Chymotrypsin and neuraminidase treatment inhibits host cell invasion by *Babesia divergens* (Phylum *Apicomplexa*)

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SUMMARY

The process of host cell invasion by *Babesia divergens* is poorly understood and improved knowledge of the mechanism involved could lead to development of measures effective in disease prevention. To investigate parasite ligands on the erythrocyte surface, *B. divergens* cultures in bovine erythrocytes were transferred into enzyme-treated bovine, human, ovine and equine erythrocytes. Parasite invasion of bovine erythrocytes was not affected by trypsin treatment while treatment with alpha-chymotrypsin led to a reduction in parasite growth of 20–40 %. Treatment of bovine and non-bovine erythrocytes with neuraminidase decreased their susceptibility to invasion by up to 97 % implicating sialic acid as an important erythrocyte ligand for babesia, but the addition of either bovine or human *N*-acetylneuraminyl-lactose to *B. divergens* cultures in bovine erythrocytes had no inhibitory effect.

Key words: *Babesia divergens*, trypsin, alpha-chymotrypsin, neuraminidase, *N*-acetylneuraminyl-lactose, sialic acid.

INTRODUCTION

The tick-borne protozoan parasite *Babesia divergens* is the main agent of bovine babesiosis in Europe and responsible for major economic losses (Kuttler, 1988). The parasite resides within the erythrocytes of its hosts where it multiplies asexually. Following division, it escapes by rupturing the old host cell and invading new ones. Several babesia rhoptry proteins and serum factors have been implicated in host cell attachment and invasion (Igarashi, Aikawa & Kreier, 1988; Vidotto *et al*. 1995), but very little is known about relevant ligands on the erythrocyte surface. However, a detailed understanding of the invasion process is likely to promote the rational development of effective therapeutics or vaccines. Kania, Allred & Barbet (1995) provide the only study on possible erythrocyte ligands of babesia using enzyme treatment of the host cell surface and competitive invasion inhibition. They showed that neuraminidase or trypsin treatment of bovine erythrocytes significantly decreased their susceptibility to invasion by *Babesia bigemina* whereas chymotrypsin had little effect. Of several potential competitive inhibitors such as red blood cell surface molecules and monosaccharides, only *N*-acetylglucosamine and *N*-acetylgalactosamine caused a reduction in parasite proliferation.

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We used the same enzymes to investigate whether they also affected erythrocyte invasion by *B. divergens*. In addition, we investigated whether the addition of bovine and human *N*-acetylneuraminyl-lactose to *B. divergens* cultures had an inhibitory effect on parasite invasion. These sialyllactoses which consist of different ratios of NeuAc(2-3)Gla(1-4)Glu and NeuAc(2-6)Gal(1-4)Glu isomers have previously been shown to differentially inhibit sialic acid-mediated haemagglutination induced by *Helicobacter pylori* (Robinson *et al*. 1990).

MATERIALS AND METHODS

In vitro adaptation of the *B. divergens* isolate used in this study (Tulla Malone or TM) has been described by Zintl *et al*. (2002). Briefly, infected bovine blood with a parasitaemia of 1·5 % was collected into EDTA, washed once in Dulbecco's phosphate-buffered saline, pH 7·2 with 15 mM EDTA, and twice in PBS without EDTA (each centrifugation at 200 ***g*** for 10 min at 4 °C). The buffy layer was removed after each centrifugation. Cultures were set up in 24-well plates with 0·05 ml of infected erythrocytes, 0·1 ml of uninfected bovine erythrocytes (suspended 1:1 in Puck's saline with 2 % extra glucose (PSGG)), 1·1 ml of HL-1 medium (BioWhittaker UK) supplemented with 2 mM L-glutamine and 15 % adult bovine serum (final PCV of 5 %). Initially, cultures were incubated at 37·5 °C in a special gas mix consisting of 5 % CO_2, 2 % O_2 and

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93 % N_2 (BOC gases). After 5–10 successful subcultures the gas mix was changed to 5 % CO_2 in air. The supernatant was replaced with fresh medium daily. Subcultures, by diluting 1 in 5, were carried out during the exponential growth phase i.e. every 2 days.

Uninfected bovine erythrocytes were taken from adult cattle reared in a babesiosis-free environment in Lyons Research Farm, Newcastle, County Dublin. Donor blood, collected by jugular venepuncture, was defibrinated by shaking with glass beads and centrifuged at 500 ***g*** for 10 min at 4 °C. The upper third of the cell pellet including the buffy layer was discarded. The lower half of the remaining pellet was washed once in PSGG and stored at 4 °C. Discarded units of human erythrocytes of type 0, rhesus +, provided by the National Blood Centre, Ireland, were washed 3 times in PSGG (180 ***g*** 10 min, 4 °C). The buffy layer was removed after each centrifugation. Equine (Lyons Research Farm) and ovine blood (Faculty of Veterinary Medicine, Dublin) was defibrinated, centrifuged at 350 ***g*** and 600 ***g*** respectively (10 min at 4 °C) and prepared in the same way as bovine erythrocytes.

For enzyme treatment, erythrocytes were suspended in an equal volume of sterile PSGG containing 2760, 27600 and 276000 U/ml TPCK-treated trypsin (from bovine pancreas; Sigma T1426). Alpha-chymotrypsin (Type I-S from bovine pancreas; Sigma C7762) was used at final concentrations of 10, 100 and 1000 U/ml and neuraminidase (from *Vibrio cholerae*; Sigma N6514) was added to erythrocyte suspensions at 10, 50 and 100 mU/ml final concentrations. The erythrocytes were incubated in each enzyme for 1 h at 37 °C with slow shaking (75 rpm). Subsequently they were washed 3 times in ice-cold PSGG and resuspended in an equal volume of PSGG.

Established cultures of *B. divergens* in bovine erythrocytes were subcultured into enzyme-treated bovine, human, sheep and equine erythrocytes; cultures were set up in 24-well plates with 0·25 ml of resuspended *B. divergens* culture, 0·1 ml of erythrocyte suspension and 1·1 ml of supplemented HL-1 medium. Three wells were set up for each erythrocyte type and enzyme concentration. Control wells contained erythrocytes incubated in PSGG alone. The following day the supernatant was changed, the cultures resuspended and transferred into 96-well plates (200 μl/well). Fifty μl of complete culture medium containing 2·5 μCi/well tritiated hypoxanthine (Amersham Pharmacia Biotech UK Ltd) were added to each well (final concentration of [^{3}H] hypoxanthine: 10 μCi/ml). Twelve replicate wells were set up for each erythrocyte type and enzyme concentration. After 24 h incubation in the presence of radioactive hypoxanthine, the cells were harvested onto glass fibre filters (Packard UK) with a cell harvester (Packard Filtermate™). Incorporation of radioactivity as cpm was determined using a direct beta-counter (Packard Matrix™ 9600).

The effects of bovine (Sigma A8681) and human (Sigma A9079) *N*-acetylneuraminyl-lactose or NeuAc-Lac were investigated by incubating *B. divergens* cultures in the presence of 0·1, 0·5 and 1 mg disaccharide/ml. NeuAc-Lac is a mixture of NeuAc (2-3)Gal(1-4)Glu and NeuAc(2-6)Gal(1-4)Glu. The variety derived from bovine colostrum is composed of 85 % of the (2-3) isomer while human milk NeuAc-Lac consists of approximately 85 % of the (2-6) isomer (Robinson *et al.* 1990). Sugars were added with 10 μCi/ml tritiated hypoxanthine 1 day after subculture. There were 12 replicate wells for each compound at each concentration. After 24 h incubation the cells were harvested and the uptake of radioactive hypoxanthine determined as described above. Control wells contained neither compound. Background levels of hypoxanthine uptake were determined by incubating uninfected erythrocytes in parallel wells.

In both sets of experiments, parasite growth was also assessed by counting the percentage of infected erythrocytes on thin Giemsa's-stained blood smears in 6 replicate wells/group. The estimates for each smear were based on a total count of 1000 erythrocytes.

In vitro culture growth in the presence of enzyme-treated erythrocytes or NeuAc-Lac is presented as a percentage of parasite growth observed under standard culture conditions. Groups were compared using one-way ANOVA and Scheffe's Post Hoc Test. The null-hypothesis was rejected at $P < 0{\cdot}05$.

RESULTS

The uptake of tritiated hypoxanthine by uninfected bovine, human, sheep, horse erythrocytes was 0·9, 2·4, 2·3 and 0·6 % of that measured in *B. divergens* cultures in the respective erythrocyte types.

Babesia cultures in bovine erythrocytes reached on average 6·5 % parasitaemia (± 0·8 S.E.) within 2 days after subculture ($n = 18$). The effects of enzyme treatment of bovine erythrocytes on parasite proliferation are summarized in Table 1. Treatment of bovine erythrocytes with trypsin had no significant effect on *in vitro* growth. Even following incubation in enzyme concentrations of 138000 U/ml, neither the uptake of hypoxanthine ($P = 0{\cdot}52$; F = 0·77; D.F. (error, group) = 44, 3) nor percentage parasitaemia ($P = 0{\cdot}1$; F = 2·39, D.F. = 20, 3) were statistically reduced. On the other hand, parasite growth was significantly decreased in bovine erythrocytes treated with alpha-chymotrypsin ($P \leqslant 0{\cdot}0001$; F = 17·41; D.F. = 42, 3 for the difference in trititated hypoxanthine incorporation; $P \leqslant 0{\cdot}0001$; F = 14·48; D.F. = 20, 3 for the difference in percentage infected erythrocytes) (Fig. 1A and B). Enzyme

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Table 1. Relative hypoxanthine uptake and relative percentage parasitaemia in *Babesia divergens* cultures subcutured into enzyme treated bovine erythrocytes

Enzyme treatment	Concentration (U/ml)	Cpm (% of control) ± S.D.	Parasitaemia (% of control) ± S.D.	Parasitaemia (% of control) ± S.D.†
Trypsin	275			23·0 ± 11·0*
	1380	95·0 ± 9·1	80·4 ± 13·1	
	2750			13·2 ± 8·3*
	13800	95·3 ± 6·4	84·1 ± 12·0	
	138000	96·8 ± 7·3	78·8 ± 23·6	
Alpha-chymotrypsin	10	80·1 ± 11·8**	78·4 ± 13·9**	
	100	78·9 ± 8·2**	68 ± 12·1**	
	1000	73·6 ± 9·0**	60·2 ± 6·8**	
	1100			91·8 ± 12·4
	11000			66·2 ± 14·1
Neuraminidase	10×10^{-3}	32·8 ± 3·7**	13·2 ± 2·9**	
	50×10^{-3}	20·0 ± 4·5**	5·5 ± 2·0**	22·9 ± 34·0*
	100×10^{-3}	13·2 ± 1·9**	2·9 ± 2·0**	3·0 ± 6·0*

† Effect of enzymatic treatment of bovine erythrocytes on the proliferation of *B. bigemina* (Kania *et al.* 1995).
* Indicates groups different from controls at the 0·05 level.
** The level of significance is given in the Results section.

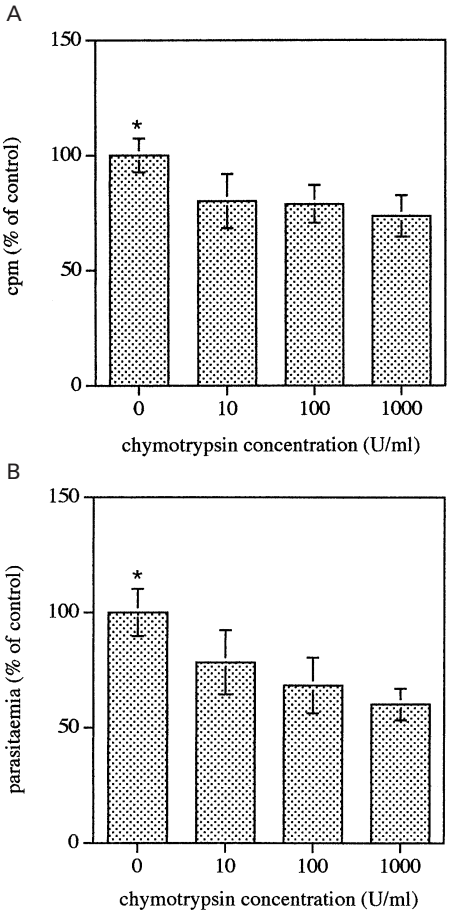

Fig. 1. Relative hypoxanthine uptake (±S.D.) (A) and relative percentage parasitaemia (±S.D.) (B) in *Babesia divergens* cultures in bovine erythrocytes treated with alpha-chymotrypsin. *Indicates groups which according to Scheffe's Post Hoc test were significantly different from all other groups. The level of significance is given in the Results section.

concentrations of 10 U/ml of erythrocyte suspension caused a decrease in parasite proliferation by approximately 20 %. Although parasite growth declined further as the enzyme concentration was raised to 100 and 1000 U/ml, there was no significant difference in the effect of the 3 enzyme concentrations.

Treatment of bovine erythrocytes with neuraminidase caused a decline of between 66 % (10 mU/ml) and 87 % (100 mU/ml) in hypoxanthine uptake ($P \leqslant 0{\cdot}0001$; F = 285·6; D.F. = 44, 3) (Fig. 2A). While cpm at 10 U/ml were significantly higher than hypoxanthine uptake at 50 or 100 U/ml, there was no significant difference between erythrocytes treated with 50 or 100 mU enzyme/ml. Percentage parasitaemia was reduced by 87–97 % ($P \leqslant 0{\cdot}0001$; F = 207·2; D.F. = 20, 3). Statistically there was no difference in the reduction of parasitaemia caused by 10, 50 or 100 U/ml (Fig. 2B).

Neuraminidase similarly affected *B. divergens* cultures in erythrocytes of other species (Fig. 2A and B). The results are again presented as a percentage of parasite growth in untreated erythrocytes of each species; parasitaemias reached on average 5·6 % ± 0·4 S.E. 2 days after subculture into human erythrocytes, 3·5 % ± 0·4 S.E. 2 days after subculture into sheep erythrocytes and 2·6 % ± 0·2 S.E. 2 days after subculture into equine erythrocytes. In human erythrocytes the uptake of hypoxanthine was reduced by 30–40 % ($P \leqslant 0{\cdot}0001$; F = 21·9; D.F. = 43, 3), percentage parasitaemia by 75–82 % ($P \leqslant 0{\cdot}0001$; F = 87·3; D.F. = 20, 3). There was no significant difference in human erythrocytes treated with 10, 50 or 100 mU enzyme. Parasite proliferation was also significantly reduced in sheep red blood cells following incubation with neuraminidase ($P \leqslant 0{\cdot}0001$; F = 104·3; D.F. = 44, 3 for the difference in tritiated hypoxanthine

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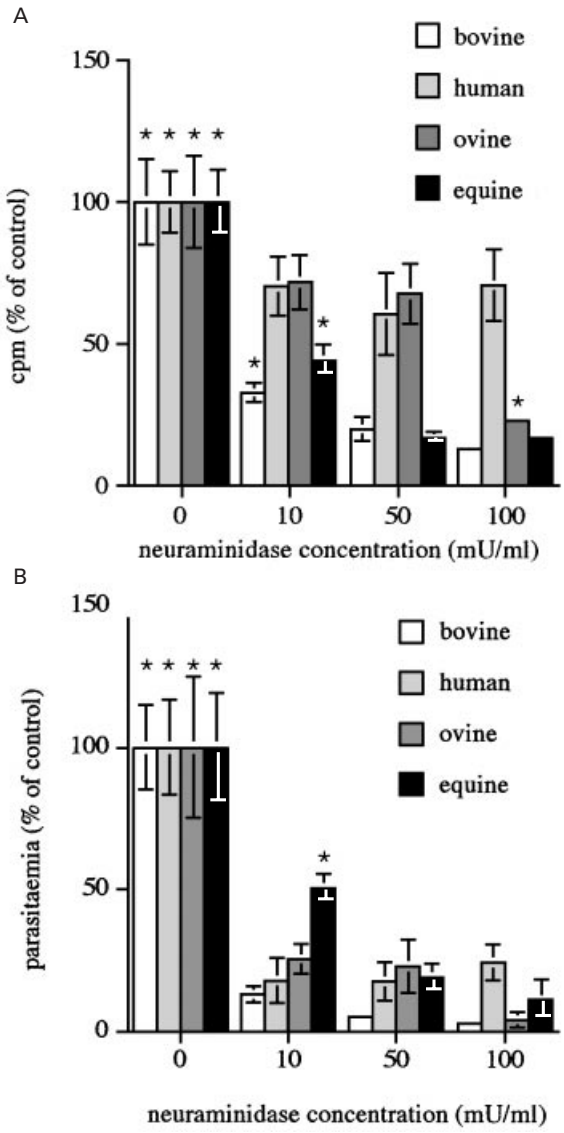

Fig. 2. Relative hypoxanthine uptake (±S.D.) (A) and relative percentage parasitaemia (±S.D.) (B) in *Babesia divergens* cultures in bovine, human, ovine and equine erythrocytes treated with neuraminidase. *Indicates groups which according to Scheffe's Post Hoc test were significantly different from all other groups. The level of significance is given in the Results section.

incorporation; $P \leqslant 0{\cdot}0001$; $F = 56{\cdot}4$; D.F. = 20, 3 for the difference in % infected erythrocytes). Uptake of hypoxanthine fell by 28–77 %, percentage parasitaemia by 75–95. While the reduction in parasite growth was similar in erythrocytes exposed to 10 and 50 mU/ml, survival decreased by a further 20–40 % when the enzyme concentration was raised to 100 mU/ml. Hypoxanthine incorporation and percentage parasitaemia in equine erythrocytes were reduced by about 50 % after incubation with 10 mU/ml neuraminidase ($P \leqslant 0{\cdot}0001$; $F = 421{\cdot}8$; D.F. = 44, 3 and $P \leqslant 0{\cdot}0001$; $F = 80{\cdot}4$; D.F. = 20, 3 respectively). Increases in enzyme concentration to 50 and 100 mU/ml led to a further significant decrease in culture growth by 30–40 %. However, there was no statistical difference between equine red blood cells incubated at 50 or 100 mU/ml.

The addition of either bovine or human NeuAc-Lac to *B. divergens* cultures in bovine erythrocytes had no effect on hypoxanthine uptake ($P = 0{\cdot}09$; $F = 2{\cdot}3$; D.F. = 64, 3 and $P = 0{\cdot}9$; $F = 0{\cdot}1$; D.F. = 65, 3 respectively) (data not shown). Percentage parasitaemia was also unaffected ($P = 0{\cdot}6$; $F = 0{\cdot}6$; D.F. = 32, 3 for cultures incubated in the presence of bovine NeuAc-Lac and $P = 0{\cdot}1$; $F = 2{\cdot}4$; D.F. = 32, 3 for parasites exposed to human NeuAc-Lac).

DISCUSSION

In our study, trypsin treatment of bovine erythrocytes did not significantly affect *in vitro* growth of *B. divergens*. Kania *et al.* (1995), on the other hand, found that trypsin decreased the susceptibility of bovine erythrocytes to invasion by *B. bigemina* by 77–87 %. The trypsin concentrations used in their study (275–2750 U/ml) were only 0·2 % and 2 % of the highest concentration used in our experiments. *B. bigemina* invasion of erythrocytes treated with 1100 and 11 000 U/ml alpha-chymotrypsin was reduced to a lesser extent (8–34 %) and was not significantly different from controls. By comparison, alpha-chymotrypsin concentrations of 10 U/ml had a significant effect on invasion by *B. divergens* reflected by 20 % reductions in hypoxanthine uptake and parasitaemia. *B. divergens* growth did not decline much further when enzyme concentrations were raised to 100 and 1000 U/ml, indicating that most available susceptible peptide bonds on the erythrocyte surface were already altered by the lowest enzyme concentration. Following incubation of bovine, human, ovine and equine erythrocytes with 10 mU/ml neuraminidase we observed a reduction in hypoxanthine uptake of between 30 and 65 % and a decline in percentage parasitaemia of between 50 and 85 %. In all but the human erythrocytes uptake of hypoxanthine fell by another significant amount when the enzyme concentration was raised from 10 to 100 mU/ml. On the other hand, percentage parasitaemia was not significantly different in erythrocytes treated with 10 or 100 mU/ml except in the case of equine red blood cells. Kania *et al.* (1995) who transferred *B. bigemina* into bovine erythrocytes treated with 50 and 100 mU neuraminidase/ml observed a decline in parasitaemia of 77 and 97 % respectively.

Neuraminidase causes the removal of 90–100 % of sialic acid from glycophorins on the surface of intact erythrocytes (Eylar *et al.* 1962; Perkins & Holt, 1988). The strong reduction of parasite proliferation observed in all erythrocyte types following neuraminidase treatment, strongly suggests that sialic acid has an important role in the host cell invasion by *Babesia* spp. Culture experiments also indicate that

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sialic acid is beneficial to *in vitro* growth (Zintl *et al*. 2002); bovine, human and ovine erythrocytes with a high sialic acid content (Eylar *et al*. 1962; Hamazaki Hotta & Konishi, 1976) all support long-term cultures of *B. divergens*, while equine erythrocytes which are low in sialic acid (Eylar *et al*. 1962; Hamazaki *et al*. 1976) do not. Moreover the variation in sialic acid on the erythrocyte surface may be reflected in the 'shelf-life' of various erythrocyte types: as erythrocytes senesce their membrane glycoconjugates desialylate (Bratosin *et al*. 1995), rendering erythrocytes with less inherent sialic acid unsuitable for culture in a shorter time-span. This may explain why human and bovine erythrocytes can be used for up to 4 weeks for *P. falciparum* (Jensen & Trager, 1977) and *B. divergens* cultures respectively, while equine erythrocytes stored for over 2 weeks become unsuitable for *in vitro* cultures of *Babesia equi* (Holman *et al*. 1994). In addition, the well-reported observation that only certain individual cattle are suitable donors for babesia *in vitro* cultures (Canning & Winger, 1987) may be because different cattle blood group phenotypes vary greatly in their sialic acid content (Hines, 1999).

Attempts to confirm the function of sialic acid as a ligand for merozoite attachment by competitive inhibition failed; neither bovine nor human NeuAc-Lac inhibited parasite invasion. Similarly, isolated glycophorin and neuraminic acid did not act as competitive inhibitors of invasion by *B. bigemina*, while the inhibitory effect of *N*-acetylglucosamine and *N*-acetylgalactosamine was probably due to their toxicity (Kania *et al*. 1995). Either the erythrocyte sialic acids used as ligands by the parasite are different from those added, or their density too high for the inhibitors to be effective at the concentrations at which they were added. Alternatively the sterical structure of sialic acid molecules in solution may be different from those bound to the erythrocyte surface to attach to the merozoites. In addition, sialic acid may not be the only erythrocyte ligand used by *Babesia* spp. It is now understood that *Plasmodium* spp. utilize a number of sialic acid-dependent and -independent pathways to invade host cells. This redundancy in the invasion process may give a selective advantage to organisms that parasitize polymorphic human populations (Dolan *et al*. 1994). The fact that trypsin treatment of bovine erythrocytes has no effect on invasion by *B. divergens* but renders them refractory to invasion by *B. bigemina* also indicate that *Babesia* spp. utilize more than one route to enter new host cells. However, the strong effect of neuraminidase on invasion of bovine, human, ovine and equine erythrocytes indicates that the sialic acid-dependent pathway is the most important one in both *Babesia* spp. Clearly, further work is required to explore these invasion pathways by identifying molecules which are able to prevent host cell invasion by competitive inhibition. By improving our understanding of babesia erythrocyte ligands, studies of host cell–parasite interactions will further drug and vaccine development.

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