Comparative analysis of red blood cell membrane proteins that are targets for protozoan merozoite invasion in different animal species

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Abstract: Erythrocyte membrane protein Band 3 and the sialoglycoproteins are implicated in what are termed as sialic acid independent and sialic acid dependent invasion mechanisms of apicomplexan merozoites, respectively. The efficiency of invasion of Red Blood Cells (RBCs) by these parasites is known to differ across species and the putative reason for this variation is the status of these proteins in the different species. This study examined physical factors of these target receptors in a number of species to appreciate some of the RBC related attributes that may influence the invasion efficiency. The apparent molecular weights and relative quantities of protein band 3 were determined as well as the sialoglycoproteins in Sodium Dodecyl Sulfate Poly-Acramide Gel Electrophoresis (SDS-PAGE) gels stained with Coomasie blue and Periodic Acid-Schiff (PAS) stain, respectively. The total sialic acid content of the membranes was also determined in each species. ANOVA using StatView 4.5J was used for statistical levels. The apparent molecular weights (kD) of band 3 were recorded and so were the relative quantities (%) of the protein in the same species. There were significant differences of apparent molecular weights in all species except between deer, cow and dog; and between cow and dog. As for the relative quantities there were significant differences in all species except between deer, cow, dog and sheep; cow, dog and sheep; and dog and sheep. As for the total sialic acid content (%) of the RBC membranes there were significant differences in most species except between cow and horse; deer and horse; and llama and humans.
This study showed the physical differences in the target RBC receptors responsible for apicomplexan merozoite invasion in different animal species and thus highlights their potential to influence merozoite parasite invasion efficiency.
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1. Introduction

Erythrocytes express an unexplained density and diversity of glycoproteins on their surfaces that have evolved as a consequence of host-pathogen interactions. These surface glycoproteins are now known to be principal receptors used by pathogens to invade the erythrocytes (2). Pathogens such as protozoan parasites form some of the most common intra-erythrocytic parasite infections of free-living animals world- wide with massive economic/health consequences. Invasion of RBCs by these parasites has been widely studied using the human malaria protozoan parasite, plasmodium falcipurum, as a model (6, 13, 22). It is now known that the parasites possess merozoite surface proteins that are required for host erythrocyte recognition and binding in order to initiate an intra-erythrocytic invasion process that starts with random attachment, re-orientation and then penetration into the erythrocyte (6, 13, 16, 19, 20, 22). This happens through two mechanisms i.e. sialic acid dependent and a sialic acid independent mechanisms. In a sialic acid dependent mechanism the sialoglycoproteins have been implicated as specific receptors of the invading parasites whereas for the sialic acid independent invasion, erythrocyte protein band 3 has been implicated (3, 5, 10, 11, 12, 16, 19, 21, 24, 26).

Despite a number of studies done to understand the invasion process, very few studies, if any, have analyzed the red blood cells (RBCs) membrane target receptor proteins in normal and in disease (10, 26). This is despite suspicion that differences in the invasion efficiency among species may be influenced by adaption differences that have evolved on these surface glycoproteins among species. A study by Gaffar et al. (10) raised a question as to whether the amount of sialic acid residues present in membrane proteins is the main factor determining invasion efficiency. Exploring differences in these target receptors offers an opportunity for understanding how these molecular differences may influence efficiency of invasion from

the point of view of the RBC membrane. This paper therefore, compares attributes of Band 3 and sialoglycoproteins in normal RBCs from a select number of species.

2. Material and Methods

Blood samples.

Blood samples were collected in heparin (10-20 IU/ml blood) from deer, cow, dog, horse, sheep, llama and humans. The samples were then put on ice and subjected to ghost cell membrane preparation within 3 hours of collection.

Ghost cell membrane preparation.

About 20 mls of blood was used for each specific preparation. The blood was centrifuged at 2000 rpms for 5 minutes at 4^0C, and then plasma and buffy coat were discarded. Five mls of RBCs were taken and diluted 10 times with cold PBS (10 mM NaPi, pH 7.4, 154 mM NaCl) and then centrifuged at 2000 rpms for 5 min at 4^0C. The supernatant together with the buffy coat were removed and the procedure repeated 3 times. The RBCs were then mixed with Hypotonic buffer (5 mM Tris-HCl, pH 7.8, 1 mM EDTA) at 10 times dilution together with 60 µl of 0.8 M PMSF (to final conc. of 1 mM) while vortexing and then left to incubate on ice for 10 min. The mixture was then ultra-centrifuged at 15000 rpms for 10 min, the supernatant removed and fluffy pellet which are RBC ghost membranes re-suspended and the procedure repeated 3 times. Finally the fluffy pellet was re-suspended in sucrose buffer (250 mM sucrose, 1 mM EDTA/Tris, pH 7.4) centrifuged at 15000 rpms for 10 min at 4^0C, the ghost membranes were collected and re-suspended in a very little amount of sucrose and stored at -80^0C after the protein concentration had been determined using the Protein Assay Kit (BioRad, Bradford Method).

Sodium Dodecyl Sulfate Poly-Acramide Gel Electrophoresis (SDS-PAGE).

RBC ghosts (15 µg/well) were solubilized in sample buffer (25% of 0.5M Tris-HCl; 20% glycerol; 10% 2-mercaptoethanol; 2.5% Bromophenol blue; 2.5% 0.1 M EDTA; 4% of SDS) to a ratio of 1:1. SDS-PAGE was performed in 10% separation polyacrylamide gels with a 4% stacking gel using the BioRad assay kit. Standards used as molecular weight markers were the BioRad precision plus protein standards. The samples were run at 150 V for an initial 10 min and then continued at 200 V for 65 min or until the marker line reached about 1 cm from the edge of the gel.

Comassie blue staining and destaining.

The polyacrylamide gels were stained in 0.05% Coomasie Brilliant Blue R250 (Sigma) in 40% methanol and 10% acetic acid for about 1 hour with constant agitation. They were then destained in 40% methanol and 10% acetic acid with constant agitation until the blue background of the gels was clear. Thereafter they were washed in distilled water until clear or in some cases left overnight before being scanned by a GS-800 Calibrated Densitometer® (BioRad).

Periodic Acid-Schiff (PAS) stain.

The gels were fixed in 10%acetic acid with 25% methanol for 1 hour to overnight at room temp then removed from the fixer in a dark room, 0.5% periodic acid added while on ice and agitated for 1 hour while in the dark. Periodic acid was drained and 0.5% Sodium metarsenite in 0.5% acetic acid added and continuously agitated for 30 min. Thereafter the gels were washed in 10% acetic acid for 20 min repeated 3 times each still in the dark room. Then the gels were stained in cold Schiff reagent on ice in the dark for 2 hours with continuous agitation. Finally the gels were washed in 10% acetic acid for a short while, removed from the dark and then scanned immediately before gels became reddish. For PAS staining examination, the concentration of RBC membrane proteins used was 50 µg per well of SDS gels.

Sialic acid content determination.

Sialic acid content of RBC membranes was determined by the periodate-thiobarbituric acid method of Denny et al. (4). Briefly membranes (1 mg membrane proteins/ml) were first hydrolyzed in 0.05 M H_2SO_4 to a final volume of 0.1 ml for 1 hour at 80^0C to release sialic acids. Standards of N-Acetylneuraminic acid (NANA) were also made ranging from 0-50 ng/ml and similarly treated to a volume of 0.1 ml with 0.05 M H_2SO_4. Both samples and standards were then incubated with 0.25 ml periodate solution (0.025 M periodic acid in 0.25 M HCl) at 37^0C for 30 minutes. After reduction of excess periodate with 0.25 ml 0.32 M sodium thiosulphate, the reaction was completed by addition of 1.25 ml 0.1 M thiobarbituric acid. The samples were heated at 100^0C for 15 minutes and cooled to room temperature with tap water. Finally 2.2 ml acid butanol was added, vigorously shaken and centrifuged at 1500 rpms in order to extract the product. The top butanol phase was then collected and assayed spectrophotometrically at 549 nm.

Densitometer and statistics.

Analysis of gel bands and their attributes was done using a GS-800 Calibrated Densitometer® (BioRad) and a software called Quantity One 1-D analysis software (BioRad). All statistical calculations (using ANOVA followed by Fishers at 5% significant difference) were performed on statistical program StatView 4.5J (Abacus).

4. Results

Protein band 3 attributes in the different species.

Coomassie Blue stains all the major erythrocyte membranes at varying intensity. Generally a similar pattern of all the major membrane proteins was seen in all the species examined. Protein band 3 was identified in all the species and its attributes notably the apparent molecular weights and the relative percentage of the protein among the membrane proteins in each species were noted as shown in the table 1. The two attributes were chosen because they generally give an indication of the available amount of the particular protein in the membranes of the species in question (relative quantity) and the physical features of the protein (molecular weight).

Table (1). The apparent molecular weights (kD) and relative quantities of the band 3 proteins of different animal species. The values are presented as mean±SD (n=4)

Species	Apparent Molecular Weight (kD) M±SD	Relative Quantity (%) M±SD
Deer	93.5 ±0.3	24.9 ±4.0
Cow	93.3 ±0.6	19.9 ±4.5
Dog	93.9 ±0.5	20.5 ±7.2
Horse	87.9 ±0.4	37.4 ±3.0
Sheep	91.4 ±0.5	26.2 ±5.5
Llama	96.2 ±0.6	47.9 ±9.7
Human	95.0±0.3	25.0±3.2

There were significant differences ($P<0.05$) in the molecular weights of band 3 in all species except between the deer and cow; deer and dog; and also between cow and dog. As for the relative quantities, there were significant differences ($P<0.05$) in almost all the species except for the deer and cow; deer and dog; deer and sheep, cow and dog, cow and sheep; and finally dog and sheep. It should be mentioned here that this data was derived from 4 good gels.

Sialoglycoproteins in the different species.

Periodic Acid-Schiff stains sialoglycoproteins. The number of sialoglycoprotein bands, apparent molecular weights and relative quantities were determined and are presented in table 2. The sialoglycoproteins show very little similarities, if any at all, in the different animal species. The number of bands observed, their apparent molecular weights, their relative staining intensities and mobilities were very different in all the species examined.

Table (2). The Apparent molecular weights (kD) and relative percentages of sialoglycoprotein bands on PAS stained SDS-PAGE gels in different animals species

Species	Number of bands detectable	Apparent Molecular weight per band (kD)	Relative quantity (%)
Deer	5	174.71 123.05 80.04 62.78 33.73	18.8 15.4 47.7 9.3 8.8
Cow	1	410.36	100
Dog	7	91.79 54.18 38.37 33.39 31.36 27.84 25.84	21.7 12.8 12.6 9.3 9.9 20.7 13.1
Horse	5	88.10 63.61 42.06 37.8 28.82	5.8 12.4 4.3 7.0 70.4
Sheep	3	45.13 27.38 22.99	28.7 17.7 53.6
Llama	4	88.7 37.72 22.34 16.04	10.7 35.0 30.2 24.1
Human	5	75.77 60.91 39.53 35.21 22.48	86.0 2.1 4.5 4.2 3.3

It was also observed that some deep PAS staining occurred in some species below the dye front in each lane. This has been attributed to lipids in a previous study (1) and therefore was not included in calculating the relative band content of sialic acid. Furthermore, protein band 3 for a number of animal species showed mild PAS staining. The animal species included dog, horse and llama.

RBC membrane sialic acid content in the different species.

The means of sialic acid concentrations for the different animal species are shown in table 3 below. Further the relative sialic acid content for each of the bands detected on PAS stains was calculated and recorded. There were significant differences ($P<0.05$) in the total sialic acid content among most animals except between cow and horse; deer and horse; and between llama and humans as shown in table 3

Table (3). The total sialic acid content and relative quantities of PAS stained SDS-PAGE gels of RBC ghost membranes from different animal species

Species	Total RBC membrane Sialic Acid (ng/mg) (M±SD) (n=8)	Band relative Molecular weight (kD)	Relative PAS stain quantity (%)	Relative Sialic Acid quantity per band (%)
Deer	49.98 ±5.04	174.71	18.8	9.40
		123.05	15.4	7.70
		80.04	47.7	23.84
		62.78	9.3	4.65
		33.73	8.8	4.40
Cow	47.72 ±3.22	410.36	100	47.72
Dog	33.29 ±4.14	91.79	21.7	7.22
		54.18	12.8	4.26
		38.37	12.6	4.19
		33.39	9.3	3.10
		31.36	9.9	3.30
		27.84	20.7	6.89
		25.84	13.1	4.36
Horse	51.06 ±6.86	88.10	5.8	2.96
		63.61	12.4	6.33
		42.06	4.3	2.20
		37.8	7.0	3.57
		28.82	70.4	35.95
Sheep	23.19 ±1.91	45.13	28.7	6.66
		27.38	17.7	4.10
		22.99	53.6	12.43
Llama	15.71 ±4.99	88.7	10.7	1.68
		37.72	35.0	5.50
		22.34	30.2	4.74
		16.04	24.1	3.79
Human	11.87 ±2.25	75.77	86.0	10.21
		60.91	2.1	0.25
		39.53	4.5	0.53
		35.21	4.2	0.50
		22.48	3.3	0.39

5. Discussions

The RBC membrane protein band 3 is a multifunctional protein with its N- and C-terminal domains on the cytoplasmic face of a lipid bilayer and approximately 14 membrane spans. The C-terminal is responsible for anion transport across the membrane whereas the N-terminal plays a crucial structural role in linking the bilayer with spectrin-based skeletal network. Apart from the above, it is now known that protein band 3 is involved in what has been termed a sialic acid independent host-parasite interaction of malaria parasites during invasion of RBCs, a mechanism common to most other apicomplexan parasites that invade a number of different animal species. Although protein band 3 has a common and major presence in RBCs of a number of species, this study was able to highlight differences in relative quantity and apparent molecular weights in different species examined. Relative quantities of protein band 3 were striking in their variability and the differences in the species examined were significant strongly indication that this heterogeneity in relative quantities is to be expected among different animal species. The differences represent adaptive mechanisms thus the interactions mediated by these proteins will differ across species relative to the different quantities of these proteins. A study by Ligi et al. (17) demonstrated that rabbit erythrocytes were able to bind a higher percentage of hemichromes than human erythrocytes because they had a comparatively higher percentage of band 3 in their membranes. This consequently had an influence on the aging rate of the cells. Hemichrome production has been closely related to plasmodium infection in humans so it is possible that a similar quantity dependent response happens to the sialic acid independent invasion mechanism carried out by band 3 in the different animal species.

Apparent molecular weight differences for band 3 were also observed among the species examined in this study thus confirming similar observations previously reported between humans and rabbit band 3 proteins (17). Other studies have shown that structural differences also exist in different mammals largely confined to the extreme amino terminal region of the band 3 protein (14, 15). Consistent with the major sequence variability near the amino-terminus has been the observation that the predominant antigenic sites were also in this region (9, 18). It is not clear whether the stated antigenic sites include those responsible for the sialic acid independent interaction however the differences at the antigenic sites would influence response to the antigens among species. Glycosylation, the commonest influence on apparent molecular weights, has been shown to influence the progression of certain diseases (25).

Many pathogens use sugar groups of surface glycoproteins, in particular sialic acid residues, as receptors for invasion (2). The sialic acid residues of glycoproteins in RBC membranes can be demonstrated on SDS-PAGE by staining with PAS stains. In humans the PAS positive bands have been extensively studied, characterized and eventually named as glycophorins. Our study demonstrated that different patterns of PAS sensitive bands could be observed for each animal species. It was not possible to find any two species that had similar band and staining intensity patterns. Similar findings were reported by Barker (1) who suggested that it would be erroneous to adopt a naming system for sialoglycoproteins like that used for humans among animals because of these differences. It was therefore deemed appropriate to use the band's apparent molecular weights for identification since each animal species had its own pattern. The sialoglycoproteins are thus identified on the basis of

their apparent molecular weights and that 50 µg/well was the most ideal concentration to load. The sialoglycoproteins in different animals were very different in character and it is thus important to investigate how these differences influence the sialic acid dependent interactions in parasitic invasions.

Sialic acids contribute the majority of the negative surface charge that prevents RBC aggregation in circulation. But as principal receptors for pathogens they have been associated with susceptibility of certain hosts to pathogens. For example high sialic acid content has been pointed as the reason indigenous cattle (7) and Nigerian ducks (23) may be resistant to trypanosomosis and Newcastle disease respectively. Comparing the sialic acid content in normal RBCs from different animal species as part of the study of the sialic acid dependent defence mechanism in different species is therefore important. This study found clear species differences in content as shown in table 3 and because of these differences and evidence from other studies studies (7, 23) it is possible to conclude that they influence the efficiency of an invasion in a species dependent manner. It should however be mentioned that the type and concentration of sialic acid side groups (i.e. O-acetyl; glycolyl) play a major part in determining susceptibility. The O-acetylated derivatives are resistant to enzymic cleavage and thus would require a much heavier infection load to respond to infection (8). Unfortunately sialic acid types were not analyzed in this study but it is possible that there is great heterogeneity in quantity and type across the species.

In conclusion, the study observed significant differences on the two target sites of apicomplexan RBC invasion and highlights the need to study their importance in host-pathogen interactions especially how they influence invasion efficiency in different animal species. Currently most research has focused on properties of invading merozoites as a basis for understanding the invasion efficiency but it is apparent from this study that molecular events on the RBCs may also provide another important avenue for understanding the invasion.

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