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Association of malaria with inactivation of α1,3-galactosyl transferase in catarrhines

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Abstract

Present-day catarrhines (old world monkeys and hominoids) lack Gal α1-3 Gal β1-4 GlcNAc-R structures (α-galactosyl epitopes) and produce the corresponding anti-galactosyl antibodies (anti-gal), while platyrrhines (new world monkeys) and non-primate mammals possess α-galactosyl epitopes and lack anti-gal. Anti-gal is shown to inhibit *Plasmodium falciparum* growth in culture in a concentration dependent manner, probably by binding to α-galactosyl epitopes on merozoite surface molecules and causing complement mediated damage. A *P. falciparum*-like malaria parasite may therefore have selected for the inactivation of an α1-3 galactosyl transferase in catarrhines. The implications of the results for the development of clinical immunity to falciparum malaria are briefly discussed.

Keywords: Anti-galactosyl antibody; α-Galactosyl epitope; Galactosyl transferase; Malaria; Primate evolution; (*Plasmodium falciparum*)

1. Introduction

Malaria due to *Plasmodium falciparum* has exerted selective pressure on human populations at the level of globin [1], major histocompatibility complex (MHC) [2,3] and glucose 6-phosphate dehydrogenase [4] genes. The MHC class I antigen HLA-Bw53, which is common among west and central Africans but rare in caucasians and orientals, affords 40% protection against cerebral malaria or severe malaria-induced anaemia [2]. The molecular basis for this protection has been ascribed to HLA-Bw53 restricted recognition of an epitope on a *P. falciparum* liver-stage specific antigen by cytotoxic T cells [3]. Gal α1-3 Gal β1-4 GlcNAc-R structures (termed α-galactosyl epitopes) are absent in old world primates including man (the catarrhines) but common in glycolipids and glycoproteins of new world monkeys (the platyrrhines) and non-primate mammals [5,6]. Correspondingly, natural antibodies to the α-galactosyl epitopes (termed anti-gal) are present in catarrhines and absent in platyrrhines and non-primate mammals [[6], Ramasamy, R. and Rajakaruna, R., unpublished data]. Inactivation of an α1-3 galactosyl transferase (α1,3 GT) in the Golgi of catarrhines is

Abbreviations: anti-gal, anti-galactosyl antibodies; α-galactosyl epitope, Gal α1-3 Gal β1-4 GlcNAC-R; α1,3 GT, α1,3 galactosyl transferase; MHC, major histocompatibility complex; mya, million years ago; rbc, red blood cells.

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responsible for the difference [7] and it has been speculated that a pathogen [7], possibly a malaria parasite [8,9], may have been the cause. We provide evidence that a *P. falciparum*-like parasite could have exerted the necessary selective pressure to inactivate α1,3 GT. A preliminary report of this work was presented at the Biochemical Society/British Society for Immunology joint congress in Harrogate, UK in December 1996.

2. Materials and methods

2.1. Preparation of anti-gal antibodies and anti-gal deficient serum

Thirty ml AB serum from donors with no known malaria exposure and 7 ml pools of sera (of unknown blood groups) from malaria-endemic Weheragala village with antibodies to *P. falciparum* merozoite surface proteins [10] were sequentially passed through a 2 ml control silica column and a 2 ml Galα1-3 Galβ1-4 GlcNAc-R Synsorb column (Chembiomed, Edmonton, Canada) for affinity purifying anti-gal antibodies. The columns were washed extensively in 0.01 M phosphate buffered saline, pH 7.2 (PBS). Proteins bound to the control silica and Gal α1-3 Gal β1-4 GlcNAc-R columns were then eluted with 3 ml 0.1 M glycine-HCl buffer, pH 3.0 to yield the control and anti-gal eluates respectively [11,12]. The two parallel eluates were neutralised with 0.1 M NaOH, dialysed extensively against the same batch of RPMI 1640 and sterilised by filtration through 0.22 μm filters. Anti-gal activity in the two eluates were determined by direct haemagglutination of 5% haematocrit rabbit red blood cells [11]. Different preparations of anti-gal and control eluates had haemagglutination titres of 32–2000 and 2–64 respectively, with the anti-gal titre being always greater than that of the corresponding control. Analysis of anti-gal by SDS-PAGE revealed mainly IgG in anti-gal with traces of IgM and other serum proteins and a total protein concentration of 32–172 μg ml^{-1} in different preparations. Control eluates contained mainly IgM with traces of IgG and other serum proteins. AB serum that had been sequentially passed through the silica and oligosaccharide columns was used as anti-gal deficient serum for parasite cultures.

2.2. [^{3}H]hypoxanthine incorporation assay for determining parasite growth inhibition

P. falciparum (3D7 isolate) was routinely maintained in culture in 0+ red cells (rbc) and RPMI 1640 supplemented with 10% AB+ human serum, 22 mM Hepes, 24 mM sodium bicarbonate and 10 μg ml^{-1} gentamicin in an atmosphere of 5% 0_2, 5% CO_2, 90% N_2 at 37°C [13,14]. *P. falciparum* was adapted to grow in anti-gal deficient AB serum for parasite growth-inhibition experiments. Late stage parasites were obtained from cultures by gelatine sedimentation [15]. For a given experiment, different eluates in RPMI 1640 were used to prepare complete medium containing 10% of the same anti-gal deficient serum to which the parasites were culture-adapted. Parasites at 0.5%–1.0% parasitaemia in different experiments were then added to the medium to yield 5% haematocrit, and 100 μl aliquots dispensed into sterile 96 well culture plates in 4–8 replicates and incubated in an atmosphere of 5% CO_2, 95% air at 37°C. Twenty four hours later, 5 μCi [^{3}H]hypoxanthine (Amersham) in 100 μl RPMI 1640 was added to each well as described [14]. At 48 h, the cells were harvested onto Millipore glass fibre filters, the DNA precipitated with ice-cold 5% trichloroacetic acid and washed in 70% ethanol, and radioactivity in the DNA determined by liquid scintillation counting.

2.3. Microscopic determination of parasite growth inhibition

Late stage 3D7 parasites were cultured as described above in the presence of anti-gal and control column eluates isolated from immune serum from another malaria endemic village, Nikawehera [16]. Smears of cultures were obtained at 24h and 48h after initiation of culture, stained with Giemsa and the proportions of different parasite stages determined by microscopy. The anti-gal used in this experiment produced 41% inhibition ($P < 0.001$) when compared to the corresponding control antibodies, in a parallel [^{3}H]hypoxanthine incorporation assay.

2.4. Requirement for complement

An aliquot of the anti-gal deficient AB serum used to grow parasites was heated to 56°C for 30 min to heat inactivate complement. Parallel cultures were

then set up as described using the complement inactivated and normal anti-gal deficient sera to each of which were added the same preparation of anti-gal and control antibodies.

3. Results

Anti-gal isolated from immune and non-immune human sera inhibited the re-invasion and growth of *P. falciparum* in cultures grown in AB serum depleted of anti-gal. The results of two experiments with seven different preparations of anti-gal are given in Table 1. Pooling results from several experiments, it was observed that anti-gal inhibited [^{3}H]hypoxanthine incorporation in 19 out of 22 tests when compared to the corresponding controls. In 11 out of 19 tests the inhibitions were statistically significant at $P \leq 0.05$ by the t test.

Microscopic determination of the numbers of rings and trophozoites/schizonts present at 24 and 48 h after adding antibodies to cultures (i.e. upto one complete cycle of rbc invasion and growth) indicated that inhibition of merozoite invasion was primarily responsible for this effect (Fig. 1). The binding of anti-gal but not the control antibodies to *P. falciparum* merozoites was demonstrable by immunofluorescence on acetone fixed late stage parasites (Ramasamy, R. and Rajakaruna, R., unpublished data).

Varying the concentration of anti-gal by serial dilution of added antibodies in RPMI 1640 showed that the anti-gal eluate inhibited parasite growth at lower protein concentrations than the control eluate (Table 2). In a related experiment, the addition of a human IgG1/kappa myeloma protein (Sigma, USA) at a concentration of 100 μg ml^{-1} in RPMI 1640 did not significantly inhibit [^{3}H]hypoxanthine incorporation into parasites in comparison to the addition of control eluate from immune serum at a protein concentration of 88 μg ml^{-1} (19 486 $\pm$ 1490 vs 19 820 $\pm$ 2075 cpm $\pm$ S.D. respectively).

Table 1
Inhibition of *P. falciparum* growth by anti-galactosyl antibodies

Addition (protein concn)		[^{3}H]hypoxanthine incorporation (mean cpm $\pm$ S.D.)	Inhibition	*P*
Experiment 1				
A	(i) Control medium	9 450 $\pm$ 316	–	–
B	(i) Control eluate from immune serum – pool A	9 991 $\pm$ 395	–	–
	(ii) Anti-gal eluate from pool A	7 644 $\pm$ 395	23%	0.002
C	(i) Anti-gal eluate from immune serum –pool B	6 117 $\pm$ 399	35%	0.0001
	(ii) Anti-gal eluate from immune serum –pool C	6 894 $\pm$ 1 403	27%	0.01
Experiment 2				
A	(i) Control eluate from immune serum –pool D (88 μg ml^{-1})	35 961 $\pm$ 2 800	–	–
	(ii) Anti-gal eluate from pool D (144 μg ml^{-1})	26 696 $\pm$ 2 275	17%	0.001
B	(i) Control eluate from non-immune AB serum –pool E (64 μg ml^{-1})	34 580 $\pm$ 3 741	–	–
	(ii) Anti-gal eluate from pool E (160 μg ml^{-1})	31 284 $\pm$ 2 261	10%	0.05
C	(i) Control eluate from immune serum –pool F (104 μg ml^{-1})	39 813 $\pm$ 2 127	–	–
	(ii) Anti-gal eluate from pool F (160 μg ml^{-1})	34 613 $\pm$ 596	13%	0.001
D	(i) Control eluate from non-immune AB serum – pool G (72 μg ml^{-1})	32 106 $\pm$ 2 251	–	–
	(ii) Anti-gal eluate from pool G (120 μg ml^{-1})	32 266 $\pm$ 1 758	0%	n.s.

The effects of anti-gal and control eluates on *P. falciparum* growth determined by [^{3}H]hypoxanthine incorporation. Different pools of sera used to prepare eluates are labelled A–G. Th immune sera were obtained from Weheragala village. The [^{3}H]hypoxanthine was used at a specific activity of 6 GBq mmol^{-1} in Exp. 1 and at 200 GBq mmol^{-1} in Exp. 2 and subsequent experiments. Protein concentrations of eluates are given where available. Significance (P) of the differences between [^{3}H]hypoxanthine incorporated in the presence of anti-gal and the corresponding control eluates were determined by Student's t test, except in 1C where anti-gal treatment was compared to parasites cultured in RPMI 1640 medium containing anti-gal deficient serum without added antibodies (control medium 1A). Data represent mean $\pm$ standard deviation for four or more determinations. n.s. = not significant, $P > 0.05$.

Table 2
Effect of anti-gal concentration on inhibition of *P. falciparum* growth

Antibody (protein concn)	[^{3}H]hypoxanthine incorporation (mean cpm ± S.D.)	Inhibition	*P*
1. Anti-gal from immune serum – pool H (108 μg ml^{-1})	49 821 ± 2200	9.5%	0.001
2. Anti-gal from immune serum – pool H (10.8 μg ml^{-1})	52 052 ± 3169	5.5%	0.05
3. Anti-gal from immune serum – pool H (1.1 μg ml^{-1})	54 256 ± 2416	1.5%	n.s.
4. Control eluate from immune serum – pool H (88 μg ml^{-1})	55 059 ± 998	–	

The effect of 10-fold dilutions of anti-gal immune Weheragala sera (pool H) on *P. falciparum* growth. The anti-gal was diluted in fresh RPMI 1640 and 100 μl aliquots of the antibodies were added to cultures. Data represent mean ± standard deviation of eight replicate cultures. Statistical analyses were done as described in Table 1.

Table 3
Role of complement in anti-gal mediated inhibition of parasite growth

Addition (protein concn)	Serum	[^{3}H]hypoxanthine incorporation (mean cpm ± S.D.)	Inhibition	*P*
1. Anti-gal from immune serum – pool H (108 μg ml^{-1})	Normal	51 639 ± 4 191	17%	0.001
2. Control eluate from immune serum – pool H (88 μg ml^{-1})	Normal	62 158 ± 3 776	–	
3. Anti-gal from immune serum – pool H (108 μg ml^{-1})	Heat inactivated	65 563 ± 3 756	0%	n.s.
4. Control eluate from immune serum – pool H (88 μg ml^{-1})	Heat inactivated	65 280 ± 1 756	–	

The effect of heat inactivating complement on anti-gal mediated inhibition of parasite growth. The anti-gal was prepared from immune Weheragala serum. Data represent mean ± S.D. of eight replicate cultures. Statistical analyses were done as described in Table 1 for differences between samples 1 vs. 2 and 3 vs. 4.

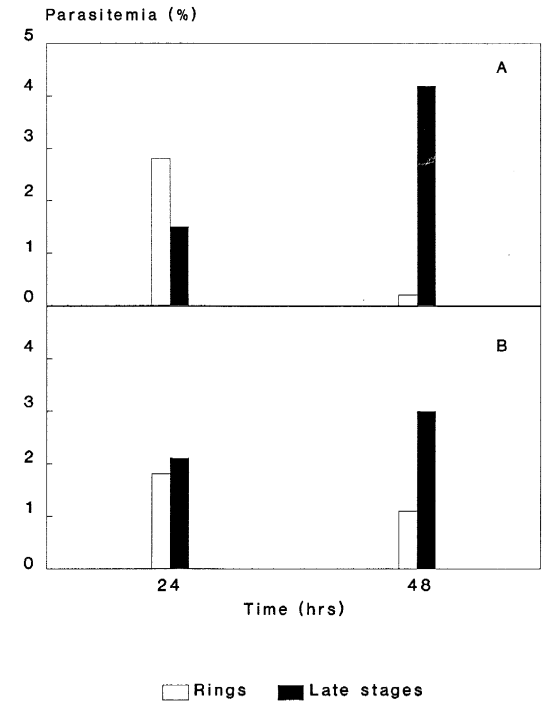

Fig. 1. Effect of control (A) and anti-gal (B) eluates from immune Nikawehera serum on growth of *P. falciparum* determined from Giemsa stained films.

Inactivating complement components by heating the anti-gal deficient serum used for culturing the parasites, abolished growth inhibition mediated by added anti-gal (Table 3).

4. Discussion

Terminal α-galactose residues are present on the 185 kDa [17] and 45 kDa [18] *P. falciparum* merozoite surface proteins in O-linked oligosaccharides [19] or glycophosphatidyl inositol membrane anchors [18]. The binding of *Bandeiraea simplicifolia* IB4 lectin to parasite membranes shows that the terminal galactose residues are present in α1-3 linkages [17]. Antibodies in non-immune [8] and immune [17,20] human sera have been previously shown to recognise α-galactosyl epitopes in *P. falciparum* by ELISA or immunofluorescence. Indeed, the binding of natural antibodies in non-immune human sera to *P. falciparum* can be inhibited by 0.5 M α-methyl galactoside [8]. The data presented here show that the bind-

ing of anti-gal to merozoite surface proteins inhibits parasite growth through complement mediated lysis of merozoites. It is probable that the in vitro growth inhibition assay underestimates the capacity of antibodies to inhibit parasite growth in vivo. Merozoites probably traverse greater distances between rbc in vivo than in the settled layer of rbc in 96 well plates and can therefore be exposed to antibodies for a longer time. Complement mediated lysis is likely to be more efficient in plasma in vivo than in culture medium containing 10% serum in vitro. There is also evidence that monocytes, macrophages and neutrophils, that were not present in the cultures, can promote antibody-dependent, cell-mediated immunity in vivo [21].

The degree of growth inhibition varied between different anti-gal preparations of similar protein concentration. Differential loss of antibody activity during preparation and storage and differences in IgG isotype composition and consequently complement fixing ability, may be some of the factors responsible for this variability. The maximum growth inhibition observed with anti-gal is less than the 96% inhibition reported with 121 μg ml^{-1} of an IgG monoclonal antibody directed against a peptide epitope on the 45kDa merozoite surface antigen [22]. The difference may partly reflect higher affinity and faster binding of anti-peptide antibodies to merozoites. Since merozoites invade rbc rapidly, the rate of antibody binding is likely to a critical factor in invasion inhibition. It is possible that a proportion of merozoites that express fewer α-galactosyl epitopes avoid complement mediated lysis and that this process can give rise to *P. falciparum* mutants lacking α-galactosyl epitopes. However the presence of α-galactosyl epitopes in FVO [17], Honduras 1 [8,17], FC27 [8] and 3D7 isolates *P. falciparum* suggests that the corresponding oligosaccharide structures may be conserved because they have a vital function in parasite biology.

Analysis of gene sequences indicates that inactivation of α1,3 GT occurred as two independent events in the two groups of catarrhines viz. old world monkeys and hominoids, no more than 17–25 million years ago (mya) [7]. *Plasmodium* genus is about 150 million years old [23]. Analysis of the sequences of small subunit ribosomal RNA and circumsporozoite protein genes show that *P. falciparum* is closely related to a chimpanzee parasite *P. reichenowi* [24,25]. These two malaria parasites form a clade that is more closely related to avian and reptilian malaria parasites (*P. gallinaceum*, *P. lophurae* and *P. mexicanum*) than to other primate and human (e.g. *P. knowlesi*, *P. vivax*, *P. malariae* and *P. ovale*) or murine (e.g. *P. berghei*) parasites [23–25]. Catarrhines diverged from platyrrhines 30–35 mya [26]. We postulate that during the Oligocene or early Miocene, a *P. falciparum*-like, non-primate (probably avian or reptilian) parasite adapted itself to infect primates in the old world which later led to the inactivation of α1,3 GT in the hosts. The driving force for inactivation of α1,3 GT may have been the ability to produce antibodies reactive with α-galactosyl epitopes of the parasite which then conferred significant protection against falciparum-like malaria without inducing autoimmunity. It is not possible to extrapolate easily from human anti-gal inhibition of *P. falciparum* growth in cultures to the selective advantage afforded by anti-gal in early catarrhines, which may have been considerably greater. Since *P. falciparum* was probably introduced into the Americas after Christopher Columbus [27], there would have been no similar selective pressure to inactivate α1,3 GT in platyrrhines. A prediction of this hypothesis viz. that *P. falciparum* infects platyrrhines more readily than old world monkeys, is already established [28]. Natural anti-gal in non-immune humans, like antibodies to blood group A and B antigens, are probably produced as a result of stimulation of the immune system by cross-reactive epitopes on the cell walls of common intestinal bacteria [29]. There is no evidence at present to support an alternative possibility that selection due to a common bacterial pathogen prevalent in the old world, but not in the new world, could have caused the inactivation of α1,3 GT in catarrhines.

The natural anti-gal clearly provides only partial protection against human falciparum malaria. However partial protection against malaria afforded by MHC molecules have produced significant genetic changes in human populations [2,3]. The relevance of anti-gal to strain transcending clinical immunity to falciparum malaria observed in adults living in highly malaria-endemic areas, and the detailed structure of parasite α-galactosyl epitopes and its relationship to the specificity of human anti-gal need to be determined. In this context it is relevant to note that higher

levels of anti-gal have been reported in persons living in malaria-endemic areas and in patients with acute falciparum malaria [30]. The human blood group B epitope has the structure Gal α1-3 (Fuc α1-2) Gal β1-4 GlcNAc-R and a subset of anti-B antibodies are reported to have anti-gal activity [31]. Hence the possibility that A and O blood groups afford some degree of protection against falciparum malaria requires investigation.

P. falciparum proteins are presently being investigated as the basis of a vaccine against falciparum malaria. Our results suggest that oligosaccharide moieties of the parasite may also be useful for this purpose.

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