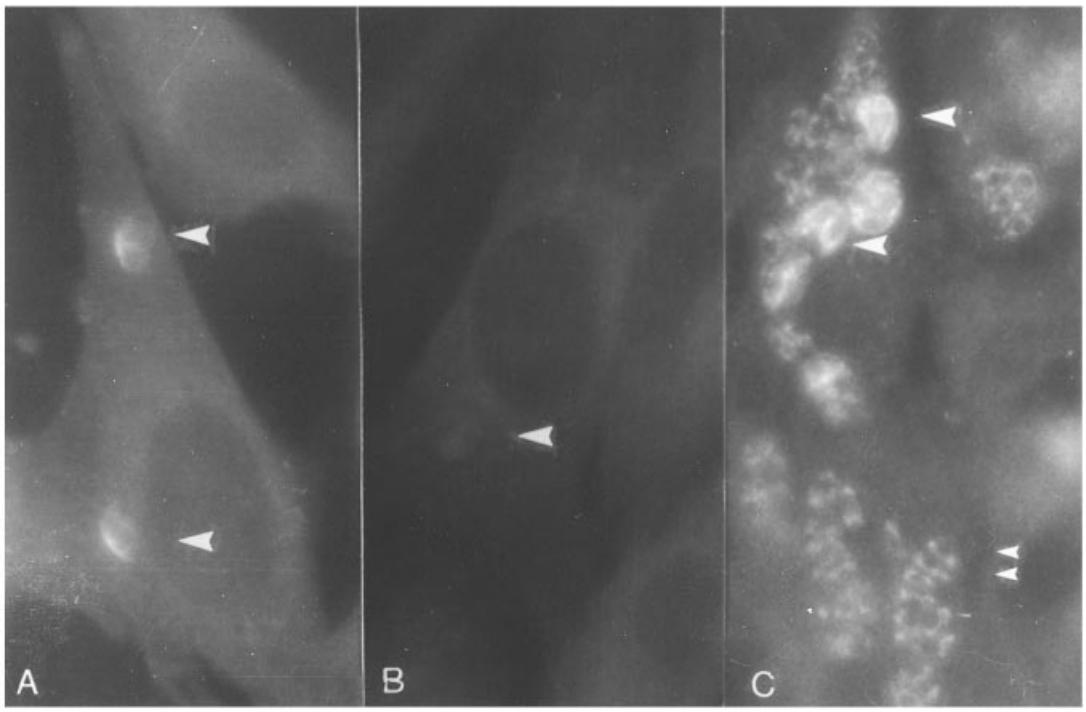

Fig. 1. (A–C) Immunofluorescence of *Neospora caninum* in baby hamster kidney cell cultures. (A) Tachyzoites (arrow heads) exposed to 1:500 dilution of polyclonal antiserum raised against dense granule antigen (NCDG1); 2 h p.i. (B) Tachyzoites (arrow heads) exposed to 1:500 dilution of normal rabbit serum; 2 h p.i. (C) Daughter tachyzoites (arrow heads) and developmental stages containing numerous tachyzoites (small double arrow heads) exposed to NCDG1; 52 h p.i. Note difference in intensity of staining.

to NRS were not (not shown). Labelling of BHK cells by anti-DG sera was comparable with labelling by NRS.

DISCUSSION

Host cell invasion by apicomplexans involves sequential activity of 3 anterior organelles, micronemes, rhoptries and DG. Each organelle has a specific function: micronemes are considered to be involved in host cell recognition, rhoptries in parasitophorous vacuole formation, and DG in remodelling of the parasitophorous vacuole for parasite development (Dubremetz *et al*. 1998).

The current study suggests that in addition to their role in remodelling the parasitophorous vacuole, DG may also be involved in the attachment or penetration of host cells, at least by *N. caninum*

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tachyzoites. At the highest dilutions used in this study (1:500), intracellular tachyzoites fluoresced brightly after exposure to both of the anti-DG, indicating a strong antigen–antibody interaction. When tachyzoites were suspended in the antisera and inoculated immediately into cell cultures, their ability to invade was reduced by 53–68% as compared with invasion by tachyzoites suspended in similar dilutions of NRS. Attachment to and penetration of host cells are 2 distinct events; attachment does not automatically lead to penetration (Hemphill *et al*. 1996). Apparently the DG antibodies were capable of interfering with one or both of these activities but, because tachyzoites that attached to the cells but did not invade were not counted, we could not distinguish between the 2 events. Fc receptor activity has been detected on *Toxoplasma gondii*, *Trypanosoma* spp. and *Leishmania* parasites (Vercammen *et al*. 1998). However, the inhibition of invasion observed in the present study with *N. caninum*, was apparently not a non-specific effect of high levels of Ig in the antiserum because (1) no clumping of the parasites occurred due to cross-linking by the antisera and (2) no inhibition of invasion occurred when tachyzoites were suspended in anti-*E. tenella* serum and inoculated onto cultures.

We do not know at this time if the antisera effect was directed specifically against the activity of the tachyzoite, host cell, or the interaction between the two. Hemphill *et al*. (1996) conducted several studies with *N. caninum* from which they concluded that (1) cellular invasion required parasite energy and was largely independent of host cell metabolism and (2) that the process was receptor-mediated, probably involving host cell surface molecules. Based on these conclusions, the inhibition of invasion observed in the present study could have been caused by the binding of antibodies to the tachyzoite, blocking either the release of DG material or the interaction between the tachyzoite and its host cell receptor molecule, or both.

Rupturing of infected host cells and release of tachyzoites in cell culture begins at approximately 72 h p.i. (Lindsay & Dubey, 1989). Therefore, cultures for quantification of development were fixed earlier (at 52 h p.i.) to ensure that most, if not all, of the developmental stages on the cover-slips originated from individual tachyzoites in the inoculum. At 52 h p.i., there was little evidence that the anti-DG sera had an effect on the development of *N. caninum* even though their target antigens were strongly expressed by the parasites at both 2 and 52 h p.i. There were no significant differences either in the percentage of tachyzoites that developed further (the number of developmental stages per total number of parasites at 2 h p.i.) or in the rate of development (the number of tachyzoites per developmental stage at 52 h p.i.).

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