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Identification of Binding Domains on Red Cell Glycophorins for *Babesia divergens*

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Abstract

BACKGROUND—Invasion of RBCs is one of the critical points in the lifecycle of *Babesia*. The parasite does not invade other host cells. Earlier work has shown that GPA and GPB function as putative receptors during parasite invasion. The primary focus of this study was the delineation of parasite binding domains on GPA and GPB.

STUDY DESIGN AND METHODS—The assay of choice to validate molecules that participate in invasion is an inhibition of invasion assay, in which changes in parasitemia are assessed relative to a wild-type assay (no inhibitors). Inhibition of invasion can be achieved by modification of different components of the assay or by the addition of competitors of the molecules that participate in invasion. In this study purified antibody fragments to various domains on GPA and GPB were tested for magnitude of inhibition of parasite invasion. Effects on invasion were monitored by assessment of Giemsa stained smears every 24 h.

RESULTS—Among ten selected antibodies directed at various epitopes on GPA and GPB, antibodies directed against GPA^M epitopes had the most severe effect (up to 35%) on inhibition of invasion, followed by antibodies directed against GPB^S epitope (up to 24%).

CONCLUSION—This study confirms the role of RBC glycophorins A and B in *B. divergens* invasion and shows that the GPA^M and GPB^S epitopes are likely to play an important role in the entry process.

Keywords

host-pathogen interaction; human babesiosis; erythrocyte invasion; apicomplexan; red cell glycophorin; transfusion transmitted disease

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Introduction

Babesiosis is a tick-borne zoonosis, a disease communicable from animals to man and an important blood-borne human parasitic infection. Human to human transmission of the parasite is well recognized to occur through blood transfusion.[1,2] Despite being a significant threat to the safety of blood transfusions, the study of this pathogen has largely been neglected.

In humans, babesiosis is caused by one of several babesial species that have distinct geographical distributions based on the presence of competent ixodid hosts.[3] In North America, babesiosis is caused predominantly by *Babesia microti*, [4] a rodent borne parasite, and also occasionally by a newly recognized species, *B. duncani.* [5] In Europe, human babesiosis is much rarer but more lethal, and is caused by the bovine pathogen *B. divergens.* [6] Disease symptoms can range from an almost silent infection to a fulminant, malaria-like disease, which can be fatal. A number of factors have contributed to the “emergence” of human babesiosis, including increased awareness among physicians, changing ecology, and an increased population of immunocompromised individuals susceptible to infection.[2] Thus, the epidemiology of human babesiosis has changed over the last 50 years, from a few isolated cases to the establishment of endemic foci in both, the United States and Europe, with significant public health impact in these regions.[7]

Host cell invasion remains the central pathogenic step in the life-cycle of all Apicomplexan parasites, like *Plasmodium* and *Babesia*. The studies detailed in this paper were done using *B. divergens* as it is the only human babesia parasite that can be propagated in human RBCs in vitro. The exceptionally high parasitemia (5–80%) seen in human infections, is also obtained in the micro-aerophilus stationary phase (MASP) *in vitro* culture.[8] The success of the parasite entry process depends on the extracellular merozoite’s ability to efficiently recognize the host cellular targets and this also serves to help them escape from the host immunological response. These parasites invade host red cells through a series of complex multi-step molecular events at the RBC–parasite interface. The first of these events includes specific recognition between parasite ligands and their cognate receptors on the RBC. Using enzymatic modification of human RBC surface membranes and clinically mutant RBCs, our laboratory has shown that *B. divergens* uses neuraminidase-sensitive receptors to enter the RBCs, of which the glycophorins A and B are the prominent ones.[9]

Determination of the binding domains on red cell receptors will facilitate our understanding of the specific receptor-ligand interactions that underlie *Babesia* invasion. In this study, we used a comprehensive set of antibodies available against various antigenic determinants on both GPA and GPB, in inhibition of invasion assays to determine regions on the glycophorin receptors that participate in invasion. Such studies are needed to identify critical domains that may interact directly with parasite proteins and help define and characterize the steps in the invasion mechanism and potentially identify new therapeutic targets.

Materials and Methods

Parasite culture

Cultures of *B. divergens* were maintained *in vitro* in erythrocytes from healthy A^+ blood volunteer donors (from New York Blood Center) collected in 10% citrate-phosphate-dextrose followed by three washes with culture medium (washed RBCs). Cells at 5% hematocrit were cultured in RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA) supplemented with 10% human serum; 50 μg/mL hypoxanthine and 7.5% (wt/vol) sodium bicarbonate solution (Invitrogen Corp.) at 37°C, using a micro-aerophilus environment of 90% carbon dioxide, 5% nitrogen, and 5% oxygen.

Antibody purification and Fab fragment preparation

A total of thirteen monoclonal antibodies (MAbs) against glycophorins A and B with MN or Ss blood group antigens specificity (Table 1) were purified using protein G-Sepharose (Protein G-Sepharose 4 Fast Flow, GE Healthcare) with IgG binding and elution buffers (Pierce) according to the manufacturer's recommendation; followed by their concentration and extensive dialysis against PBS. They were 2B14, 2B52, 2B53,2B54, 2B62 [10,11]; K92; 6A7[12]; MIMA103, MIMA174, MIMA175, MIMA179, MIMA181 and MIMA221[13]. All MAbs were provided and referenced by the Immunochemistry Laboratory, New York Blood Center, NY, USA.

Univalent Fab fragments were prepared from purified IgGs that were digested by a commercial kit (Pierce Mouse IgG_1 Fab and $F(ab')_2$;Thermo Scientific preparation Kit) according to the manufacturer's instruction. The purified F(ab) fragments were dialyzed against PBS and concentrated. Protein concentrations were measured by Bradford's colorimetric assay. The efficiency of digestion of the purified material was evaluated by SDS-PAGE (10% acrylamide gels stained with Coomassie Blue). Antibody reagents were stored in polypropylene tubes at 4°C until used.

Characterization of Erythrocytes

Multiple erythrocyte parameters such as the membrane deformability, the mean corpuscular volume (MCV) and the cell volume variation index (RDW, red cell volume distribution width) were assessed to evaluate the influence of each monoclonal antibody on the red blood cell characteristics.

Membrane deformability was evaluated by ektacytometry, a laser diffraction method previously described by Narla and colleagues.[14] Washed RBCs were suspended in PBS (vol/vol) containing 4% polyvinylpyrrolidone and the intact MAbs were assayed after 30–60 min incubation, at room temperature. The cells were subjected to increasing stress of 0–200 rpm/min, over a 10-min period. The change in their laser diffraction pattern from circular to elliptical was measured. Each curve generated provided a measure of membrane deformability that was compared with the controls and expressed as a deformability index. The concentration of each intact MAb used in these assays was identical to that used in the invasion assays (50–100 μg/mL). Untreated red cells were used as comparative controls.

MCV and RDW measurements were obtained using an automated hematology analyzer (Advia 120 multispecies hematology analyzer; Bayer Healthcare).

B. divergens growth-inhibition assay

Erythrocytes infected with *B. divergens* merozoites were grown until 50–60% parasitemia was reached. At the start of each assay, the parasitemia was adjusted to 1–4% using fresh RBCs. 200 μL cultures (5% hematocrit) were set up and were followed for 48–72h in the presence or absence of specific Fab fragments. Each experiment was conducted in triplicate. The culture medium was changed daily and the infectivity of the merozoites was monitored every 24h.

The efficiency of the invasion process in controls and test samples was expressed as percentage of infected cells by analyzing Giemsa-stained smears. Student's t-test was carried out to compare the parasitemia in test and control samples. The level of significance was set at $p \leq 0.05$.

Results

Effect of monoclonal antibodies on the structural erythrocyte variation

In order to map the region of glycophorins binding to the parasite, various MAbs against different epitopes of the glycophorins A and B were exploited. It has been postulated that MAbs against red cell membrane proteins may alter membrane properties, which, in turn, may alter receptor function.[15] In such a study, it is therefore important to discriminate between changes in RBC deformability due to the antibody and specific effects of the MAbs on the interaction of the *Babesia* merozoite and the red cell. Thirteen MAbs (Table 1) were thus, first evaluated for their effect on RBC membrane deformability so that we could identify specific inhibitory effects of the antibodies on parasite invasion. In order to achieve this, red cell deformability studies and various red cell measurements were assessed as parameters to evaluate the effect of these Mabs on both the RBC membrane and overall RBC physiology.

Figure 1 shows the effect of MAbs on the erythrocyte membrane deformability as measured by ektacytometry. In comparison with the control (untreated cells), only two MAbs (MIMA221 and 2B62) induced a decreased membrane deformability of the RBC as seen by a much larger value of applied shear stress to obtain equivalent extent of the deformability index. When the RBCs were incubated with the other eleven MAbs there was no change in erythrocyte membrane deformability (Fig. 1) and the deformability index versus shear stress curves for these MAbs were identical to that of the control red cells (data not shown).

Effect of MAb on red cell volume

We also studied various RBC parameters: cell volume, MCV and RWD in order to ensure that treatment with various antibodies did not induce significant red cell alterations during the invasion assays. As can be seen in Table 1, MCV and RDW values remained relatively stable for the majority of MAbs tested at concentrations 50 μg/mL and 100 μg/mL. MCV values from 10 of 13 antibody treatments of RBC assays (2B14; 2B53; 2B54; 6A7;

MIMA103; MIMA174; MIMA175; MIMA179; MIMA181; MIMA221) showed a basal variation (88–91 fL) similar to the variation observed for the controls of two independents experiments (88–90 fL). This baseline variation seen in the majority of MAb treatments represents a change of ~1–3% in red cell volume as compared to the untreated red cell. For the same 10 MAbs, the parameter RDW reflecting heterogeneity of cell volume also showed low variation, with the values ranging from 0% to <10% in relation to the control. However, three MAbs (2B52; 2B62; K92) induced an increase in the mean corpuscular volume and/or RBC distribution width, and this effect appeared to be concentration-dependent. 2B52, which in comparison with 2B62 and K92 had a weaker effect on the morphology of the RBC, did not induce structural cell changes when used at a lower concentration. As can be seen from Table 1, in comparison with the control untreated RBCs, 2B52 at a concentration of 50 μg/mL showed no change in RDW values of cells; and MCV values were within the basal variation limits (3% higher than control). This was not the case with 2B52 at 100 μg/mL however, as both MCV and RDW values increased in comparison with the control. At this antibody concentration, MCV was 7% higher than untreated cell (MCV: 90fL control; 96 fL test) and RDW showed a startlingly 92% increase in value, in comparison to the RDW obtained for untreated erythrocytes (RDW: 13 control/25 test). Cells incubated with 2B62 and K92 exhibited an increased MCV for both antibody concentrations. (10–12% larger than the control) (2B62-MCV: 100 fL and 100 fL; K92-MVC: 99fL and 102 fL for 50 μg/mL and 100 μg/mL MAb respectively; and Controls-MCV: 88fL and 90 fL from two independent experiments); and the RDW increased 100–130%, more than double for K92 in comparison with control (B62-RDW: 26 and 29; K92-RDW: 28 and 30 for the two different MAbs concentrations; Control-RDW: 13).

Based on these results obtained from ektacytometry and analyses of RBC volume, we have found significant changes in all cellular parameters investigated, when RBCs were treated with the following antibodies: 2B52, 2B62 and K92. Thus, these antibodies were eliminated from further analysis and studies on parasite invasion focused on the remaining 9 of the original 13 MAbs, 2B14; 2B53; 2B54; 6A7; MIMA103; MIMA174; MIMA175; MIMA179; MIMA181. An exception was made for MIMA221, a monoclonal antibody with EnaFs specificity that induced only a mild change on red cell deformability but not on the other erythrocyte parameter evaluated here. So, in addition to the other MAbs with specificity to MNS antigens, MIMA221 (with EnaFs specificity) was also included in the assay and, in total, 10 antibodies were tested for effects on invasion in parasites cultures.

Effect of Specific antibodies on parasite invasion

In order to avoid crosslinking of surface molecules or RBC agglutination in parasite cultures, univalent Fab fragments were prepared from the 10 selected MAbs which showed no effect on RBC membrane stability and which were directed to various epitopes of GPA and GPB, (2B14; 2B53; 2B54; 6A7; MIMA103; MIMA174; MIMA175; MIMA179; MIMA181). For the reasons mentioned above, Fab-MIMA221 was also prepared, and presented an additional epitope to be tested. The inhibitory potential of these selected MAbs on *B. divergens* erythrocyte invasion was assayed with Fab concentration ranging between 50–100 μg/mL (depending on availability of sufficient antibody). Use of these high Fab

concentrations, enabled us to achieve near saturation of the available epitopes on the glycophorin receptors.

Inhibition of parasite invasion in antibody treated cultures varied according to the specific antibody tested and also depended on the length of time the parasites were treated with the specific antibody. All cultures were initiated at approximately 1–4% parasitemia (zero time). Table 2 show the average percentage of parasitemia obtained from each invasion assay (mean of 3 inhibition of invasion assays). As can be seen by parasitemia levels achieved, inhibitory effects of the MAbs in the invasion assay were noted as early as 24 hours of culture with most of the antibodies (Table 2). While some antibodies induced inhibition at the early time points (2B53, MIMA175, MIMA179 and MIMA181), there were some antibodies that exerted a constant inhibition through the entire 72 h of testing (6A7) and those that seemed to have a cumulative inhibitory effect with the degree of inhibition increasing at each time point (MIMA-103). Some antibodies like 2B54 showed a significant inhibitory effect only at the last time point of assay (72h, $p<0.05$).

Magnitude of Inhibition

The same antibodies when classified according to the magnitude of inhibition fell into three classes of activity: (1) No or negligible effect; (2) mild effect on invasion but not found to be significant; and (3) moderate effect on merozoite cell invasion, found to be significant at specific time points of culture (Table 2).

In the first class are 2B14 and MIMA174 antibodies (Table 2). 2B14 treatment showed no effect on parasite growth and MIMA174 showed a negligible effect (at most 6% inhibition) with antibody treated and control cultures showing similar parasitemia at all time points tested.

A mild inhibition on cell invasion was observed with 2B53, MIMA175, MIMA179 (Table 2), at least at one time point of testing. Although we found that the specific MAbs interfered with parasite invasion, the difference between the parasitemia of treated and untreated cells was not statistically significant ($p\geq 0.5$) representing up to 17% inhibition of red cell infection (Table 2).

A moderate effect on invasion was observed only with 2B54, 6A7, MIMA103, MIMA181 treatments (Table 2). MIMA103 was the most inhibitory antibody to reduce parasite growth. The parasitemia at 48 h were 15% compared to 24% in control wells (inhibition of 38%) and at 72h was 43 % in the treated sample compared to 66% in the control (inhibition of 35%), showing a significant difference between test and control cultures ($P\leq 0.05$) (Table 2). All four antibodies showed significant difference in the parasitemia between treated and untreated samples ($P\leq 0.05$), with inhibitions observed at 19–38% as recorded in Table 2.

Epitope Specificity of Inhibitory Antibodies

In order to define what regions of GPA and GPB may play a role in parasite binding, we looked at the specific epitopes of the inhibitory antibodies, that have been mapped in previous studies. [10–13]. We found that all of the three antibodies that caused the highest inhibition, (2B54, 6A7 and MIMA103; inhibition of 20–35%, $p\leq 0.05$) were specific for

GPA^{M} epitope (see materials and methods). Additionally, MIMA-181, specific for a GPB$^{S\text{-like}}$ epitope, also resulted in a moderate but significant inhibition of invasion (up to 24%, $p \leq 0.05$). Another antibody (2B53), also against an epitope at the amino terminal of GPA (GPA, Gly^{5}) showed only a mild effect on merozoite invasion (10% inhibition; $p > 0.05$). However, this antibody was assayed at a lower concentration (50 μg/mL) in comparison to the others which were assayed at 100 μg/mL (Table 2) as the available amount of this antibody was limited. Epitopes on GPB^{s}, GPB^{U} and Enas^{FS} may not play a role in parasite invasion as antibodies specific for these epitopes did not have an effect on parasite cell invasion. 2B14, an antibody directed to an epitope localized to the transmembrane region of the glycophorins (GPA 119-124) (Table 1), showed no effect on parasite invasion (Table 2). This is a region on the receptor, almost impossible for the parasite to access and hence serves as a suitable control for our invasion assays. Unfortunately the use of binding domains on the N blood group antigen could not be tested in parasite cultures because both available antibodies (2B62-GPA-N^{leu1} and K92-Gp^{N}) had a severe effect on erythrocyte membrane deformability.

Microscopic Evidence of Inhibition of Invasion

We also found an interesting correlation with microscopic images of cultures treated with the antibodies and the severity of inhibition of invasion. The Giemsa stained smears of cultures treated with those antibodies that significantly interfered with *B. divergens* ability to invade cells, showed the presence of free merozoites whose numbers appeared to relate to time of antibody treatment. This fact is illustrated in Figure 2 where cultures treated with MIMA103, showed after 24h treatment, a small number of extracellular, free parasites outside the cells and this number increased after 72h. On the other hand, few extracellular parasites were observed in untreated cultures at all evaluated time periods (Fig. 2).

Discussion

From a blood safety perspective, transfusion-transmitted infections involving *Babesia* spp have become increasingly problematic, with progressively more cases reported each year. [1,16] Invasion of the human red cell is the central pathogenic step in the life-cycle of *Babesia*. The blood stage *Babesia* parasite is designed for one major role: to locate, bind to and invade host red blood cells. This is a very specific interaction as *Babesia* does not invade other host cell types.[3,17] This specificity implies the presence of a receptor(s) on the erythrocyte, which is recognized by a complementary parasite ligand(s). Earlier work in our laboratory focused attention on GPA and GPB as potential receptors for the parasite[9] and here we have delineated what appear to be important binding epitopes on these receptors for parasite entry. Among the ten selected antibodies directed at M, S-like, s, U and Ena^{FS} epitopes on these blood group antigens, that were pre-selected based on their ability to leave the RBC membrane unperturbed, we found that antibodies directed at GPA^{M} and GPB$^{S\text{-like}}$ had the highest degree of inhibition, while antibodies directed to epitopes on GPB^{s}, GPB^{U} and Enas^{FS} did not impair invasion.

It is well established that some red cell antibodies by themselves may alter erythrocyte properties, such as membrane rigidity. This is considered a critical factor of the host cell's

ability to support invasion of related parasites such as *P. knowlesi* and *P. falciparum.*[15] Specifically extracellular ligands against GPA and Band 3 can modulate transmembrane signaling and interfere in the erythrocyte membrane deformability[18], promoting strong or transient interactions between integral membrane proteins which can lead to increased rigidity of RBC membrane, [18,19] which in turn inhibits parasite invasion. To avoid such a misinterpretation of the antibodies' role in parasites invasion, antibodies such as 2B-62 (GPA^N, Leu^1); K92, (Gp^N) and 2B52 (GPA^M, Ser^1) which were found to change RBC membrane properties were not assayed in culture.

Very little is known about the interaction between parasites of all *Babesia* species and their host cells, especially the nature of host cell receptors used for entry. Studies have mainly focused on vaccine and diagnostic fields by searching for novel antigenic parasite ligands to develop new methods for disease control or pathogen identification[7,20,21]. In contrast, research related to parasite ligand-erythrocyte receptor interactions of related Apicomplexan parasites *Plasmodium* and *Toxoplasma* have accelerated, [22] giving us a comparative platform to analyze the invasive process in *B. divergens. Plasmodium* invades human red cells through a complex multi-step mechanism which involves different pathways that exploit multiple ligand-receptors interactions.[23] These pathways have been broadly classified as sialic acid (SA) dependent or independent. The SA dependent pathways mainly rely on the sialylated glycophorins (GPA, GPB, GPC) for parasite entry.[24] The alternative SA-independent pathway comprises of receptor-ligand interactions not fully characterized yet (X, Y and Z receptors) but these molecules are also considered important "entrance doors" on the red blood cell surface for parasite invasion.[25,26] Recently, CR1 (complement receptor 1) and basigin have been identified as two of these alternative pathway receptors[26,27] and mediators of SA-independent erythrocyte invasion in *P. falciparum* field isolates.

This wide variety of proteins available on the surface of the RBC used by other apicomplexan parasites as receptors for invasion may explain why the different antibodies assayed in this study that blocked distinct epitopes on GPA^M and $GPB^{S\text{-like}}$, were able to inhibit the *B. divergens* invasion. These results corroborate our earlier findings demonstrating that GPA and GPB function as receptors for *B. divergens* invasion when mutant cells lacking GPA or GPB did not support efficient invasion.[9] The results in this paper also confirm a greater role for GPA in parasite invasion relative to GPB as found in our earlier study[9] as antibodies against GPA^M (2B54; 6A7; and MIMA103) had a more potent inhibitory effect (up to35% inhibition, $p \leq 0.05$) than anti- $GPB^{S\text{-like}}$ antibodies like MIMA181 (17% inhibition, $p \leq 0.05$) (Table 2). It is also reasonable to speculate that copy numbers of the various proteins on the RBC surface may be tied to their functioning as parasite receptors. GPA is larger than GPB (43,000 kDa vs 25,000 kDa) and there are 800,000 molecules on each red cell, four times the amount of GPB molecules available per cell.[28]

Interestingly, a geographic association between GPB*S/s polymorphism and the susceptibility to *P. falciparum* infection has recently been described, where the GPB^{S+} variation shows at a higher frequency among patients with malaria disease in contrast to GPB^{s-} variant which has a higher frequency among uninfected individuals.[29] This

indication that the S epitope may participate in malaria parasite entry is in line with our results as MIMA181, a $GPB^{S\text{-like}}$ (Table 2) induced moderate inhibition of *B. divergens* invasion while antibodies directed at other GPB-epitopes (GPB^{s}, MIMA174 and MIMA175; and GPB^{U}, MIMA179) showed no effect on merozoite invasion (Table 2). Our results, thus suggest that *B. divergens* may share some aspects of the malaria SA-dependent pathway, preferentially exploiting GPB^{S} as the domain of binding on GPB instead of GPB^{s} or GPB^{U}.

In conclusion, results presented in this paper suggest that GPA^{M} and GPB^{S} epitopes of the glycophorins play an important role in *B. divergens* invasion. This appears to be via a SA-dependent pathway, and substantiates results obtained previously demonstrating that neuraminidase treatment of the RBC ablates parasite invasion [9]. As found in our previous study, GPA in comparison to GPB seems to be preferentially used by merozoites in the invasion process. Epitopes relating to GPB^{s}; GPB^{U} and Ena^{FS} were not found to play a major role in invasion since no inhibition was observed when these epitopes were blocked by the corresponding antibodies. Similar to related Apicomplexa, *B. divergens* may also exploit multiple receptors on the host cell for entry since the inhibition caused by GPA epitope blocking was merely 30% of normal invasion. This could be an important survival tactic of the parasite in terms of parasite adaptation to ensure successful RBC invasion independent of host genotype. The inhibition of RBC invasion by the *Babesia* merozoites would prevent infection and consequently disease. A detailed study of the molecular interactions between parasite ligands and their cognate RBC receptors will be critical to understand the mechanisms of parasite invasion. This in turn would provide powerful platforms to test strategies to block parasite entry and consequently lower the impact of this transfusion transmitted pathogen.

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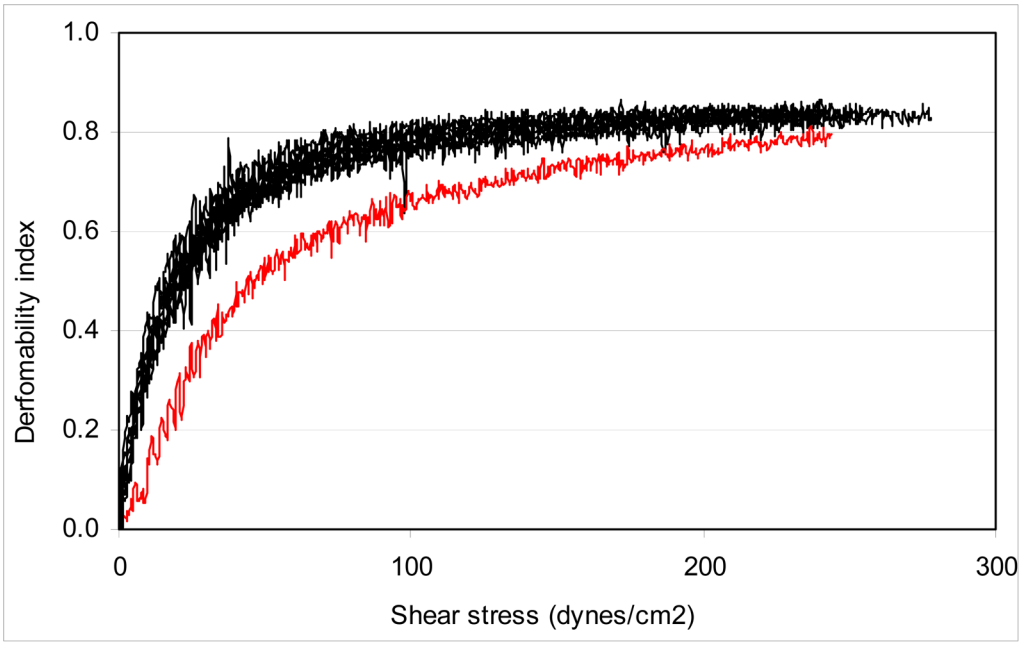

Fig. 1. Effect of MAbs on the erythrocyte membrane deformability as measured by ektacytometry

Black curves depict the absence of any effect on erythrocyte membrane deformability in RBC treated with the following MAbs: 2B52; 2B53; 2B54; 2B14; 2B15; MIMA103; MIMA174; MIMA175; MIMA177; MIMA179; MIMA181; K92; 6A7, as the control and untreated red cells. Red curve depicts the erythrocyte membrane deformability after treatments with 2B62- and MIMA221-MAb.

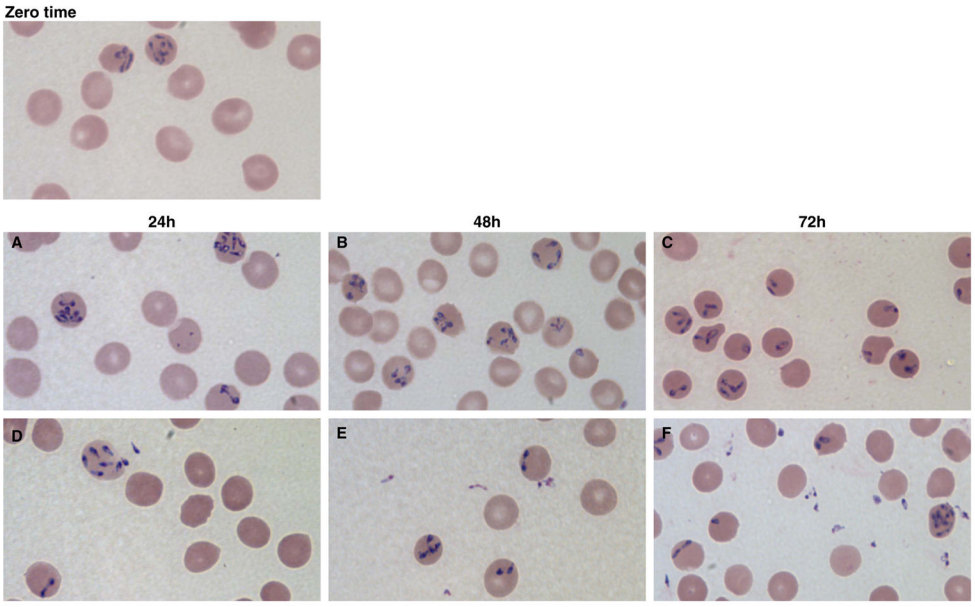

Fig. 2.

Giemsa stained smears demonstrating the inhibition of red cell invasion by the inhibitory antibodies. An increased number of extracellular *B. divergens* merozoites is seen in cultures exposed to MIMA103, after 72h treatment. Panels (A, B, C) Control cultures, no Ab Panels (D, E, F) Cultures treated with MIMA103, at different time points.

Table 1

Effect of different GPA and GPB MAbs on the morphology of RBCs.

MAbs/Fab Identification	Epitope[Reference]	Ig Class	µg/µL	MCV[(a)] (fL)	RDW[(b)]
2B14*	GPA 119-124[10, 11]	IgG1*k*	100	89	14
2B52	GPA-M Ser1[10, 11]	IgG3*k*	50 100	91 96	13 25
2B53*	GPA-Gly5[10, 11]	IgG1*k*	50 100	91 88	13 14
2B54*	GPA-M[10, 11]	IgG1*k*	50 100	89 89	13 14
2B62	GPA-N Leu1[10, 11]	IgG1*k*	50 100	100 100	26 29
6A7*	GPA-M[12]	IgG1*k*	100 100	91 88	13 14
K92	N[*pc*]	IgG2a	50 100	99 102	28 30
MIMA103*	GPA-M[13]	IgG2a*k*	100	89	14
MIMA174*	GPB-s[13]	IgG2a	50 100	90 89	13 14
MIMA175*	GPB-s[13]	IgG2a	50 100	91 89	13 13
MIMA179*	GPB-U[13]	IgG1*k*	100 100	89 91	13 13
MIMA181*	GPB/S-like[13]	IgG2b	50 100	91 88	13 13
MIMA221*	EnaFS[13]	IgG2a*k*	100 100	88 91	14 13

(RDW) Red Blood Cells distribution width;

(MCV) mean corpuscular volume;

*Fab was prepared and assayed in parasites culture;

[(a)]MCV-Controls (untreated cells) from two independent experiments ranged between 88–90 fL;

[(b)]RDW-Control value for all experiments: 13;

[*pc*]Halverson, Personal communication.

Table 2

Parasitemia of cultures at 24h, 48h and 72h in the presence of specific antibodies.

Antibodies	24h			48h			72h		
	P (SEM)	C (SEM)	I	P (SEM)	C (SEM)	I	P (SEM)	C (SEM)	I
GPA									
2B53	4.5 (±0.1)*	5.4 (±0.2)	**17**	30 (±0.9)	34 (±1.4)	**12**	53 (±2.8)	59 (±1.9)	**10**
2B54	5.4 (±0.1)	5.4 (±0.6)	**0**	31 (±1.9)	33 (±1.4)	**7**	40 (±0.7)*	59 (±1.9)	**32**
6A7	4.8 (±0.1)*	6.1 (±0.2)	**22**	26 (±0.3)*	32 (±0.8)	**19**	43 (±3.2)*	54 (±2.3)	**20**
MIMA103	5.4 (±0.3)	6.1 (±0.3)	**11**	15 (±2.1)*	24 (±0.6)	**38**	43 (±2.5)*	66 (±1.7)	**35**
2B14	6.0 (±0.3)	6.1 (±0.2)	**0**	33 (±1.1)	33 (±0.8)	**0**	nd	-	-
MIMA221	9.4 (±0.5)	10 (±0.1)	**6**	19 (±0.8)	22 (±2.6)	**14**	29 (±1.3)	31 (±2.0)	**7**
GpB									
MIMA174	6.0 (±0.4)	6.1 (±0.2)	**1**	31 (±1.2)	33 (±0.8)	**6**	54 (±2.0)	54 (±2.3)	**0**
MIMA175	5.1 (±0.6)	6.1 (±0.2)	**16**	32 (±2.0)	33 (±0.8)	**3**	52 (±2.6)	54 (±2.3)	**4**
MIMA179	6.6 (±0.4)	7.8 (±0.5)	**15**	33 (±1.2)	38 (±1.3)	**13**	51 (±1.8)	51 (±4.4)	**0**
MIMA181	4.6 (±0.4)*	6.1 (±0.2)	**24**	30 (±0.4)	33 (±0.8)	**9**	45 (±0.4)*	54 (±2.3)	**17**

Antibodies were assayed at 100 μg/μL, except for 2B53 and 2B54 assayed at 50 μg/μL;

*Level of significance by Student's t-test, p≤ 0.05;

(C) Parasitemia of Control cultures;

(P) Parasitemia of treated culture;

(I) Inhibition of invasion relative to the control;

(SEM) Standard Error of the Mean.