in mice [37]; in bacterial meningitis robust inflammatory response has been identified as harmful and contributing to tissue damage [38].

The major limitation of this study is the low incidence of symptomatic malaria [22]. The study was conducted in an area with low but perennial malaria transmission with the intention of identifying clearly individuals susceptible or resistant to the symptomatic malaria phenotypes. Nevertheless, this study has highlighted clearly that the TLR9 gene polymorphisms significantly influence susceptibility to symptomatic malaria. It is therefore worthwhile to carry out more studies in areas with different malaria transmission and using a larger sample size, so that the role of TLR9 polymorphisms on different malaria phenotypes can clearly be deciphered.

Abbreviations

TLR: Toll like receptor; SNPs: Single nucleotide polymorphisms; WHO: World Health Organization; HWE: Hardy-Weinberg's Equilibrium; LD: Linkage disequilibrium.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AHO participated in conception and designing of the study, carried out the molecular genetic studies, the luciferase reporter gene assay, performed data analysis, interpretation, and drafted the manuscript; YM participated in conception and designing of the study, data analysis, interpretation and revising the manuscript critically; YA participated in designing of the study, data collection and analysis; HS assisted during the luciferase reporter gene assay experiment; MFO and BDA participated in designing of the study, and data collection; SMN participated in designing of the study and revising manuscript critically; KM participated in designing of the study and data analysis; KH participated in conception and designing of the study, data analysis, interpretation, revising manuscript critically and gave final approval of the version to be published. All authors read and approved the final manuscript.

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Author details

[1]Department of Immunogenetics, Institute of Tropical Medicine (NEKKEN) and Global COE Program, Nagasaki University, 1-12-4 Sakamoto, Nagasaki, 852-8523, Japan. [2]Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana. [3]Center for International Collaborative Research (CICORN), Nagasaki University, 1-12-4 Sakamoto, Nagasaki, 852-8523, Japan.

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The rs150311303 Polymorphism in FcγRIIa Enhances IgG Binding Capacity

A. H. Omar*[1], H. Shibata*[1], M. Yasunami*, A. Yamazaki*, M. F. Ofori†, B. D. Akanmori†, M. N. Shuaibu*, M. Kikuchi*‡, & K. Hirayama*

*Department of Immunogenetics, Institute of Tropical Medicine (NEKKEN) and Global COE Program, Nagasaki University, Nagasaki, Japan; †Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana; and ‡Center for International collaborative Research (CICORN), Nagasaki University, Nagasaki, Japan.

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Correspondence to: K. Hirayama, MD PhD, Department of Immunogenetics, Institute of Tropical Medicine (NEKKEN), Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan. E-mail: hiraken@nagasaki-u.ac.jp

[1]These authors contributed equally to this work.

Abstract

Fc gamma receptor (FcγR) provides an important link between humoral and cellular immune responses. FcγRIIa-H131R polymorphism has been associated with differential binding to IgG subclasses and susceptibility to severe malaria phenotypes among different populations in the malaria endemic world. In this study, the effect of *FCGR2A* gene polymorphisms on susceptibility to symptomatic malaria among Ghanaian cohort children was investigated. Blood samples from four hundred and 29 (429) healthy Ghanaian children were genotyped for *FCGR2A* polymorphisms by direct DNA sequencing. Attributable and relative risks to symptomatic malaria were calculated for the polymorphic variants. Two major *FCGR2A* polymorphisms, rs1801274A/G (FcγRIIa-H131R) and rs150311303 (FcγRIIa-ins170L), were identified in the study population, and assessment of their risks did not show significant association with susceptibility to symptomatic malaria. The functional significance of these polymorphisms was also examined by evaluating their binding abilities to IgG subclasses using flow cytometric analysis of HEK cells transfected with the FcγRIIa haplotype variants. The binding assay revealed the rs150311303, which was observed only among carriers of the FcγRIIa-131RR genotype for the rs1801274 to consistently enhance binding capacities to all IgG subclasses. Thus, of the three FcγRIIa haplotype variants observed in this study population, the FcγRIIa^{RL} haplotype variant was observed to have the highest binding ability to IgG1, IgG3 and IgG4.

Introduction

Malaria is one of the most prevalent infectious diseases in the world with an estimated 216 million cases and 655,000 deaths in the year 2010, with almost all the deaths occurring among children under 5 years of age in sub-Saharan Africa (WHO world malaria report 2011). Malaria has been endemic for many years, and it has placed immense selective pressure on the human genome leading to acquisition and retention of various abnormalities like hemoglobinopathies (HbS, HbC and HbE), thalassemias, loss of some erythrocyte surface antigens (Duffy antigen) and enzymes like Glucose-6-Phosphate dehydrogenase, which provide significant protection against severe malaria infection in all over the malaria endemic world [1, 2].

Fc gamma receptor (FcγR) provides an important link between humoral and cellular immunity by mediating interaction between antibodies and effector cells [3]. FcγRIIa is a low-affinity receptor, which binds IgG1 and IgG3 in a complexed or an aggregated form [4]. FcγRIIa is the most widely distributed FcγRs class that is expressed on most types of blood leucocytes, Langerhans' cells, endothelial cells, dendritic cells, macrophage and platelets [4]. FcγR polymorphisms influence ligand-binding affinity and thus affect the efficacy of cellular immune responses and hence may affect susceptibility, severity and outcome of infectious diseases [5]. A single-nucleotide polymorphism in *FCGR2A* gene leads to either arginine (R) or histidine (H) amino acid substitution at position 131 in the ligand-binding domain of FcγRIIa (H131R), affecting receptor-binding affinity and specificity for IgG subclasses [6, 7]. Although both FcγRIIa-HH131 and -RR131 variants bind IgG1 and IgG3, the HH131 genotype displays a higher binding affinity for IgG3 and is capable of binding IgG2 most effectively, while none of the two variants bind IgG4 efficiently [4, 6]. However, Bruhns *et al.* [7]have shown

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that the HH131 genotype has a higher binding affinity for IgG1 and IgG2 and has a similar binding affinity for IgG3 subclass with the RR131 genotype.

The asexual blood stages of the malaria parasites are responsible for clinical malaria. Antibodies against asexual blood stages of malaria have long been recognized to provide protection against symptomatic and severe malaria [8–10], and the cytophilic IgG subclasses (IgG1 and IgG3) predominantly produced in response to merozoites antigens are believed to play an important role in protection against clinical malaria [9, 11]. However, other studies have associated IgG2 levels in individuals with FcγRIIa-HH131 genotype with protection against *Plasmodium falciparum* malaria [12, 13]. Therefore, the FcγRIIa-H131R polymorphism that shows preferential binding to IgG subclasses may play an important role in malaria pathogenesis.

Previous studies conducted to investigate the role of FcγRIIa-H131R polymorphism on susceptibility/resistance to malaria phenotypes produced varied results. The FcγRIIa-RR131 genotype was significantly associated with protection against high-density parasitaemia among Kenyan infants [14, 15], while the FcγRIIa-HH131 genotype was associated with susceptibility to severe malaria in Gambian children [16]. Moreover, the FcγRIIa-HH131 genotype in combination with FcγRIIIB-NA2 was significantly associated with susceptibility to cerebral malaria among Thai patients [17] and also with severe malarial anaemia among Kenyan children [18]. In contrast to these findings, other studies have found that the FcγRIIa-HH131 genotype to be more prevalent among the Fulani (who are less affected by symptomatic malaria) than other non-Fulani tribes of eastern Sudan [19] and it was also associated with protection against severe malaria manifestation among some populations living in areas with unstable malaria transmission [13, 20, 21]. The FcγRIIa-RR131 genotype was also associated with susceptibility to severe malaria among Sudanese and Indian populations [13, 20]. Although detailed analysis is worthwhile, the discrepancies between these studies may be due to the differences in study designs, population genetics, malaria transmission levels and the phenotypes studied. However, the findings of these studies indicate that the role of FcγRIIa-H131R polymorphism in susceptibility to malaria phenotypes is still not clearly understood. Therefore, in this study, we investigated the possible influence of *FCGR2A* polymorphisms on susceptibility to symptomatic malaria among healthy Ghanaian cohort children and also determined the functional significance of the FcγRIIa haplotype variants identified in this study population.

Materials and methods

Study area and subjects. Details about the study area, population, set-up of the cohort and the follow-up protocol have previously been described [22]. In brief, the cohort study was conducted at Asutuare, a sub-district of Dangme West District in the Great Accra region of Ghana. The district has two rainy seasons annually from April to July and October to December, and malaria transmission is seasonal, with the peak transmission coinciding with the period of the major rainy seasons while the dry seasons having low malaria transmission [23]. It is estimated that individuals in Dodowa (the district headquarter for Dangme West district) are exposed to about 20 infective bites per year, and 98% of the infections are because of *P. falciparum* [23].

Four hundred and 29 (429) healthy Ghanaian children, aged 3–11 years, were enrolled into a 1-year prospective cohort study, from June 2007 to July 2008, which spanned across three (3) rainy seasons. Only one child per household was recruited to avoid inclusion of closely related individuals. The study participants were actively followed up for clinical malaria symptoms with regular home visits of 2-week intervals. During the visits, data on the health status of the participants for the previous 2 weeks were collected using a standard questionnaire and their body temperatures measured. Individuals suspected of clinical malaria were referred to the community health centre where medical examinations and blood smear for malaria were carried out and treatment given according to the recommendation of the Ghanaian Ministry of Health.

Phenotype definition. In this study, a participant was considered to be suffering from symptomatic mild malaria, if he or she has a fever that is ≥38.0 °C, with a parasite load that is equal to or >5000 parasite per microlitre (μl).

Informed consent was obtained from each participant's parents/guardian after a detailed explanation of all procedures in the study. The study was approved by the Ghanaian Ministry of Health, Institutional Review Board (IRB) of Noguchi Memorial Institute of Medical Research, University of Ghana and IRB of Institute of Tropical Medicine, Nagasaki University, Japan.

DNA extraction and polymerase chain reaction (PCR). Sample collection and storage has been described in detail elsewhere [22]. Genomic DNA was extracted from blood sample using *QIAamp* DNA blood mini kit (Qiagen, Tokyo, Japan). The PCR amplification of Exon 4 of the *FCGR2A* gene was carried out using forward primer *FCGR2Ae4F2* (CAAGTTCTGTGAGTAACGTAC) and reverse primer *FCGR2Ae4R* (CCCATAGCAGCAAAATTGGG). The PCR mixture was 1.0 μl of × 10 buffer, 6.4 μl of water, 0.8 μl of dNTP mix, 0.4 μl of forward primer, 0.4 μl of reverse primer, 0.06 μl of Hs Taq polymerase and 1.0 μl of DNA, and the PCR conditions used was as follows: One cycle at 95 °C for 5 min, 35 cycles at 95 °C for 1 min, 35 cycles at 58°C, and 35 cycles at 72°C. The PCR product was then analysed by agarose gel electrophoresis.

Genotyping of FCGR2A *polymorphisms.* Direct sequence of the PCR amplicons was carried out to identify polymorphic variants in Exon 4 region of the *FCGR2A* gene

using forward primer *FCGR2Ae4F2* (CAAGTTCTGTGA GTAACGTAC). The sequencing was carried out using 3730 DNA Analyzer sequencing machine (Applied Biosystems, Foster city, CA, USA) and Assign™ sequencing analysis software (Conexio genomics Pty Ltd, Fremantle, WA, Australia) used to analyse the DNA sequence data.

FcγRIIa haplotype variants constructions in plasmid vector. Four types of FcγRIIa haplotype variants with A or G for rs1801274 (FcγRIIa-H131R) in combination with either insertion CTT (ins170L) or wild type for rs150311303 were constructed in pGEM (Promega Co., Madison, WI, USA) plasmid vector. The cDNA template was synthesized using reverse transcript polymerase (Primescript^{R} RT; Takara Bio, Shiga, Japan), by mixing the random hexamer and oligo DT primers in a 1:1 ratio according to the manufacturer's instructions, from mRNA of human spleen as the sources of DNA (Clontech, Takara Bio). The cDNA was then used as a template for full length of *FCGR2A* gene and PCR amplification carried out using the forward primer *FCGR2A-N* (AGAT CTGGGATGACTATGGAGACC CAA) and reverse primer *FCGR2A-C* (GTCGACTTA GTTATTACTGTTGACATGGTC). The PCR amplicons were confirmed by direct sequencing method (Applied Biosystems) and then cloned into pCR Blunt II Topo® cloning vector (Invitorogen Co., Carlsbad, CA, USA). To generate ins170L for rs150311303, the pCR Topo plasmid vectors containing amplicons with A or G for rs1801274 were amplified using two sets of primers: forward primer *FCGR2A-N* and reverse primer mutCD32insR (GGTAC CTGAAGGACAGTGATGGTCACAGG), and forward primer mutCD32insF (GGTACCCAGCATGGGCAGCT CT) and reverse primer *FCGR2A-C*. Then, four PCR amplicons (designated as WT, MT1, MT2 and MT3) (Fig. 1) were obtained, treated with *KpnI* and *SacI* restriction enzymes (New England biolab Co, Ipswich, MA, USA) and BAP (Toyobo, INC., Osaka, Japan) and then ligated into pGEM®T easy vector (Promega Co., USA). The sequences of all the resultant clones were confirmed by sequencing. Thereafter, pGEM vectors containing the four FcγRIIa haplotypes variants were ligated into *NheI* and *NotI* restriction sites of pcDNA5/FRT vector (Invitrogen Co.) for expression and functional experiments.

Stable FcγRIIa expression on Flip-in™-HEK 293 cells. Flip-in™-HEK 293 cells (Invitrogen Co.) were cultured in DMEM medium supplemented with 10% foetal calf serum and Zeocin (Invitrogen Co.) following the manufacturer's instructions. The cells were passaged three times and after achieving more than 90% confluence in a cell culture flask, 5×10^5 cells/well in 24-well plate were transfected using Fugene HD (Promega Co), with 0.2 μg of each of the pcDNA5/FRT-FcγRIIa haplotype variants and 1.8 μg of pOG44 vector (Invitrogen Co.), according to the manufacturer's recommendations. Subsequently, the transfected Flip-in™-HEK 293 cells were cultured and selected

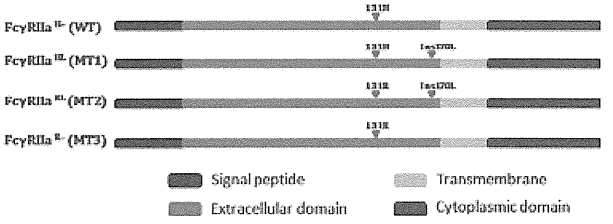

Figure 1 Schematics of Fc gamma receptorIIa (FcγRIIa) haplotype plasmid constructs. The four plasmid constructs varied at the rs1801274 A/G and rs150311303 (-/CTT) polymorphic sites.

in the presence of Hygromycin B (Invitrogen Co.) and Zeocin for FcγRIIa expression. The cells were stained with PE-labelled anti-human CD32 mAB (Santa Cruz biotechnology, Inc., Santa Cruz, CA, USA) and analysed using flow cytometric analysis (MoFlo, Beckman Coulter, Inc., Brea, CA, USA).

IgG adsorption to polystyrene beads. Polyclonal human antibodies IgG1-4 subclasses (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) were adsorbed to polystyrene beads (Polybead Polystyrene Microsphere 1.00 μm; Polysciences, Inc.,Warrington, PA, USA) according to the manufacturer's protocol. Briefly, polystyrene beads were washed twice with binding buffer (0.1 M boric acid, pH 8.5) and then the beads were incubated with 300 μl of each IgG subclass (1 mg/ml) (IgG1, IgG2, IgG3 and IgG4) and bovine serum albumin (BSA) at room temperature for over 20 h. The remaining active sites of the IgG-coated beads were blocked with 7.5% BSA in 0.1 M boric acid (pH 8.5) and suspended in storage buffer (0.1 M of phosphate buffer, 0.15 M of NaCl, 1% of BSA, 5% of glycerol and 0.1% azide, pH7.4) at 4 °C until use.

IgG-coated polystyrene beads binding assay. To study the binding ability of the four FcγRIIa haplotype variants, 5×10^5 HEK 293 cells were incubated with 2.0×10^7 polystyrene beads coated with IgG subclasses (a ratio of 1:40 cell to beads) at 37 °C for 4 h as previously described [24] with some modifications. The cells were stained with PE-labelled anti-human CD32 at 4 °C for 30 min. For quantification of the IgG binding capacity, HEK 293 cells stably transfected with the four FcγRIIa haplotype variants were analysed using flow cytometric analysis (MoFlo; Beckman Coulter, Inc.) with Kaluza software (Beckman Coulter, Inc.). All measurements of the binding indices for the FcγRIIa haplotype variants and negative control (HEK 293 cells transfected with empty vector) were adjusted for the background (BSA coated beads). The results are presented as the median fluorescence intensity (MFI) and are representatives of two (2) independent experiments.

Statistical analysis. All data were analysed using GraphPad prism (Software version 5.00, Inc; San Diego, CA, USA). Genotype and allele frequencies for the *FCGR2A*

polymorphisms were calculated by direct counting and their consistencies with Hardy–Weinberg equilibrium (HWE) determined by comparing the observed and expected frequencies under the HWE for the estimated allele frequency [25].

The incidence, attributable risk, relative risk and 95% confidence intervals (CIs) for symptomatic malaria were calculated as previously described [26], and their significance tested using *t*-statistics. *P* value of <0.05 was considered to be significant after Bonferroni's corrections were made for multiple tests. Haplotypes for FcγRIIa variants were estimated from non-phased genotype data with maximization-likelihood algorithm by the use of PHASE version 2.1.1 [27, 28] and pairwise linkage disequilibrium determined for the two major *FCGR2A* polymorphisms using the software Haploview [29].

Results

Genotyping of *FCGR2A* polymorphisms

To identify polymorphic variants in exon 4 of the *FCGR2A* gene, full sequencing of the exon was carried out, and five polymorphic variants, namely rs4986941A/G (M104V), rs145979252A/G (T119A), rs1801274A/G (H131R), rs141094947T/A (F160Y) and rs150311303 (-/CTT) (ins170L), were identified and their genotype frequencies found to be in line with the HWE (Table 1). The minor allele frequencies for the M104V, T119A, H131R, F160Y and ins170L were calculated as 0.7%, 0.8%, 38%, 0.5% and 8.4%, respectively. Pairwise linkage disequilibrium analysis for the major variants, rs1801274 and rs150311303, showed no linkage disequilibrium for the two major polymorphisms in the cohort subjects (r^2 = 0.05). Two loci haplotype inference was performed by maximization-likelihood algorithm using the PHASE software (University of Washington, Seattle, WA, USA) for the two major SNPs identified in this study: FcγRIIa-H131R and FcγRIIa-ins170L. The FcγRIIa-ins170L was observed only among individuals with FcγRIIa-RR131 genotype but none among FcγRIIa-HH131 genotype carriers. The haplotype frequencies for $Fc\gamma RIIa^{RL}$ (FcγRIIa-RR131 + ins170L), $Fc\gamma RIIa^{R-}$ (RR131 + Wt) and $Fc\gamma RIIa^{H-}$ (HH131 + Wt) were found to be 8.5%, 53.6% and 37.7%, respectively.

Association with malaria phenotypes

The two major *FCGR2A* polymorphisms, rs1801274A/G (H131R) and rs150311303 (ins170L), were further examined for their possible influence on susceptibility to symptomatic malaria by assessing the attributable and relative risks of each of their genotypes. It was observed that the rs1801274 GG (RR131) genotype increased attributable risk for symptomatic malaria by 0.032 events per person per year, with an incidence of 0.055 versus 0.091 for the rs1801274 AA versus GG genotypes, respectively, and a relative risk of 1.5. However, this was not statistically significant (*P* = 0.25) (Table 2). The effect of rs150311303 leucine insertion (ins170L) on susceptibility to symptomatic malaria also showed similar trend as the RR131 carriers with an increased attributable risk of 0.035 events per person per year, incidence of 0.066 versus 0.100 for ins170L carriers versus the wild type, respectively, and a relative risk of 1.5 that was also not statistically significant (Table 2). The $Fc\gamma RIIa^{RL}$ haplotype variant also showed no association with susceptibility/resistance to symptomatic malaria (Table 2).

The effect of rs1801274 and rs150311303 genotypes on parasitaemia was also examined, but no significant influence on level of parasitaemia was observed for both polymorphisms examined in the study population (data not shown).

Table 1 Showing genotype frequencies of *FCGR2A* polymorphisms among Ghanaian cohort children and the expected genotype frequencies

Polymorphism	Observed frequency (%)	Expected frequency	OR
rs4986941A/G (M104V)			
AA	422 (98.6)	422	
AG	6 (1.4)	6	
GG	0	0	
rs145979252A/G (T119A)			
AA	421 (98.4)	421	
AG	7 (1.6)	7	
GG	0	0	
rs1801274A/G (H131R)			
GG	161 (37.6)	165	OR = 1.0; *P* = 0.8
GA	209 (48.8)	202	OR = 1.1; *P* = 0.7
AA	58 (13.6)	62	OR = 0.9; *P* = 0.8
rs141094947T/A (F160Y)			
TT	424 (99.1)	424	
TA	4 (0.9)	4	
AA	0	0	
rs150311303 (-/ins170L)			
Wt/wt	356 (83)	359	OR = 0.9; *P* = 0.9
Wt/ins170L	72 (17)	67	OR = 1.1; *P* = 0.6
Ins170L/ins170L	0	3	OR = 0.1; *P* = 0.2

Wt: Wild type for rs150311303; Ins170L represent CTT insertion for rs150311303.

IgG binding assay for FcγRIIa haplotype variants

To examine the functional relevance of *FCGR2A* polymorphisms identified in this study, four plasmid variants representing the FcγRIIa haplotype variants, that is, $Fc\gamma RIIa^{H/L,\ H/-,\ R/L\ \&\ R/-}$ (Fig. 1), were constructed and their binding capacities to IgG subclasses assessed. Stable transfectants of HEK 293 cells expressing FcγRIIa haplotype variants were incubated with polystyrene beads-bound IgG subclasses (IgG1-4) and flow cytometric analysis performed to determine their binding abilities. The

Table 2 Risk assessment of *FCGR2A* polymorphisms on susceptibility to symptomatic malaria among Ghanaian children.

Genotype	Incidence	Relative risk	Attributable risk	Confidence interval (95% CI)	*P* value
rs1801274 (H131R)					
AA versus AG+GG	0.055	0.736	−0.020	(−0.09 to 0.05)	0.56
GG versus AG+AA	0.091	1.529	0.032	(−0.023 to 0.086)	0.25
rs150311303 (-/ins170L)					
Wild/ins170L[a] versus wild/wild[b]	0.100	1.532	0.035	(−0.041 to 0.11)	0.36
FCGR2A haplotypes					
R/L[c] versus non-R/L	0.100	1.531	0.035	(−0.04 to 0.11)	0.37
R/-[d] versus non-R/-	0.068	0.083	−0.012	(−0.067 to 0.044)	0.68
H/-[e] versus non-H/-	0.054	0.723	−0.021	(−0.086 to 0.045)	0.54

[a]Wild/ins170L, heterozygous for rs150311303 CTT insertion.
[b]Wild/wild, homozygous for rs150311303 wild type.
[c]R/L, Fc gamma receptorIIa (FcγRIIa) haplotype variant with RR131 and ins170L.
[d]R/-, FcγRIIa haplotype variants with RR131 but no ins170L.
[e]H/-, FcγRIIa haplotype variant with HH131 but no ins170L.

expression levels and IgG subclass binding indices of the FcγRIIa haplotype variants are given in supplementary Figure S1.

Analysis of the binding indices for two independent experiments showed that the FcγRIIa haplotype variants had a similar binding pattern for IgG1, IgG3 and IgG4 (H/L > R/L > R->H-) (Fig. 2A–C), with the FcγRIIa$^{H/L}$ and FcγRIIa$^{R/L}$ variants having a higher binding ability for IgG1, IgG3 and IgG4 (MFI ≥ 149,741.72, MFI ≥ 167,872 and MFI ≥ 105,258.67 for IgG1, IgG3 and IgG4, respectively) compared with the FcγRIIa$^{R/-}$ and FcγRIIa$^{H/-}$ variants (MFI ≤ 127,684.93, MFI ≤ 141,026.47 and MFI ≤ 94,705.78 for IgG1, IgG3 and IgG4, respectively).

On the other hand, the binding pattern of the FcγRIIa haplotype variants for IgG2 was observed to be distinctively different between carriers of FcγRIIa-RR131 and -HH131 genotypes (H/L > H- > R/L > R-) (Fig. 2D). The FcγRIIa$^{H/L}$ and FcγRIIa$^{H/-}$ variants exhibited a higher binding ability to IgG2 (MFI ≥ 103,840.01) in comparison to the FcγRIIa$^{R/L}$ and FcγRIIa$^{R/-}$ variants (MFI ≤ 85,942.86).

Discussion

In the present study, *FCGR2A* polymorphisms were investigated among participants of our malaria cohort study in Asutuare, Ghana. Two major *FCGR2A* polymorphisms, rs1801274 (H131R) and rs150311303 (-/CTT) (ins170L), were identified and their possible influence on susceptibility to symptomatic malaria was investigated. No significant influence of the rs1801274 (H131R) and rs150311303 (ins170L) polymorphic variants on susceptibility to symptomatic malaria was observed among the Ghanaian cohort children. To the best of our knowledge, there is no previous report on association between rs150311303 (FcγRIIa-Ins170L) and malaria or any other disease. On the other hand, our finding on FcγRIIa-H131R polymorphism is in agreement with the finding of a recent study, which reported lack of influence of this polymorphism on susceptibility to uncomplicated malaria in Sudan [30]. Previously, a number of studies examining the effect of FcγRIIa-H131R polymorphism on different malaria phenotypes (severe malaria, mild malaria and non-malaria (asymptomatic) controls) have reported significant association of FcγRIIa-H131R polymorphism with susceptibility to severe malaria [13,20,16]. However, none of these studies reported any significant association of the polymorphism with susceptibility to mild malaria in comparison to non-malaria (healthy) controls. Therefore, it is possible to believe that although FcγRIIa-H131R polymorphism may play an important role in influencing susceptibility to severe malaria, it may not have such an influencing effect on susceptibility to symptomatic (mild) malaria.

The FcγRIIa-H131R genotypes have also been shown to have differential binding ability for IgG subclasses [4, 6, 7]. In the present study, the functional relevance of *FCGR2A* polymorphisms on binding to IgG subclasses was investigated. HEK 293 cells stably transfected with FcγRIIa haplotype variants were evaluated for their binding abilities to polystyrene beads-bound IgG subclasses (IgG1-4) using flow cytometric analysis. The results of these analyses revealed that the FcγRIIa haplotype variants had a similar binding pattern for IgG1, IgG3 and IgG4 (H/L> R/L> R/-> H/-), while the binding pattern for IgG2 subclass was observed to show a distinctively higher binding capacity for FcγRIIa-HH131 carriers in comparison to FcγRIIa-RR131 carriers (H/L> H/-> R/L> R/-). Our findings are in agreement with previous reports which showed that the FcγRIIa-HH131 and -RR131 genotypes bind to IgG1 and IgG3, while only the HH131 genotype

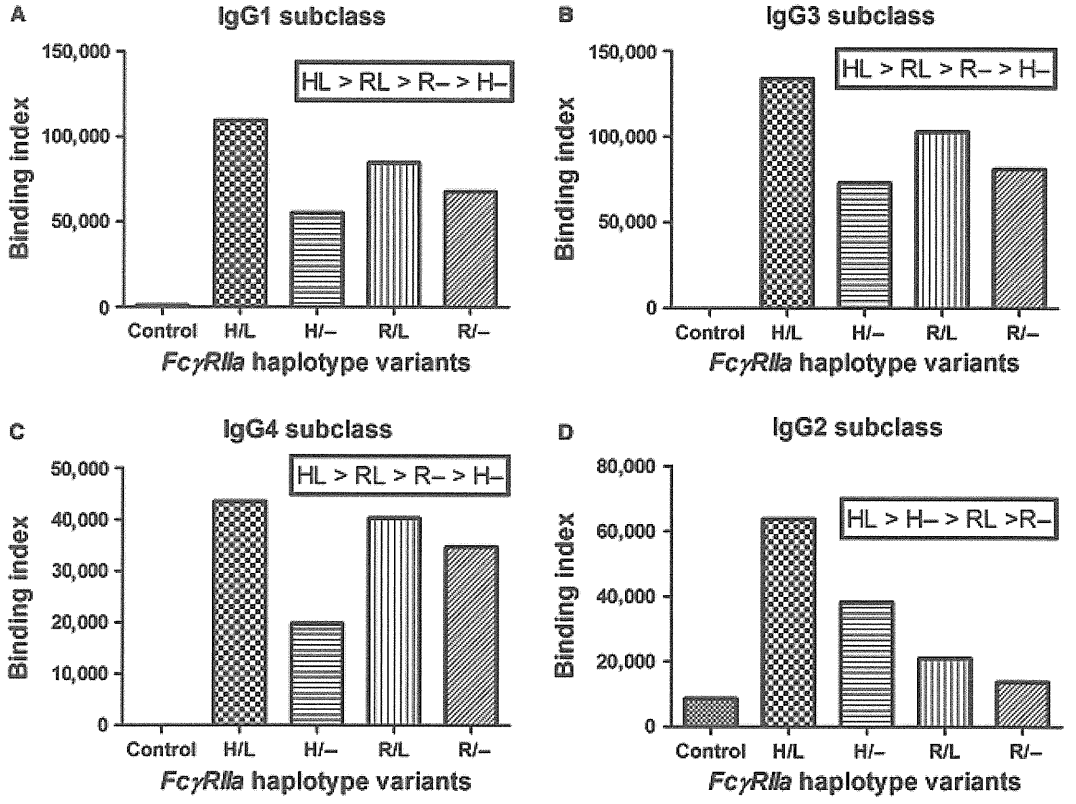

Figure 2 Flow cytometric analysis of HEK cells expressing Fc gamma receptorIIa (FcγRIIa) haplotype variants and their binding capacities for IgG subclasses. Stable transfectants of HEK 293 cells expressing FcγRIIa haplotype variants were incubated with polyclonal IgG subclasses coated beads, stained with anti-human CD32 antibody and their binding capacities evaluated by flow cytometric analysis. Figure 2A–D are bar chart representations of the FcγRIIa haplotype variants binding index for the IgG1-, IgG3-, IgG4- and IgG2-coated beads, respectively. The binding index is a reflection of the median fluorescent intensity, which represents the number of beads bound to the cells expressing each of the FcγRIIa haplotype variants. The results given are from one measurement that is representative of two independent experiments.

efficiently binds to IgG2 [4, 6, 7], with the HH131 genotype having a higher binding affinity for IgG1 [7] and IgG3 [4, 6] compared with the RR131 genotype.

Besides these confirmatory findings, we also observed that the FcγRIIa-ins170L mutation consistently enhanced the IgG subclass binding abilities of both FcγRIIa-HH131 and RR131 genotype carriers. When this observation is interpreted in the context of our study, whereby only three FcγRIIa haplotype variants for rs1801274 (H131R) and rs150311303 (ins170L) (i.e. $Fc\gamma RIIa^{R/L,\ R/-\ \&\ H/-}$) were identified among the cohort children, we find that $Fc\gamma RIIa^{R/L}$ haplotype variant carriers have a higher binding capacity for IgG1, IgG3 and IgG4 as compared with the $Fc\gamma RIIa^{R/-\ \&\ H-}$ variants. The IgG binding advantage gained by carriers of FcγRIIa-RR131 genotype (in the case of $Fc\gamma RIIa^{R/L}$ haplotype variant) could be a case of functional compensation for the carriers of this genotype. FcγRIIa-RR131 genotype has been shown to have poor binding capacity for IgG2 and with no advantage over the HH131 carriers in binding to IgG1 and IgG3.

The difference in IgG2 binding affinity has been reported to influence susceptibility to infections with encapsulated organisms, as human IgG2 is the main IgG subclass induced in response to encapsulated bacterial infections [31, 32]. Individuals with the FcγRIIa-RR131 genotype have been associated with increased susceptibility to invasive pneumococcal infections [33] and severe meningococcal meningitis [34–37] in different populations. It has also been reported that African populations have a higher RR131 to HH131 genotype ratio [16, 38] compared with other populations like the Japanese and Chinese who have a lower RR131 to HH131 genotype ratio [39, 40]. However, the possible advantage possessed by FcγRIIa-RR131 genotype carriers that have managed to maintain this genotype at a stable frequency among the African populations despite the deleterious consequences of susceptibility to encapsulated bacterial infections is still not well elucidated.

In this study, FcγRIIa-ins170L was observed only among individuals with the FcγRIIa-RR131 genotype and it consistently enhanced the binding ability to all IgG subclasses

leading to the FcγRIIa^{RL} haplotype variant having the highest binding capacity for IgG1, IgG3 and IgG4. It is, therefore, possible to speculate that the rs150311303 leucine insertion is a functional mutation acquired by individuals with the FcγRIIa-RR131 genotype and could have a role in protection against some deleterious life-threatening conditions like severe malaria. This could be possible as the FcγRIIa^{RL} haplotype variant has an enhanced binding ability for IgG1 and IgG3, which are the two major IgG subclasses believed to be involved in providing protection against blood stage infections of *P. falciparum* [41]. The RR131 genotype has previously been associated with protections against severe forms of malaria among different African populations [13, 15, 16, 18], and thus, we believe that it may be worthwhile to carry out more studies to investigate the role of rs150311303 leucine insertion and its influence on susceptibility or protection against severe forms of malaria and other infectious diseases.

In conclusion, this study investigated the role of *FCGR2A* polymorphism in symptomatic malaria and found no significant association of the rs1801274 (H131R) and rs150311303 (ins170L) genotypes with symptomatic malaria among Ghanaian cohort children. The study also examined the functional relevance of *FCGR2A* polymorphisms and their effect on binding capacity to IgG subclasses and showed that the rs150311303 leucine insertion (FcγRIIa-ins170L) enhanced the binding capacity of IgG subclasses among carriers of FcγRIIa-RR131 genotypes.

Competing interest

The authors declare that they have no competing interests.

Authors' contributions

AHO participated in conception and designing of the study, carried out the molecular genetic studies, performed data analysis, interpretation and drafted the manuscript; HS performed IgG binding assay, data analysis and revising the manuscript; MY participated in conception and designing of the study, data analysis, interpretation and revising the manuscript critically; AY participated in designing of the study, data collection and analysis; MFO and BDA participated in designing of the study and data collection; MNS participated in designing of the study and revising manuscript critically; MK participated in designing of the study and data analysis; KH participated in conception and designing of the study, data analysis, interpretation, revising manuscript critically and gave final approval of the version to be published

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Supporting Information

Additional supporting information may be found in the online version of this article.

Figure S1 The expression levels and IgG binding capacities of FcγRIIa haplotype variants in HEK 293 cells. Flow cytometric analysis of stably transfected HEK 293 cells with FcγRIIa haplotype variants stained with PE-labeled anti-human CD32 mAB. All panels on the left in figure S1a-e shows FcγRIIa haplotype variants expressions levels. All panels on the right in figure S1a-e shows the binding pattern for each of the FcγRIIa haplotype variants. The expression levels and binding capacities were evaluated separately for; (a) BSA coated beads, (b) IgG1 coated beads, (c) IgG2 coated beads, (d) IgG3 coated beads and (e) IgG4 coated beads. Tables displayed below the graphs show the median fluorescent intensity (MF1) and the degree of variability (coefficient of variation) for each measurement. The experiment shown is representative of two independent experiments. PA#1: HEK 293 cell transfected with empty vector; PA#2: HEK 293 cell transfected with $Fc\gamma RIIa^{H-}$ haplotype variant; PA#3: HEK 293 cell transfected with $Fc\gamma RIIa^{HL}$ haplotype variant; PA#4: HEK 293 cell transfected with $Fc\gamma RIIa^{RL}$ haplotype variant; PA#5: HEK 293 cell transfected with $Fc\gamma RIIa^{R-}$ haplotype variant.

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MALARIA JOURNAL

METHODOLOGY Open Access

A simple and inexpensive haemozoin-based colorimetric method to evaluate anti-malarial drug activity

Tran Thanh Men[1,2], Nguyen Tien Huy[3*], Dai Thi Xuan Trang[1], Mohammed Nasir Shuaibu[3], Kenji Hirayama[3,4] and Kaeko Kamei[2*]

Abstract

Background: The spread of drug resistance in malaria parasites and the limited number of effective drugs for treatment indicates the need for new anti-malarial compounds. Current assays evaluating drugs against *Plasmodium falciparum* require expensive materials and equipment, thus limiting the search for new drugs, particularly in developing countries. This study describes an inexpensive procedure that is based on the advantage of a positive correlation between the haemozoin level of infected erythrocytes and parasite load.

Methods: The relationship between parasitaemia and the haemozoin level of infected erythrocytes was investigated after converting haemozoin into monomeric haem. The 50% inhibitory concentration (IC_{50}) values of chloroquine, quinine, artemisinin, quinidine and clotrimazole against *P. falciparum* K1 and 9A strains were determined using the novel assay method.

Results: The haemozoin of parasites was extracted and converted into monomeric haem, allowing the use of a colorimeter to efficiently and rapidly measure the growth of the parasites. There was a strong and direct linear relationship between the absorbance of haem converted from haemozoin and the percentage of the parasite ($R^2 = 0.9929$). Furthermore, the IC_{50} values of drugs were within the range of the values previously reported.

Conclusion: The haemozoin-based colorimetric assay can be considered as an alternative, simple, robust, inexpensive and convenient method, making it applicable in developing countries.

Keywords: Anti-malarial, Assay, Haemozoin, Malaria

Background

Malaria is more than just a problem for tropical countries, it also is a major global public health concern. Annually, there are approximately 300 million new malaria infections and millions of deaths worldwide due to malaria [1,2]. Because a vaccine for malaria is not available, chemotherapy is the main treatment. However, the rapid spread of resistance to current quinoline anti-malarials has made malaria a major global and important problem. In addition, artemisinin, from a Chinese herb (*Qinghaosu*) that has been used in the treatment of fevers for more than a thousand years, is now considered an essential component of artemisinin-based combination therapy against drug-resistant malaria [3,4]. However, the malaria parasites have recently been found to be resistant to artemisinin [5-7]. The alarming spread of drug resistance and the limited number of effective drugs for treatment indicates how important it is to find new anti-malarial compounds.

For decades, the anti-malarial activity of a drug has been measured *in vitro* by quantifying the uptake of radioactive substrates by a parasite as a measure of growth and viability in the presence of the test drug [8,9]. Although several *in vitro* methods exist, the ^{3}H-hypoxanthine method [8] is a popular test for novel anti-malarial drugs, but it is labelled with radiation that presents a potential risk to safety, and it relies on

* Correspondence: tienhuy@nagasaki-u.ac.jp; kame@kit.ac.jp
[2]Department of Biomolecular Engineering, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan
[3]Department of Immunogenetics, Institute of Tropical Medicine (NEKKEN), Nagasaki University, Nagasaki, Japan
Full list of author information is available at the end of the article

relatively expensive radio-isotopes and includes multi-step procedures that become increasingly problematic and impractical when the incidence of testing is increased. The other methods, including the PicoGreen [10,11] and the SYBR Green I [12,13] methods, are also considered to be expensive approaches regarding equipment and chemicals. In addition, there are other methods that are based on enzymatic reaction and/or antibodies that specifically detect the presence of histidine-rich protein II or parasite lactate dehydrogenase [14-16]. However, these methods involve multiple complex steps that are too expensive for developing countries, which makes them ill suited for screening potential anti-plasmodial drugs.

During the development and proliferation stage in host erythrocytes, the malaria parasites degrade haemoglobin for use as a major source of amino acids. This is accompanied by the release of free haem. With haem as a prosthetic group of haemoglobin, the iron is in the ferrous state, but free haem loses one electron and assumes the ferric state. This ferric haem could be oxidatively active and toxic to both the host cells and malaria parasites, and can even cause parasite death. Moreover, due to the absence of haem oxygenase, the parasite is unable to cleave haem into an open-chain free haem, which is necessary for cellular excretion [17]. To protect itself, it is necessary for the parasite to convert haem to non-toxic metabolites. Principally, the parasite detoxifies free haem via neutralization with histidine-rich protein 2 [18,19] and degradation with reduced glutathione [20,21], but mostly with crystallization into haemozoin, which is a water-insoluble malarial pigment produced in the food vacuole [19,22]. Therefore, a simple and inexpensive *in vitro* assay was developed based on the colorimetric quantification of haemozoin in infected red blood cells to evaluate the anti-malarial activity of drugs.

Methods

Materials

Chloroquine diphosphate, quinine sulphate, primaquine, clotrimazole, haemin chloride (haem), RPMI 1640 medium, hypoxanthine, and gentamycin were purchased from Sigma Aldrich Chemical Company (Tokyo, Japan). Albumax II (Gibco), and the other chemicals used in the present study were of a high grade. *Plasmodium falciparum* K1 (chloroquine resistant) and 3D7-9A (chloroquine susceptible) strains [23] were provided from Dr Osamu Kaneko and Dr Shusuke Nakazawa, respectively, from the Institute of Tropical Medicine, Nagasaki University, Japan.

Plasmodium cultivation

Plasmodium falciparum K1 and 9A strains were maintained *in vitro* with continuous culture according to a previously described method with a slight modification [24]. The culture medium consisted of RPMI 1640 supplemented with 0.025 mg/ml gentamicin, 0.01 mM hypoxanthine, 23.8 mM $NaHCO_3$, 11 mM glucose and 0.5% albumax II, and adjusted to a pH of 7.3 to 7.4. The parasite was cultured and maintained in a tissue culture flask with complete culture medium containing 5% human erythrocytes. The parasite density was maintained at about 1.5% parasitaemia under an atmosphere of an AnaeroPack sachet (Mitsubishi Gas Chemical Company Inc, Tokyo, Japan) to create 20% CO_2 and remove O_2 (<0.1%) 37°C [25]. Every two days, infected erythrocytes were transferred into fresh medium containing 5% human erythrocyte. The level of parasitaemia was determined by light microscopy on a Giemsa-stained thin blood smear, and parasitized erythrocytes were diluted when parasitaemia was higher than 5% in erythrocytes contained at 5% in culture medium, in order to lower parasitaemia and allow continuous growth. Parasite culture was diluted with fresh uninfected erythrocytes and culture medium to achieve a starting parasitaemia of 2% and a haematocrit of 5%. This final parasite culture was immediately used for anti-malarial assay.

The relationship between parasitaemia and haemozoin level

A culture of the *P. falciparum* K1 strain was serially diluted with uninfected erythrocytes in complete medium to yield a haematocrit of 5% and parasitaemia ranging from 0 to 10%. The serial culture containing 200 μl was prepared independently in triplicate in microtubes, followed by the addition of 800 μl of 2.5% sodium dodecyl sulphate in 0.1 M sodium bicarbonate pH 8.8, then the samples were mixed at room temperature for 15 min. After centrifugation at 13,000 rpm for 10 min, the supernatant was removed. The pellet was washed twice with 800 μl of 2.5% sodium dodecyl sulphate in 0.1 M sodium bicarbonate (pH 8.8), then 200 μl of 5% sodium dodecyl sulphate was added to 50 mM NaOH to convert the haemozoin into haem. After incubation at room temperature for 30 min, the sample (200 μl) was transferred to a 96-well microplate and scanned at 405/750 nm ($A_{405\ nm}$ minus $A_{750\ nm}$) using an IMark microplate reader (Bio-Rad). After the background absorbance of haemozoin was purified of uninfected erythrocytes (5% haematocrit) then subtracted, the amount of haemozoin in the infected erythrocytes was presented as the absorbance at 405/750 nm and then plotted against parasitaemia.

Evaluating the anti-malarial activity of drugs using the haemozoin-based spectrophotometric method

The *P. falciparum* K1 and 9A strains were used to evaluate the anti-malarial activity of quinine, chloroquine,

pyrimethamine, artemisinin and clotrimazole by using the haemozoin-based spectrophotometric method. Stocks of drugs were prepared in dimethyl sulphoxide or phosphate buffer saline (for chloroquine) and were then serially diluted with complete culture medium. To each well of a microplate, 10 μl of serially diluted drug solution was added into 200 μl of final asynchronous parasite culture. Dimethyl sulphoxide or phosphate buffer saline were also tested by adding a similar amount to control wells. The microplates were cultured 72 hr under the conditions described above. The haemozoin of infected erythrocytes was extracted, purified, and quantified, as described above. The 50% inhibitory concentration (IC_{50}) value was calculated by non-linear fitting of the absorbance at 405/750 nm against the logarithm of the drug concentration using the GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Sigmoidal doses with variable slope models were used with the following equation:

$$y = \min + \frac{\max - \min}{1 + 10^{(\{\log IC_{50} - x\} \times Hill\ slope)}}$$

where y is the absorbance at 405/750 nm; x is the logarithm of the drug concentration, min is the absorbance at 405/750 nm measured at time zero (starting point of assay), and max is the maximal absorbance of a particular drug. The Hill slope is the steepness of the curve. The logarithm of the concentration at zero was defined at 2 log lower than the lowest concentration of a particular drug.

Results and discussion

Relationship between the absorbance of the haem content of haemozoin and parasitaemia

The relationship between the haemozoin amount in a parasitized erythrocyte and parasitaemia was revealed by measuring the absorbance of the haem content of the haemozoin obtained from the parasitized erythrocytes after degradation to monomer haem. As shown in Figure 1, the absorption of converted haem showed a direct and linear correlation with the level of parasitaemia. Low and unsynchronized parasitaemia (about 1%) could be detected using this haemozoin-based colorimetric method. The results indicate that this novel assay of parasites is applicable for monitoring parasite growth and for screening new antimalarial compounds.

Haemozoin-based colorimetric assay to determine the IC_{50} values

The IC_{50} values of some anti-malarial drugs were determined with a dose-response experiment using a haemozoin-based colorimetric assay. The result showed that increasing the concentration of anti-malarial drugs resulted in a decreased absorbance at 405/750 nm (Figure 2). The data was best fitted by a typical sigmoidal dose-response model with a variable slope (four parameters) that agreed well with previous reports [26,27].

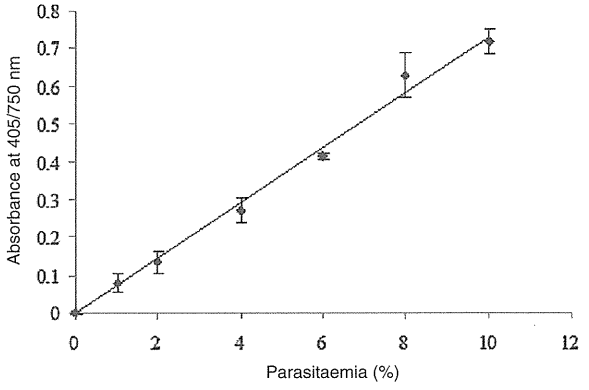

Figure 1 The linear relationship between the haemozoin level of a parasite and parasitaemia. Haemozoin concentration of infected erythrocytes is presented by the absorbance at 405/750 nm of monomeric haem after conversion from haemozoin using an NaOH solution. Absorption values (means ± standard errors of triplicate wells) are plotted against parasitaemia. A well correlated, linear relationship (R^2 = 0.9929) is strong evidence of the sensitivity of the method.

Table 1 summarizes the results of the assay to determine IC_{50} using the haemozoin-based colorimetric method. The *P. falciparum* K1 strain was primarily observed for quinine, chloroquine, clotrimazole, pyrimethamine and artemisinin, with IC_{50} values of 0.258,

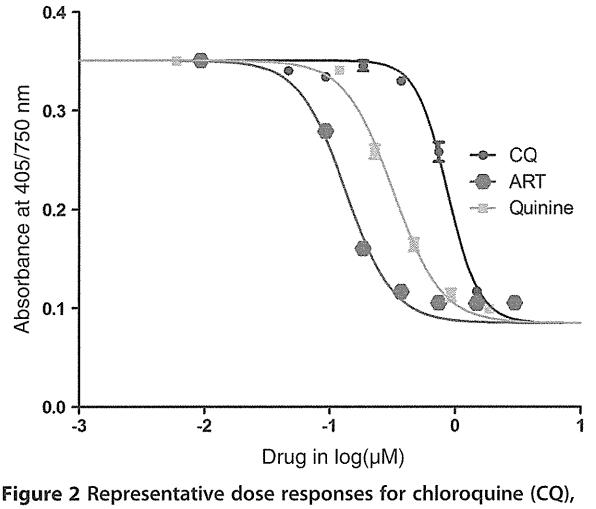

Figure 2 Representative dose responses for chloroquine (CQ), artemisinin (ART) and quinine against the *Plasmodium falciparum* K1 strain. Parasite growth after incubation of parasitized erythrocytes for 72 hr with a drug was measured using a haemozoin-based colorimetric method. The symbols and error bars are the average absorption values at 405/750 nm and standard deviations in triplicate, respectively. The sigmoidal dose response model with a variable slope is the best fit to the data.

Table 1 *In vitro* anti-malarial activities of drugs against chloroquine-susceptible (9A) and -resistant (K1) strains of *Plasmodium falciparum*

Drugs	Mean IC_{50} and 95% CI (μM)	
	P. falciparum K1 strain	*P. falciparum* 9A strain
Quinine	0.258 (0.242 - 0.275)	0.398 (0.307 - 0.516)
Chloroquine	0.873 (0.824 - 0.926)	0.132 (0.082 - 0.211)
Clotrimazole	0.805 (0.689 - 0.939)	1.67 (1.18 – 2.37)
Pyrimethamine	23.03 (18.36 - 28.90)	Not done
Artemisinin	0.139 (0.124 - 0.155)	0.483 (0.376 - 0.622)

Parasitized red blood cells were incubated with different concentrations of drugs for 72 hr and parasite growth was evaluated using the haemozoin-based colorimetric method. The IC_{50} and its 95% confidence interval (95% CI) were calculated from the concentration-response curve of the haemozoin level *vs* the log concentration of a drug.

0.873, 0.805, 23.03, and 0.139 μM, respectively. For the *P. falciparum* 9A strain, pyrimethamine was not evaluated, and the IC_{50} values were 0.398, 0.132, 1.67 and 0.483 μM in succession for quinine, chloroquine, clotrimazole and artemisinin, respectively. The IC_{50} values for quinine, chloroquine, clotrimazole, and pyrimethamine were in a range that was similar to those observed in previous reports [28,29]. On the other hand, the IC_{50} values for artemisinine were higher than previous reports, probably due to asynchronous cultures, ring stage-specific target of artemisinine, accumulation of released hemozoin in the continuous cultures, or several rounds of continuous cultures and cloning of parasite strains in our laboratories. Therefore, further studies are required to compare the novel assay with recent developed methods to validate the accuracy in the screening new antimalarial compounds [30]. Another limitation of the novel method is that it is not easily adaptable for a high throughput screening of anti-malarial drug candidates, which is under-developed using 96-well filter plates [31].

In recent years, the number of laboratories, diagnosis centres and research institutes has risen in developing countries. However, most of them lack the modern equipment and expensive chemicals to apply new methods for screening anti-malarial candidates. In addition, some methods have potential risks of toxicity, so it is prudent to wear disposable gloves at all times when proceeding. Another obstacle is that many laboratories lack the facilities to treat toxic contamination before the toxin is discarded in the environment. The novel anti-malarial assay is safe, non-expensive and easy to apply in laboratories.

Conclusions

The standard curve obtained in this study was strongly linear between the absorbance of monomeric haem converted from haemozoin and the percentage of parasitaemia. The IC_{50} values of chloroquine and quinine obtained from the haemozoin-based colorimetric method are similar to other methods. Even though this report describes specific conditions, the current experiment has introduced an assay that is adaptable to a wide range of conditions. The results also show that using this method has several advantages over using current methods. First, the method is fast and is based on a simple technique that uses a microplate reader, which is available in most laboratories. Second, the assay is based on inexpensive chemicals with no requirement of cold storage. Last but not least, the assay of the inhibition of *P. falciparum* growth using a haemozoin-based colorimetric method is feasible, reproducible, non-toxic, and more convenient than other assays, which makes it particularly useful for developing countries in the screening of novel anti-plasmodials, as a useful high throughput screening method for anti-malarial drug candidates.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NTH and KK developed the idea for the project. TTM and NTH conceived and designed the experiments. TTM, NTH and MNS carried out the laboratory work. TTM, NTH, DTXT, KH, and KK analysed and interpreted the data. TTM, NTH, MNS, KH and KK contributed reagents/materials/analysis tools. . TTM, NTH, DTXT and KK wrote the paper. All authors had full access to all data in the study, read and approved the manuscript.

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Author details

[1]Department of Biology, College of Natural Science, Cantho University, Cantho City, Vietnam. [2]Department of Biomolecular Engineering, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan. [3]Department of Immunogenetics, Institute of Tropical Medicine (NEKKEN), Nagasaki University, Nagasaki, Japan. [4]Global COE program, Nagasaki University, Nagasaki, Japan.

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Original article

Immunoproteomics Identification of Major IgE and IgG4 Reactive *Schistosoma japonicum* Adult Worm Antigens Using Chronically Infected Human Plasma

Daniel Boamah[1], Mihoko Kikuchi[1], Nguyen Tien Huy[1], Kenta Okamoto[1], Honggen Chen[2], Irene Ayi[3], Daniel Adjei Boakye[3], Kwabena Mante Bosompem[3] and Kenji Hirayama[1]*

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Abstract: Immunoepidemiological studies from endemic areas have revealed age-dependent resistance correlation with increased level of IgE and decreased level of IgG4 antibodies in responses to schistosomes' soluble worm antigen. However, there have been limited studies on analyses of major antigens that provoke IgE and IgG4 immune response during chronic stage of schistosomiasis. In this study, for the first time, immunoproteomics approach has been applied to identify *S. japonicum* worm antigens in liquid fractions that are recognized by IgE and IgG4 antibody using plasma from chronically infected population. ProteomeLabPF 2D fractionated 1-D and 2-D fractions of SWA antigens were screened using pooled high IgE/IgG4 reactive plasma samples by dot-blot technique. In 1-D fractions, IgE isotype was detected by fewer antigenic fractions (43.2%). The most recognized isotype was IgG3 (79.5%) followed by IgG1 (75.0%) and IgG4 (61.4%). Liquid chromatography MS/MS protein sequencing of reactive 2-D fractions revealed 18 proteins that were identified, characterized and gene ontology categories determined. 2-D fractions containing proteins such as zinc finger, RanBP2-type, domain-containing protein were strongly recognized by IgE and moderately by IgG4 whereas fractions containing proteins such as ubiquitin-conjugating enzyme and cytosolic II 5'-nucleotidase strongly recognizing by IgG subclasses (IgG1, IgG3 and IgG4) but not IgE. By this study, a simple and reproducible proteomic method has been established to identify major immunoreactive *S. japonicum* antigens. It is anticipated that this will stimulate further research on the immunogenicity and protective potential of proteins identified as well as discovery of novel compounds that have therapeutic importance.

Key words: *Schistosoma japonicum*, IgE, IgG4, Proteome, Mass Spectrometry, Genome

INTRODUCTION

The pathophysiology of schistosomiasis is mainly due to the immune response against tissue trapped eggs with consequent clinical manifestations being typical of the species infecting, intensity of worm burden as well as the immunity of the infected host. The variety of antigens released by dead worms or secreted by the worms or shed during the various developmental stages of the worm life cycle (cercariae, schistosomula, adult male and female, and eggs) provide strong sustained stimuli to the host's humoral and T-lymphocyte-mediated immune responses [1]. In recent years, immune response regulation by the schistosomes during infections has been a topic of great concern. Particularly, the role of antibodies in resistance to reinfection. In schistosomiasis, the balance between IgE and IgG4 antibody isotypes is thought to play a role in resistance or susceptibility to infection. Immunoepidemiological studies from endemic areas have revealed age-dependent resistance correlation with specific antibody isotype responses to the schistosome antigens, particularly IgE responses to *Schistosoma mansoni* adult worm antigens (AWA). The IgE levels are low in children and high in adults, whereas for IgG4 the reverse has been reported [2–4]. Furthermore, since IgE and IgG4 can exhibit parallel specificity profile, it has been suggested that IgG4 subclass acts as a blocking

[1] Department of Immunogenetics and Global Centre of Excellence, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki City, Nagasaki 852-8523, Japan
[2] Jiangxi Provincial Institute of Parasitic Disease, Nanchang 330046, Jiangxi, PRC, China
[3] Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana, Legon, Post Office Box LG 581 Legon, Accra, Ghana
*Corresponding authors:
E-mail: hiraken@nagasaki-u.ac.jp
Abbreviations: BCA, Bicinchoninic acid; TCEP, Tris (2-Carboxyethyl) Phosphine Hydrochloride; n-OG, n-Octylglucoside

antibody against killing of the parasites by inhibiting IgE antibody-dependent cellular cytotoxicity (ADCC) mediated by monocytes, platelets or eosinophiles. Similar effect has also been suggested for IgM and IgG2 antibodies [2, 5–8]. The IgG3 antibody level also correlated with susceptibility to and biomarkers in liver fibrosis [6]. The production of IgE is stimulated by interleukin-l3 (IL-13) and IL-4, and modulated by IL-12 and interferon-gamma (IFN-γ) while the production of IgG4 is also stimulated by IL-4 [4]. The IL-4-dependent production of IgE and IgG4 is blocked by IFN-γ, though the level required to block IL-4-dependent IgE production is much lower than that needed to block IgG4. In the sequential events of class switching, IgG4 is synthesized thereafter IgE, caused by sequential involvement of different lymphokines raising the possibility that development of protection against schistosomes would depend on population of lymphocytes producing cytokine [4, 9, 10].

In spite of many studies demonstrating importance of antibody-mediated protection against re-infection of schistosomes both in experimental and epidemiological models, many of the human schistosome vaccine research based on antibody-mediated protection have not progressed to the phase III clinical trials. This in part might be due to the limited understanding of protective anti-schistosome response against specific proteins [11]. Relatively, limited target antigens have been analyzed in the context of selective antibody isotype recognition for IgE or IgG4 especially in *S. japonicum* infection [2–4, 6]. Antigens that are IgE, IgG4 or both antibodies preferred can be very useful for studying mechanisms associated with antibody related resistance to schistosomiasis.

Many of the antigenic substances produced by the schistosomes at the various life cycle stages consist of proteins, glycoproteins and polysaccharides in nature [12]. So far, characterization of schistosome antigens has involved studying crude parasite extracts that had no detailed characteristics of reactive immunoglobulins. Some studies have also focused on proteins or glycoprotein components of schistosomes either directly or by cloning in bacteria systems [5, 13]. Although, elevated IgE level is important for development of resistance to reinfection in schistosomiasis, only a limited number of studies have been conducted to isolate and characterize IgE-specific antigens from *S. mansoni* [14] with a homologous antigen identified in *S. haematobium* [15] and *S. japonicum* [16]. Therefore, the antigenic source of variation in IgE antibody isotype-specific response to *S. japonicum* is limited.

The mass spectrometry (MS) based proteomics has facilitated identification of large numbers of proteins from complex biological systems. Proteomics has in recent years achieved improvements in platforms and the standard proteomics approaches rely on the second dimensional (2-D) separation of complex protein mixtures using second dimensional gel electrophoresis (2-DE) [17, 18]. In some cases, the 2-DE may be combined with difference in gel electrophoresis (DIGE) as a profiling platform and proteins are identified by ESI-MS/MS of trypsin-derived peptides. However, 2-DE has a number of shortcomings including limited loading capacity; inability to resolve proteins of extreme pI values; limitation in resolution of hydrophobic proteins and inability to resolve proteins of smaller molecular weights. Therefore, fractionating complex protein mixtures while maintaining intact proteins by liquid chromatography (LC) is most desirable for downstream analyses (top-down proteomics) [19].

The proteomeLabPF 2D instrument introduced by Beckman-Coulter (Beckman Coulter, Fullerton, CA, USA) features a rapid semi-automated 2-D HPLC system that uses two different methods to separate proteins; ion-exchange in the 1-D and non-porous reversed phase in the 2-D chromatography [20–22]. Unlike gel electrophoresis, it offers an added advantage that collected fractions are in liquid phase and can be utilized directly for any of various analytical procedures, such as enzymatic digests, mass spectrometer analysis, additional fractionation, western blot, or a combination of analytical tests. Additionally, it has been shown to be suitable for high-throughput large-scale analysis of intact proteins [23–26] and high loading capacity (up to 5 mg) than with gel electrophoresis, thus significantly increasing the sensitivity of protein identification. Liquid-based fractionation and separation systems offer great flexibility and can be suitable for large-scale proteomic profiling in a quantitative analysis [25, 26].

This study focused on isolating, identifying, and characterizing immunogenic *S. japonicum* proteins that are preferentially detected by IgE and IgG4 antibodies using serological proteomics approach. Identifying and characterizing antigenic proteins detected by the isotypes studied would contribute to understanding of schistosome-specific adaptive immunity. This also, highlights the importance of vaccine research focusing on induction of protective isotype-specific antibody response to specific peptides as a single protein from the parasite might possess undetermined antigenic determinants capable of stimulating various antibody productions.

MATERIALS AND METHODS

Soluble Worm Antigen Preparation

Soluble worm antigen (SWA) extract was prepared from frozen Chinese strain *S. japonicum* adult worms fol-

lowing the procedures previously described [27] with slight modifications. Briefly, adult worms (600 mg) were homogenized in 3.25 ml cold Diethyl Ether (Wako Pure Chemical Industries, Ltd. Osaka, Japan). The homogenate was centrifuged at 2,000 *g*, 5 min to remove lipids together with the diethyl ether. Thereafter, the pellet was freeze-thawed several times in 3.5 ml of lysis buffer (6 M Urea, 2 M Thiourea, 10% Glycerol, 50 mM Tris-HCl, pH 7.8, 2% n-OG, 5 mM TCEP) mixed with 0.1 mM PMSF and 2 μg/ml Leupeptin. This was dialyzed in PBS (pH 7.5) containing 8 M Urea at 4°C with stirring. The homogenate was centrifuged at 20,000 *g* for 1 hr at 4°C and then filtered through 0.22 μm filter (Millex GP Filter Unit, Millipore Ireland Ltd. Tullagreen, Carrigtwohill Co Cork, Ireland). Protein concentration was determined by BCA Protein Assay Kit (Bio-Rad Laboratories Inc., Tokyo, Japan) and stored at –80°C until used.

Measurement of Anti-worm Antibody Levels

An ELISA was carried out using SWA to screen plasma samples obtained from individuals with liver fibrosis ($n = 31$ grade 0; $n = 62$ grade 1; $n = 91$ grade 2 and 3 individuals) due to schistosomiasis japonica as previously described [28, 29]. The project proposal including the reuse of the stored samples was processed to the Institutional Review Board at NEKKEN and was approved (No. 12081793). Five plasma samples originally confirmed by microscopy and ultrasound were included as positive controls. Three plasma samples were also included as negative controls which were obtained from healthy Japanese individuals without schistosomiasis history. Briefly, plates (Nunc-Immuno Plate, Nunc, Denmark) were coated with 5 μg/ml of SWA. After washing unbound antigens two times (2×) with PBS containing 0.05% Tween 20 (PBST, pH 7.4), the plates were blocked with 5% non-fat skimmed milk in PBST for 60 min at room temperature (RT) followed by 2× washing. Plasma samples were diluted 1:20 for detection of IgE and IgG4 and 1:800 for detection of IgG1 and IgG3 with 1% blocking solution at followed by incubation at 37°C for 60 min and then 3× washing. The procedure continued with 60 min incubation (37°C) with horseradish peroxidase-conjugated mouse anti-human IgG1, IgG3 (Southern Biotechnology Associates Inc., Birmingham, AL, USA), IgG4 (MP Biomedicals. LLc, France) or biotin-conjugated goat anti-human IgE (Invitrogen Corporation, Camarillo, CA, USA) in 1% blocking solution at 1:1000, 1:1000 1:400 or 1:400 respectively. For detection of IgE, the plates were further treated with 1:400 horseradish peroxidase-conjugated streptavidin (DakoCytomation, Copenhagen, Denmark). Finally, plates were developed with stabilized chromogen (SB01, Invitrogen) in the dark followed by addition of stop solution (1N H_2SO_4, WAKO). The OD was measured at 450 nm (iMark Microplate Absorbance Reader, Bio-Rad laboratories, Inc. Japan). The mean ODs obtained were Log-transformed after subtracting the mean ODs of the negatives and samples within the upper quartile (95 percentile) (51/184) were pooled for dot-blot reactivity against 1-D and 2-D fractionated SWA (Fig. 1A).

Buffer Exchanging and Chromatofocusing

The PD-10 desalting column containing 8.3 ml of Sephadex G-25 medium (85 to 260 μm particle size), (GE Healthcare Bio-Sciences K. K. Tokyo, Japan) was applied in buffer exchange of SWA before chromatofocusing following the manufacturer's recommendation. Briefly, the PD-10 column was equilibrated with proprietary buffer, "ProteoSep Start" buffer (Eprogen, Darien, IL, USA) by allowing it to enter the packed bed completely. The flow-through was discarded. This was repeated with a total of 25 ml "ProteoSep Start" buffer. The 2 mg of SWA was resuspended in 1.25 ml of "ProteoSep Start" buffer, loaded onto the equilibrated PD-10 column and allowed to enter the column completely. The flow-through was again discarded. Elution was performed with 3.5 ml "ProteoSep Start" buffer added onto the column and the eluent collected into a new 15 ml tube under gravity and applied in 1-D Chromatofusing.

Chromatofocusing was performed using the ProteomeLab PF 2D protein separation system (Fig. 1B) with 32 Karat user interface software. The pH gradient was formed using two proprietary buffers: "ProteoSep Start" buffer ([essentially contained urea, Tris-HCl and n-OG at pH of 8.5) and "ProteoSep Elution" buffer (Eprogen, Darien, IL, USA) (essentially Urea, Polybuffer 74-HCl, n-OG and iminodiacetic acid, pH 4.0). The High Performance Chromatofocusing Column (A51685 ProteoSep HPCF Column, 250 mm × 2 mm, Eprogen. Darien, IL, USA) was treated according to the manufacturer's instructions. Briefly, the column was washed with 10 volumes of autoclaved MilliQ water at a flow rate of 0.2 ml/min for 45 min and then equilibrated with 30 volumes of "ProteoSep Start" buffer for 130 min at 0.2 ml/min, ambient temperature. The buffer exchanged SWA sample was introduced with a manual injector into the column. Proteins bound to the strong anion exchanger in the HPCF column were eluted with a continuous decreasing pH from 8.5 to 4.0. Twenty minutes after sample injection, the valve automatically switched from "ProteoSep Start" buffer to "ProteoSep Elution" buffer at a flow rate of 0.2 ml/min over 95 min. The pH began to decrease after about 45 min. Fractions were automatically collected every 0.3 pH units into a 96-well deep-plate

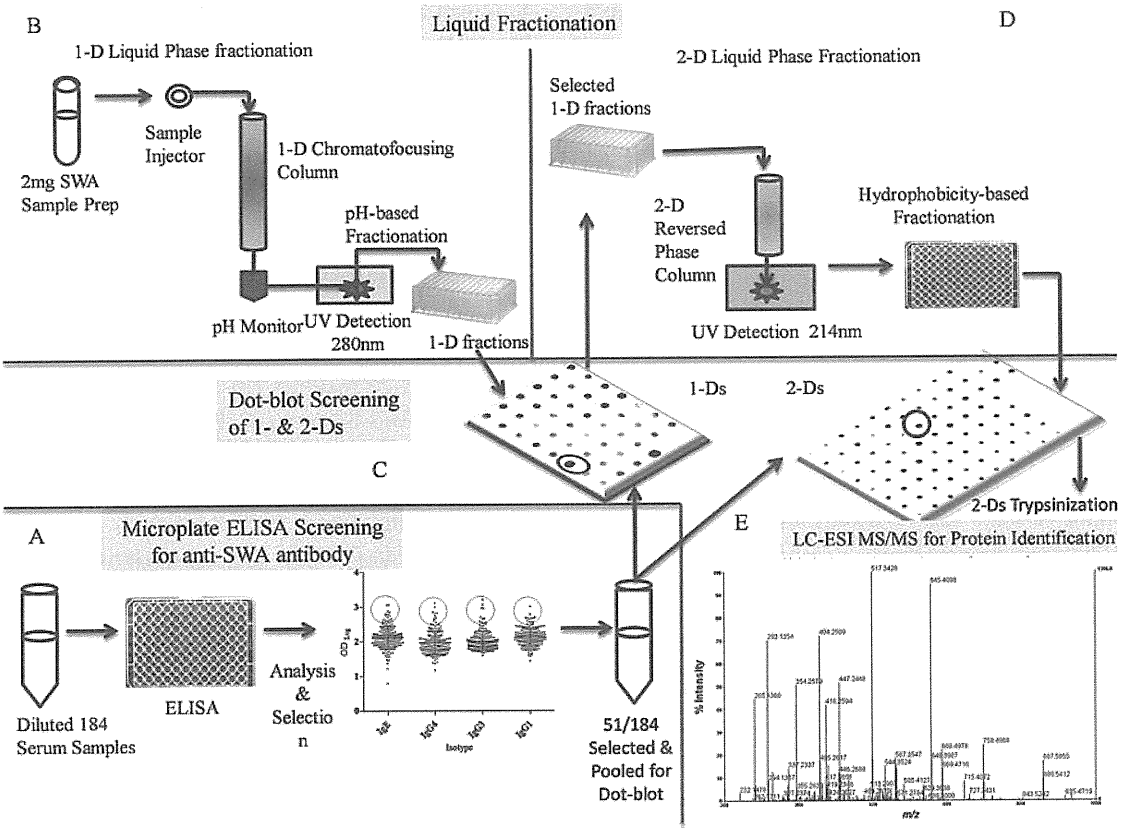

Fig. 1. Workflow of the experiment. In order to obtain anti-worm antibody, plasma from chronically *S. japonicum* infected individuals were screened in ELISA system (A) and highly reactive samples pooled for dot-blot screening. Soluble worm antigen (SWA) was fractionated by Chromatofocusing (B) using ProteomeLab PF 2D (Beckman Coulter, Fullerton, CA, USA) followed by dot-blot screening (C). Reactive fractions were further fractionated by reversed phase chromatography which was again screened by dot-blot and reactive fractions trypsinized for ESI-MS/MS protein identification (E).

(Part No. 26700J, Beckman Coulter, Fullerton, CA, USA). At 170 min, the HPCF column was washed for 45 min with 10 column volumes of a third buffer of high ionic strength solution (1 M NaCl) and re-stored by 10 column volumes of distilled water for 45 min. The absorbance of the column effluent was monitored at 280 nm with an online pH flow cell. The percentage concentration eluted over the different pH conditions was estimated using the peak area of the fraction monitored at 280 nm, a wavelength at which the peak area is directly proportional to the quantity of the proteins [30]. The 1-D fractions obtained were screened by dot-blot assay and selected reactive fractions directly applied to the 2-D reversed phase unit.

Second Dimension Reversed Phase Chromatography

The 2-D separation (Fig. 1D) was performed using Reversed Phase High Performance Column (391106 PF 2D HPRP Column, Beckman Coulter, Fullerton, CA, USA) and two solvents, 0.1% TFA in water (Solvent A) and 0.08% TFA in ACN (Solvent B). At the end of each run, equilibration of the column was achieved with initial mobile phase (Solvent A) for 10 min followed by Solvent B for 5 min prior to each injection. All 2-D chromatography was conducted at a column temperature of 50°C and buffer flow rate of 0.75 ml/min with the absorption of the effluent monitored at 214 nm. From the selected 1-D fractions, 200 µl was automatically injected into the PF 2D HPRP column and ran for 6 min. The column was eluted at a flow rate of 0.75 ml/min with a 0–100% linear gradient of solvent A and solvent B for 35 min. Thereafter, Solvent B was continued for 5 min, followed by re-equilibration with 100% Solvent A for 10 min. The fractions were collected at a flow rate of 0.18 min into 96-well microplate (Product code 3363, Corning International K. K. Tokyo, Japan) placed in an automated fraction collector (Gilson FC 204 Fraction Collector, M & S Instruments Inc. Osaka, Japan). The 2-D fractions were stored at –80°C while some screened by dot-blot and some.

Dot-blot Screening

The 44 1-D fractions as well as 80 2-D fractions (derived from each 1-D fraction) obtained were screened for reactivity against circulating anti-schistosome IgE, IgG4, Ig3 and IgG1 antibody isotypes in dot-blot assays in search of novel reactive proteins. The dot-blot was conducted using Bio-Dot SF Micro filtration apparatus (Bio Rad Laboratories, Inc., CA, USA) as previously described with modifications [27]. Briefly, 30 µl 1-D fraction was loaded onto polyvinylidene fluoride (PVDF) membrane (Amersham Hybond-P PVDF Membrane, GE Healthcare Bio-Sciences K. K. Tokyo, Japan) imbedded in transfer buffer (192 mM Glycine, 25 mM Tris, pH 7.4) [31] and fixed into the Bio-Dot SF Micro filtration apparatus. Following blocking, 30 µl of diluted pooled plasma (51 samples) (IgG1, 1:4000; IgG3, 1:4000; IgG4, 1:100; or IgE, 1:800) in TBS washing buffer (20 mM Tris, 137 mM NaC1, 0.01% Tween 20, pH 7.6) was applied to each respective well blotted with the fractions. Bound antibodies were incubated with respective conjugated enzymes using horseradish peroxidase-conjugated mouse anti-human IgG1, IgG3 (Southern Biotechnology Associates Inc.), IgG4 (MP Biomedicals, LLc) or biotin-conjugated goat anti-human IgE (Invitrogen) in dilutions of 1:16,000, 1:16,000 1:1,000 or 1:4,000 respectively. The IgE antibody bound membrane was further treated with 1:6,000 horseradish peroxidase-conjugated streptavidin (DakoCytomation). Blocking (5% skimmed milk/TBS washing buffer) and conjugate reaction of the membranes were conducted in a separate container. The reactivity was revealed by ImmunoStar Reagents (WAKO Pure Chemicals Industries, Ltd. Osaka, Japan) for chemiluminescence detection following the manufacturer's protocol. Digital images were obtained by the Las-4000EPUV Mini with an interface Las-4000 Image Reader (Fujifilm Corporation. Tokyo, Japan). The acquired antigenic spots were further transformed into pixels units for quantification of the recognition intensity using ATTO Lane and Spot Analyzer 6.0 software (ATTO Corporation. Tokyo, Japan). In screening of 2-D fractions by dot-blot assay, similar steps were followed. Briefly, 50 µl of each fraction in transfer buffer after drying to remove most of the ACN and TFA to prevent interference of membrane blotting was loaded onto the PVDF membrane. Test plasma were diluted for IgG1, 1:25,000; IgG3, 1:15,000; IgG4, 1:400; and IgE, 1:1,000 and applied to each respect 80 wells blotted with the 2-D fractions. Bound antibody was again incubated with conjugated enzymes using horseradish peroxidase-conjugated mouse anti-human IgG1, IgG3, IgG4 and biotin-conjugated goat anti-human IgE accordingly, in dilutions of 1:30,000, 1:30,000 1:3,000 or 1:6,000 respectively. Two crude SWA and BSA (Sigma A4503, Sigma-Aldrich Co. MO, USA) spots were included in the entire test for positive and background control respectively. The IgG1 and IgG3 were included to aid in selection of IgG4 and IgE preferred fractions.

For the analysis of all the antigenic spots intensity, the background intensity was subtracted and then the value obtained divided by that of the positive control to generate a relative reactivity index of '1' for positive control, '0' for negative control. Using these criteria each antigenic spot was designated 'not-reactive', 'weakly reactive', 'moderately reactive' or 'strongly reactive' with respect to isotype recognition using the pixel analysis and manual verification. These were calculated for each isotype separately so that the spot intensity was standardized within the membrane. The intensity scoring rather than absolute values could be compared among the fractions. This approached was applied since it was not expected for any fraction to have equal intensity by the four isotypes for determining reactivity intensity across the antibody isotypes.

In-solution Tryptic Digestion

Fractions from basic, neutral and acidic regions were treated with trypsin prior to protein sequencing. Briefly, liquid fractions (50 to 150 µl) were precipitated at –80°C overnight in about 10-bed volume of pre-chilled acetone (WAKO) followed by centrifugation at 20,000 *g* for 30 min at 4°C. After removing most of the supernatant, the samples were speed-vacuumed to eliminate the remaining ACN and TFA together with the acetone. Then 15 µL of denaturation solution (8 M urea; 500 mM Tris-HCl, pH 8.5; 2.5 mM EDTA) was added and incubated for 10 min at 100°C followed by cooling at RT. Addition of 5 µl of reduction solution (40 mM DTT), (WAKO) in 25 mM NH_4HCO_3 (WAKO), incubated 1 hr at 56°C with shaking followed. Alkylation reaction performed with 5 µL of 250 mM iodoacetamide (Tokyo Chemical Industry Co, Ltd, Tokyo, Japan) in 25 mM NH_4HCO_3 and incubated in the dark for 45 min at 25°C with shaking. A volume of 180 µL 50 mM NH_4HCO_3 was added to dilute out the urea and to terminate all the reactions prior to trypsin proteolytic digestion. Trypsin proteolysis was conducted using 50 µl (10 µg/ml) sequence grade modified trypsin (Promega Corporation, Madison, WI, USA) in 50 mM NH_4HCO_3 overnight at 37°C followed by addition of 5 µl 5% LC-MS grade Formic Acid (WAKO) in LC-MS grade ultra pure water (WAKO) to terminate the reaction. The trypsinized peptides were again speed-vacuumed and resuspended in 25 µl of 0.3% formic acid before filtered in Spin-X centrifuge tubes (0.22 µm Nylon, Costar Corning Inc., Corning, NY, USA) which was span at 2,300 *g* for 30 sec. The filtrate was transferred into MS vials (0.3 ml, TPX Snap Vial, GL Sciences Inc, Tokyo, Japan) for loading onto the ESI-MS/MS system.