Anemia amelioration by lactose infusion during trypanosomosis could be associated with erythrocytes membrane de-galactosylation

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ABSTRACT

African trypanosomosis is a potentially fatal disease that is caused by extracellular parasitic protists known as African trypanosomes. These parasites inhabit the blood stream of their mammalian hosts and produce a number of pathological features, amongst which is anemia. Aetiology of the anemia has been partly attributed to an autoimmunity-like mediated erythrophagocytosis of de-sialylated red blood cells (dsRBCs) by macrophages. Lactose infusion to infected animals has proven effective at delaying progression of the anemia. However, the mechanism of this anemia prevention is yet to be well characterized. Here, the hypothesis of a likely induced further modification of the dsRBCs was investigated. RBC membrane galactose (RBC m-GAL) and packed cell volume (PCV) were measured during the course of experimental trypanosomosis in mice infected with *Trypanosoma congolense* (stb 212). Intriguingly, while the membrane galactose on the RBCs of infected and lactose-treated mice (group D) decreased as a function of parasitemia, that of the lactose-untreated infected group (group C) remained relatively constant, as was recorded for the uninfected lactose-treated control (group B) animals. At the peak of infection, the respective cumulative percent decrease in PCV and membrane galactose were 30 and 185 for group D, and 84 and 13 for group C. From this observed inverse relationship between RBCs membrane galactose and PCV, it is logical to rationalize that the delay of anemia progression during trypanosomosis produced by lactose might have resulted from an induction of galactose depletion from dsRBCs, thereby preventing their recognition by the macrophages.

1. Introduction

Trypanosomes are blood parasites and the causative agents of protozoan diseases known as nagana in animals, sleeping sickness and Chagas' disease in humans. In addition to *Trypanosoma brucei*, other causes of nagana include *Trypanosoma congolense* and *Trypanosoma vivax*, which are all transmitted to the mammalian host by a parasite-carrying tsetse during its blood meal (Jamonneau *et al* 2004; Kraisur, 2009). Sleeping sickness and nagana are a major health problem in sub-Saharan Africa, and the economic impact of the animal disease is a severe constraint against the development of the region (Fenwick *et al.*, 2005).

Anemia has long been established as a prominent pathological feature during trypanosomosis (Woodruff *et al.*, 1973; Jennings *et al.*, 1974; Salgado *et al.*, 2011). It is largely attributed to the rapid loss of red blood cells (RBCs) from circulation, rather than a defective erythropoiesis (Biryomumaisho and Katunguka-Rwakishaya, 2007). A number of hypotheses have been put forward to explain the precise mechanism of this increased erythrocytes loss/destruction. They include: immuno-destruction, resulting from adsorption of parasites' antigenic material on to RBCs membranes (Woo and Kobayashi, 1975); membrane-lipid peroxidation, leading to oxidative damage of the RBCs (Igbokwe *et al.*, 1996; Wolkmer *et al.*, 2009); and phagocytosis of de-sialylated RBCs (Esiebo *et al.*, 1982). The last mechanism is the best-understood and widely accepted cause of the anemia (Guegan *et al.*, 2013).

The erythrocyte membrane of human and animal species is heavily sialylated (Yaari, 1969; Mehdi *et al.*, 2012), with the sialic acids (SAs) occupying terminal positions and masking an underlying galactosyl residue of cell membrane sialoglycoproteins (Varki, 2001). Membrane SAs have been shown to be a critical anti-recognition molecule, protecting RBCs from phagocytosis (Lee *et al.*, 1988; Bratosin *et al.*, 1998) by cells of the mononuclear phagocyte

system (MPS). Removal of SAs usually by actions of sialidases results in exposing the previously masked penultimate galactosyl residue on RBC membrane, which becomes recognisable by the galactose specific lectins on macrophages, leading to erythrophagocytosis/sequestration of the asialo-RBCs, hence, the anemia (Brastosin *et al.*, 1998; Guegan *et al.*, 2013). Sialidases have been reported in different species of trypanosomes; their hydrolytic capacity on RBC membrane SAs, and the role in anemia pathogenesis are well studied (Nok and Balogun, 2003; Nok *et al.*, 2003; Buratai *et al.*, 2006; Coustou *et al.*, 2012). As a result, the use of sialidase inhibitors has been thought to hold great prospect for chemotherapy or at least anemia prevention during trypanosomosis (Antoine-Moussiaux *et al.*, 2009). However, there is yet to be any successful effort towards treatment of trypanosome-infected animals with sialidase inhibitors. The only effort in this direction is focused on the use of lactose infusion to ameliorate the anemia.

Although, the amelioration of anemia by lactose infusion during trypanosomosis is documented (Umar *et al.*, 1998; Fatihu *et al.*, 2008), there is a dearth of information on this, and the mechanism of the slow anemia progression remains inconclusive. To date, the only suggested mechanism is a competition for the galactose-specific lectins on MPS by lactose. However, due to the abundance of RBCs with exposed galactose during the disease as a result of the high parasite sialidase activity, it is logical to suppose that competition alone, between infused lactose and the circulating asialo-RBCs cannot account for such magnitude and efficiency of RBCs' protection from MPS. This has led to a speculation for the existence of additional mechanism(s) of anemia prevention by lactose infusion during trypanosomosis. In the present study, the presence of a potential glycoconjugate-modifying enzyme during lactose infusion to trypanosome-infected mice was examined. We previously reported an elevated serum

glycosidase activity in the lactose-infused animals (Nok and Balogun, 2003), and herein, for the first time discuss its possible role in further modification of desialylated RBCs as a possible mechanism of asialo-RBCs' protection from recognition and sequestration by the MPS.

2. Materials and methods

Same-colony 5-6 weeks old female BALB/c mice, weighing 20–22 g, were procured from the animal unit of Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria-Nigeria. They were maintained on commercial pellet diet and water *ad libitum* under standard housing conditions. All animals used in this work were treated in accordance with the approved guidelines for animal experiments as reviewed by Ahmadu Bello University animal care and use committee. The animals were randomly separated to four groups (A-D) of four mice each. Starting from day 0; group A were not infused and not infected, group B were infused but not infected, group C were not infused but infected, and group D were infused and infected. Infection was by a single intraperitoneal inoculation with 10^4 *Trypanosoma congolense* (stb 212) cells, while infusion was twice-a-day intraperitoneal administration of lactose in phosphate buffered saline (PBS) solution at a dosage of 0.5 g per Kg body weight throughout the animal survival period.

Parasitemia was monitored daily by microscopic examination of thin blood smears, and in order to evaluate the loss of RBC that is often associated with the infection, microhematocrit method was used to monitor packed cell volume (PCV) at two days interval. To examine the relationship between PCV profiles and RBC surface modifications in trypanosome-infected animals and to investigate if the modifications were affected by lactose infusion, RBC ghosts were prepared as described in Natala *et al.* (2013), and analysed. We have previously reported a

113 correlation between parasitemia and trypanosome sialidase activity with RBC desialylation and
114 decreased PCV in *T. congolense*-infected animals (Nok and Balogun, 2003). To investigate the
115 possible further modifications occurring on RBCs during infection, susceptibility of the exposed
116 galactosyl residues on membranes of de-sialylated RBCs to the released glycosidase was
117 evaluated. A modified method of Stibler *et al.* (1983) was used to quantify RBC membrane
118 galactose. Briefly, protein concentration of homogenous suspension of prepared RBC
119 membranes of all mice groups (A-D) was determined according to Lowry *et al.*, (1951).
120 Normalized by constant protein amount (0.3 g/l), triplicate samples of the RBC membranes were
121 hydrolysed in 200 µl reaction containing 0.1 M HCl at 95 °C for 20 h. Mineral oil was overlaid
122 on each reaction to prevent evaporation. The subsequent steps were as described in Stibler *et al.*
123 (1983) except that amounts of NAD and D-galactose dehydrogenase per reaction were reduced
124 to 100 nmol and 50 mU, respectively. RBC membrane SAs (RBC m-SAs) was quantified as
125 described in Nok and Balogun (2003), using the prepared ghost cells. Two-tailed independent
126 t-test was used to analyse the differences in PCV, RBC m-SAs, and RBC m-GAL between the
127 groups.
128

3. Results and Discussion

The rate of parasitemia development varied in all the infected mice but all had above 10^6 parasites/mL of blood by day 5 post-infection. The parasitemia progressed, demonstrating the classical waves of infection (data not shown). From our previous observation, significant number of mice died during the fourth wave, therefore, the peak of infection was taken as a level of approximately 10^8 parasites/mL that was attained at the third parasitemic bout.

Similar to our previous report (Nok and Balogun, 2003) and that of Fatihu et al., (2008), lactose infusion to the infected animals significantly maintained PCV by a value of over 10% above the non-lactose treated infected group, even at peak of parasitemia (Fig. 1). Surprisingly, this difference in PCV could not be accounted for by the difference in RBC membrane sialic acids (RBC m-SAs), considering that the values were comparable for the lactose-treated and untreated infected groups (Table 1). These values were 60% of that for uninfected lactose-treated and untreated control groups (Table 1), implying that terminal RBC m-SAs were cleaved by trypanosome sialidase even in the lactose-treated animals, hence the erythrocytes protection was not because dsRBCs were not produced, but for other reason(s). However, in contrast to the similar RBC m-SAs in the infected mice, there was a coincidental significant depletion of RBC membrane galactose (RBC m-GAL), to a difference of about -30% in the lactose-treated group with reference to the untreated group. The RBC m-GAL of the untreated group was similar to the uninfected controls (Table 1).

To further elucidate the variations in RBC m-GAL at the different stages of parasitemia, the RBC ghosts of the animals were analysed. Results revealed that while the RBC m-GAL value remained fairly constant through the course of infection in the untreated group, it began to get depleted when trypanosome number reached 10^4 parasites/mL (Fig 2). The depletion continued

and was negatively correlated ($r = -0.73$, $P < 0.003$) to parasitemia level (Fig 2), resulting in about -180% relative cumulative change. However, the depletion was no longer proportional to trypanosome load beyond 10^7 parasites/mL, as seen from the relative similarity in the value of RBC m-GAL at parasitemia level between 10^7 and 10^8 parasites/mL (Fig 2). This result suggests that the infusion of lactose to the infected mice led to an accelerated depletion of galactose from the membrane of RBCs.

In order to obtain a meaningful interpretation of the relationship between RBC m-GAL and PCV at the different parasitemia level, these data for the lactose-treated and untreated groups were plotted, subjected to linear regression analysis, and compared. The regression equation for treated group is given by the formula $PCV = -2.196 \log_{10}(N) + 56.28$ ($P=0.005$) and that for the untreated group as $PCV = -4.366 \log_{10}(N) + 57.55$ ($P<0.001$), where N is the parasite number per mL (Fig. 3a and b). A visual inspection of the plotted data points clearly reveals that PCV percent decreases as the parasitemia level increases in both cases. The regression equations quantify these observed relationships. Values of the regression coefficients; -2.1964 for the lactose-treated and -4.3661 for the untreated animals, imply that the rate of drop in PCV in the untreated group is twice that for the lactose-treated animals. Conversely, judging from the RBC m-GAL curves and their regression coefficients; -0.1313 for the lactose-treated and -0.0045 for the untreated (Fig 3a and b), there was no significant change in the RBC m-GAL values ($P > 0.25$) for the untreated group, while that for treated animals dropped considerably (at a rate approximately 30 times higher) as parasitemia increased ($P < 0.001$). These findings suggest that the relative maintenance of PCV in the lactose-treated animals is accompanied by a sharp decrease in the amount of their erythrocytes membrane galactose.

174 The observed inverse relationship between the amount of RBC m-GAL and PCV in
175 trypanosome-infected animals that receive lactose treatment has revealed an interesting
176 phenomenon that may help to provide an insight into the mechanism of anemia amelioration.
177 From the aforementioned, coupled with our previous observation that the serum of this group of
178 animals possessed an increased ability to cleave galactose from an artificial substrate,
179 4-Methylumbelliferyl galactose (Nok and Balogun, 2003); we can infer that lactose infusion
180 resulted in an increased amount of a secreted galactose hydrolyzing enzyme. Moreover, a
181 separate group reported that *Trypanosoma evansi* contained beta galactosidase (Sallau et al.,
182 2008). Although we could not locate any open reading frame (ORF) for galactosidase gene in the
183 genome of *T. congolense*, four putative glucosidase ORFs were identified from the TritrypDB
184 (<http://tritrypdb.org/tritrypdb/>). This could mean that at least one of the genes' products is a
185 non-specific glucosidase that also cleaves galactose, hence; we refer to it broadly as a
186 glycosidase. It is logical to therefore rationalize that elevation of the glycosidase in the
187 lactose-treated infected animals modified the surface of the dsRBCs further by removing the
188 exposed galactose, making them unrecognizable by the galactose-specific lectins on
189 macrophages, hence reducing their rates of removal from circulation and leading to a reduction
190 in the rate of anemia progression. On the contrary, there was probably low plasma glycosidase in
191 the lactose-untreated animals (as suggested by higher galactose content of their dsRBCs);
192 resulting in an unchanged surface (exposed galactose) of the dsRBCs, which were rapidly
193 recognized and sequestered by the immune cells-erythrophagocytosis. To conclude, our present
194 observation revealed a likely mechanism of anemia suppression by lactose infusion to
195 trypanosome-infected animals. Lactose induced the cleavage of exposed galactose on the
196 membrane surface dsRBCs that are produced by the trypanosomal sialidases, making them

unrecognisable by the galactose-specific lectins of macrophages, and preventing their sequestration by these immune cells, thus, reducing the rate of anemia progression.

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Table and Figure captions

Table 1. Erythrocytes membrane galactose (RBC m-GAL) and sialic acids (RBC m-SAs).

Results are mean $\pm$ standard deviation (SD). Different letters in the same column indicate a significance difference ($P < 0.05$).

Fig. 1. **Effect of lactose infusion on packed cell volume (PCV) of *Trypanosoma congolense* infected mice (four per group).** Bars represent averages of two readings at peak of parasitemia, while error bars are $\pm$ SD. Different letters indicate a significance difference ($P < 0.05$).

Fig. 2. **The variation of RBC m-GAL at different level of parasitemia.** Galactose was quantified in erythrocytes ghosts prepared from blood of *Trypanosoma congolense* infected mice that were treated (group D; solid line) and untreated (group C; dotted line) with lactose. Plots were prepared using averages of three determinations, and error bars represent $\pm$ SD.

Fig. 3. **Comparison between the variations of RBC m-GAL (diamond lines) and PCV (cube lines) induced by lactose infusion to *Trypanosoma congolense* infected mice.** (A) is lactose treated group (D), while (B) is the untreated group (C). The respective regression lines are shown as dotted and solid lines. The generated regression equations suggest that maintenance of PCV in group D mice is attributable to RBC m-GAL depletion from the membrane of dsRBCs. Each plot is an average of three experiments, but for the purpose of clarity, $\pm$ SD were not indicated.

Table 1: Erythrocytes membrane galactose (RBC m-GAL) and sialic acids (RBC m-SAs).

Animal groups	Protein (g/L)	RBC m-GAL (mmol/g Prot)	RBC m-SAs (μmol/g Prot)
Not infected, not lactose-treated (A)	28.8 ± 0.5	1.37 ± 0.05 ^a	81.5 ± 1.8 ^c
Not infected, lactose-treated (B)	25.9 ± 0.6	1.38 ± 0.05 ^a	80.9 ± 2.1 ^c
Infected, not lactose-treated (C)	28.6 ± 0.8	1.35 ± 0.06 ^a	49.5 ± 1.9 ^d
Infected, lactose-treated (D)	26.3 ± 0.6	0.65 ± 0.06 ^b	48.1 ± 1.9 ^d

RBC m-GAL: red blood cells membrane galactose; RBC m-SAs: red blood cells membrane sialic acids

Fig. 1

Fig. 2

Fig. 3