

NATURAL HUMAN IgG SUBCLASS ANTIBODIES TO *PLASMODIUM FALCIPARUM* BLOOD STAGE ANTIGENS AND THEIR RELATION TO MALARIA RESISTANCE IN AN ENDEMIC AREA OF THAILAND

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Abstract. The immunoglobulin G (IgG) subclass antibodies to *Plasmodium falciparum* blood stage antigens in the sera of 181 individuals living in malaria endemic area in Kanchanaburi Province, western Thailand, were determined by enzyme-linked immunosorbent assay (ELISA). In this study, IgG3 and IgG1 were shown to be the predominant subclasses. Generally, IgG2 were coexpressed with IgG1 and IgG3 while IgG4 was found to coexpress with other three IgG subclasses. The levels of specific IgG1, IgG2, and IgG3 increased significantly with age ($r = 0.295$, $p = 0.000$; $r = 0.416$, $p = 0.000$; $r = 0.320$, $p = 0.000$, respectively). The data indicate that the higher antibody production required continuous stimulation under natural condition. Furthermore, the levels of specific IgG1, IgG2 and IgG3 increased in immune individuals without clinical malaria, reported in adolescents and adults, were associated with malaria resistance. Similar results were found in children with different patterns of IgG subclasses in which the specific IgG2 and IgG3, but not IgG1 was related to resistance.

INTRODUCTION

In both humans and mice, passive transfer of anti-*Plasmodium falciparum* antibodies from naturally immune individuals dramatically reduces parasitemia (White *et al*, 1991; Edozein *et al*, 1962). The IgG isotypes have been implicated as important components of such acquired immunity. In *in vitro* study, human antibodies efficiently inhibit *P. falciparum* merozoite proliferation and mediate opsonization of infected erythrocyte (Groux and Gysin, 1990). It has been reported that in sera of adults and children living in malaria endemic area in Africa and Thailand the predominance

of IgG1 and IgG3 in individuals to crude schizont extract were associated with protection against *P. falciparum*, whereas predominance of antibodies in other IgG subclasses and of IgM antibodies or overall low antibody levels were associated with disease susceptibility (Bouharoun-Tayoun *et al*, 1992). A recent study in Solomon Islanders also showed that the antibodies to crude schizont extract were distributed among four subclasses with IgG1 and IgG3 predominating (Rzepczyk *et al*, 1997). However, antibodies to MSA2 in Gambian sera were predominantly of IgG3 subclass (Taylor *et al*, 1995) which might contribute to the development of clinical protective immunity to malaria (Taylor *et al*, 1998). Similarly in a study in Senegal, IgG3 antibody to *P. falciparum* blood-stage extract was reported in association with lower risk of malaria attack, and recovery from severe malaria (Aribot *et al*, 1996; Sarthou *et al*, 1997). In contrast to the study in Madagascar,

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the levels of total IgG and IgG subclass to *P. falciparum* extract were higher in non-protected subjects than in protected ones (Dubois *et al*, 1993). However, in infants, the level of IgG1 to MSP1 was shown to be negatively correlated with protection (Branch *et al*, 2000), while IgG2 was related to a decrease in the risk of *P. falciparum* infection (Deloron *et al*, 1997). So far, the mechanisms that are intimately involved in the secretion of an IgG subclass which confer protection are not yet understood. It may depend upon many factors including the parasite strain, the parasite antigens used in the analysis, and the host genetic background.

The aim of the present study was to investigate in peoples living in a malaria endemic area in western Thailand whether they develop IgG antibodies directed against blood stage *P. falciparum* extract. The distribution of IgG subclass patterns was determined and the influence of age as well as malaria attack on the level of each IgG subclass were also evaluated.

MATERIALS AND METHODS

Study area and population

The Bongty subdistrict, Saiyok district, Kanchanaburi Province is situated in a rural area of Thailand 150 km west of Bangkok along the Thai-Myanmar border where malaria is endemic. The area of study covered four villages with a total population of 689: 406 children (58.9%) and 283 adults (41.1%). The climate in this region is warm and humid with the temperature ranging from 28°C to 35°C. The rainy season goes from May to October. The villages are located on the bank of a permanent fresh water river. In 1999, approximately 9% and 3% of the population were infected with *P. falciparum* and *P. vivax*, respectively.

Sample collection

Sample collection was carried out in June 1999. Blood samples were obtained from 408

individuals with complete data, aged 3 to 107 years (mean 20.8), living permanently in the villages during the survey. Most of these subjects were children aged 3 - 15 years (62.7%), together with adults aged 16 to 107 years (37.3%). Informed consent was obtained individually from all participants or their parents. A questionnaire was completed with information from each participant regarding name, age, sex, febrile status, and clinical malaria experiences. Approximately 2 ml of blood was obtained by venipuncture in heparinized tubes, which were preserved in a container with ice until arrival the Hospital for Tropical Disease, Faculty of Tropical Medicine, Mahidol University, Bangkok, where sera were collected and kept at -20°C until use. Control sera samples were obtained from 30 individuals residing in Bangkok where malaria is non-endemic. They had no history of malaria exposure, denied traveling to any endemic area in the past two years and hence would be most unlikely to have been exposed to malaria during the time of study.

Plasmodium falciparum antigens

The parasites (K1 strain, kindly provided by Dr Praon Wilairatana) were cultured *in vitro* according to the method of Trager and Jensen (1976), and harvested when parasite density had reached 5-10% hematocrit. After two washes in PBS pH 7.2, erythrocytes were suspended in PBS at 10% hematocrit. Fractions were placed into conical tubes on top of 2.5 ml of 60% Percoll (Pharmacia, Sweden) and centrifuged for 15 minutes at 1,500g giving a distinct band, containing 50-100% parasitized erythrocytes of late trophozoites and schizont as well as free parasites. After washing twice with PBS, the parasites were pooled, sonicated and stored at -20°C until use.

Determination of anti - *P. falciparum* IgG and IgG subclasses

The anti-*P. falciparum* IgG and their IgG subclasses in sera were determined by ELISA modified from that described elsewhere (Wahlgen *et al*, 1986). Briefly, the well of

microtiter plates (Costar, Cambridge, MA, USA) were coated overnight at 37°C with 50 µl of 10 µg/ml antigen suspended in 0.1 M carbonate buffer pH 9.6. The unbound site was blocked for 1 hour at room temperature with blocking buffer containing 0.5% BSA in PBS and 0.05% Tween 20 (PBS-T). The plates were washed twice with PBS-T, then 50 µl of test sera diluted 1:40 with PBS-T were added. After incubation overnight at 4°C, the plates were washed and incubated with rabbit anti-human IgG antibody conjugated with alkaline phosphatase (Dakopatt, Denmark) at room temperature for 1 hour. The color reaction was developed using p - nitrophenylphosphate substrate. The optical density (OD) was measured at a wavelength of 410 nm.

For determination of anti-*P. falciparum* IgG subclasses, the appropriate dilution of tested sera at 1:40 was used for IgG1, IgG2, IgG3 and 1:4 for IgG4. After incubation at 4°C overnight, mouse anti-human IgG1, IgG2, IgG3 and IgG4 monoclonal antibodies (Zymed, USA) were added. The plates were incubated at room temperature for 3 hours, washed, then reacted with goat anti-mouse IgG conjugated with alkaline phosphatase (Dakopatt, Denmark) at room temperature for the another 3 hours. After washing, the p-nitrophenylphosphate substrate solution was added to develop the color reaction. The optical density (OD) was measured at a wavelength of 410 nm.

Statistical analysis

The total IgG and the level of IgG subclass greater than the mean optical density of control sera plus one standard deviation (OD+1SD) were considered as positive. The IgG subclasses antibodies were presented as mean optical density ± one standard error (OD ± SE). The correlation between the antibody levels and their age were determined by Spearman's rank correlation test.

RESULTS

The distribution of *P. falciparum* specific IgG subclasses was done in 181 out of 408

sera positive for total *P. falciparum* specific IgG (44.4%). Among these, individuals who have developed malaria attack at least one were 76.8% (139 of 181), whereas those who have never developed a malaria attack were 23.2% (42 of 181).

P. falciparum IgG subclasses and their pattern expression

All 181 sera that contained anti-*P. falciparum* IgG were reactive to at least one subclass. The prevalent rates of anti-*P. falciparum* IgG subclasses varied from 67.4% for IgG1, 48.1% for IgG2, 58.6% for IgG3 and 51.9% for IgG4. Although the numbers of IgG4 containing sera were relatively high, however, the levels of such antibody were very low when compared to the other three subclasses. Among these, only 36.6% was positive to all four subclasses. The anti-*P. falciparum* specific IgG1 and IgG3 were the

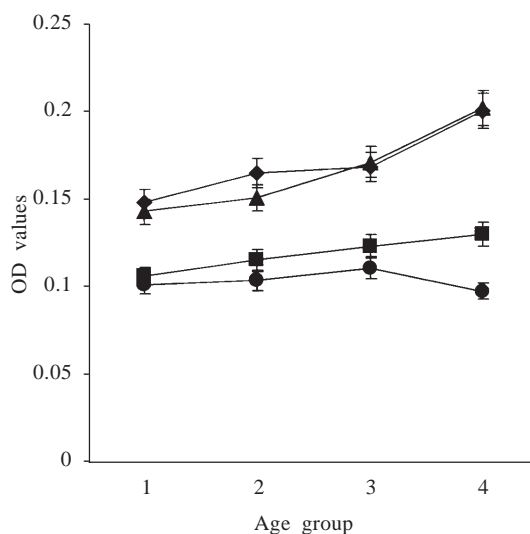


Fig 1—Mean optical density (OD) values of anti-*P. falciparum* IgG antibodies to crude schizont extract in sera of individuals defined in different age groups. Age groups: 1, 6-10 years, n = 42; age group 2, 11-25 years, n = 57; age group 3, 26-40 years, n = 43; age group 4, > 40 years, n = 39. (◆) IgG1; (■) IgG2; (▲) IgG3; (●) IgG4. Bars show the mean OD ± standard errors.

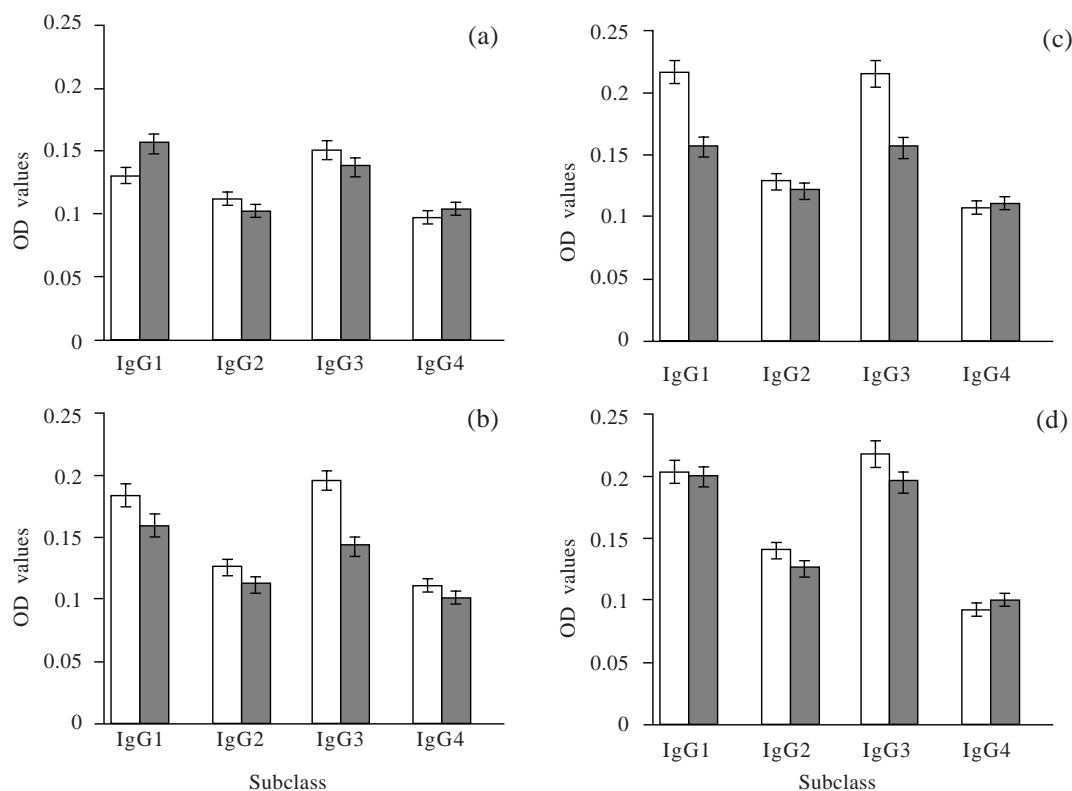


Fig 2—Mean optical density (OD) values of anti-*P. falciparum* IgG subclass antibodies to crude schizont extract in sera of individuals who have never developed a malaria attack (□ column) or who have developed malaria attack (■ column) categorized in different age groups. (a) age group 1, 6-10 years (individuals who have never developed a malaria attack, n = 14; individuals who have developed a malaria attack, n = 28) (b) age group 2, 11-25 years (individuals who have never developed a malaria attack, n = 8; individuals who have developed malaria attack, n = 49) (c) age group 3, 26-40 years (individuals who have never developed a malaria attack, n = 10; individuals who have developed a malaria attack, n = 33) (d) age group 4, > 40 years (individuals who have never developed a malaria attack, n = 10; individuals who have developed malaria attack, n = 29). Vertical lines represent standard errors of the mean.

most predominant subclasses since 51.4% of sera contained both IgG1 and IgG3 only or coexpressed with other subclasses. Of these, 14% of sera were found to contain only IgG1 coexpressed with IgG3. In addition, the IgG2 was generally coexpressed with IgG1 and IgG3 by 31.2%. Similar to IgG2, the IgG4 in 18.3% of sera was shown to coexpress with IgG1 and IgG3.

Age dependent anti *P. falciparum* IgG subclass expression

In general, the levels of *P. falciparum*

specific IgG1 and IgG3 subclasses were more elevated than IgG2 and IgG4. The levels of IgG subclasses were found to differ as age dependent. The levels of specific IgG1 and IgG3 antibodies tended to increase with age, whereas that of specific IgG2 antibodies only slightly increased. By statistical analysis, the OD values of the IgG1, IgG2 and IgG3 subclasses in sera were significantly correlated with age ($r = 0.295$, $p = 0.000$; $r = 0.416$, $p = 0.000$; $r = 0.320$, $p = 0.000$, respectively). In contrast, no significant correlation between IgG4 OD value and age was found

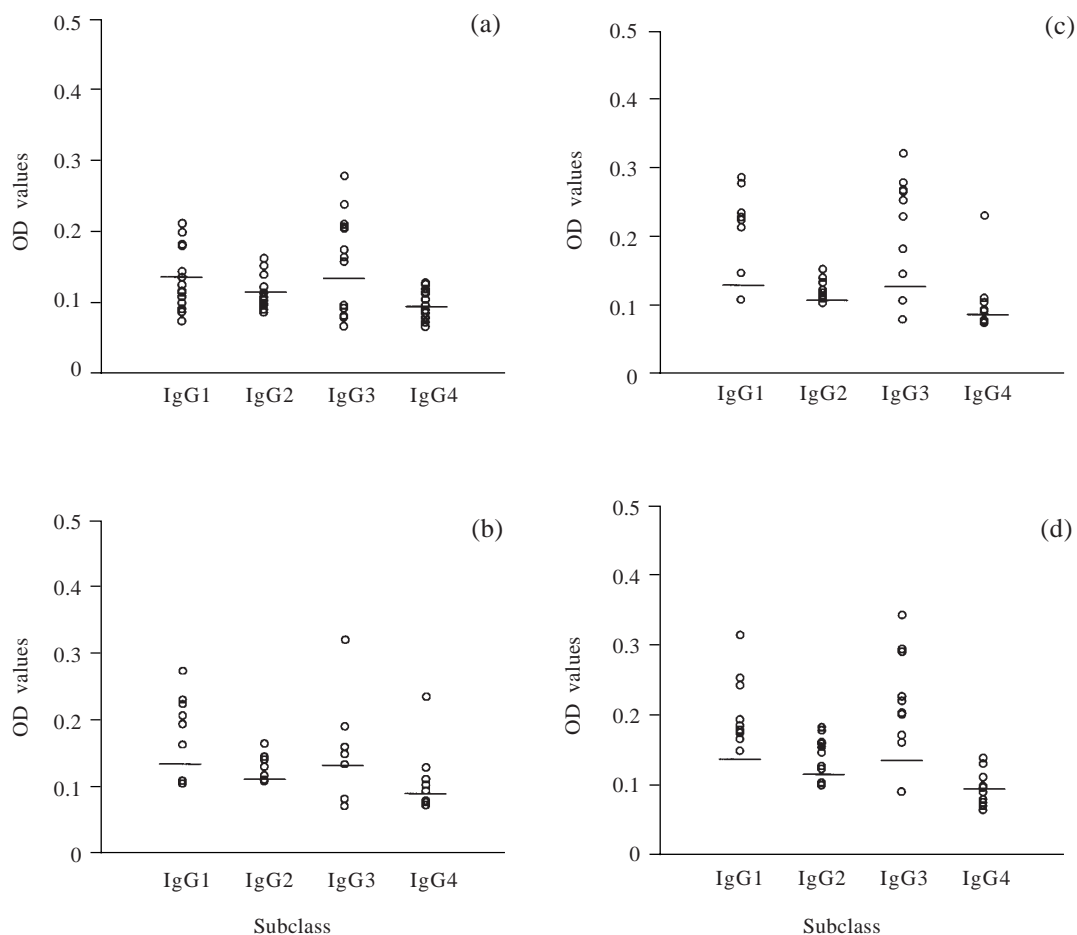


Fig 3—Dot plots of anti-*P. falciparum* IgG subclass antibodies to crude schizont extract in serum of each individual who has never developed a malaria attack. (a) age group 1, 6-10 years (n = 14); (b) age group 2, 11-25 years (n = 8); (c) age group 3, 26-40 years (n = 10); (d) age group 4, > 40 years (n = 10). Horizontal lines represent the mean + 1 standard deviations of 30 healthy control sera.

($r = 0.027$, $p = 0.723$) (Fig 1).

Relationship of age group, anti *P. falciparum* and malaria attack

The relationship between levels of anti-*P. falciparum* specific IgG subclasses with malaria attack was evaluated. Based on the clinical reports, the specific IgG subclasses antibodies in individuals who have developed malaria attack were compared with those who have never developed a malaria attack. Four different age groups were defined: 6-10 years, 11-25 years, 26-40 years, and >40 years of

age. The mean level of each anti-*P. falciparum* specific IgG subclass in individuals who have never developed a malaria attack were higher than those who have developed malaria attacks in almost all age groups, except in children of 6 -10 years with malaria attack, whose sera contained higher IgG1 than those without a malaria attack (Fig 2). No different IgG4 mean levels were found among these two defined groups of individual at all ages. The levels of anti-*P. falciparum* IgG1, IgG2, IgG3 and IgG4 in each individual who had never developed malaria attack are shown in Fig 3.

DISCUSSION

In the present study, we evaluated the IgG response as well as IgG subclass distribution and patterns of *P. falciparum* specific antibodies against crude schizont extract among individuals living in malaria endemic area, western Thailand. The effects of age and malaria attack on IgG responses and their subclasses were investigated. The measurement of total IgG response alone may be inadequate to assess immunity, the ability to mount an appropriate subclass response to this parasite may be crucial in protection from infection.

The results on the *P. falciparum* specific IgG subclass expression in individual sera showed that the majority of sera contained IgG1 and IgG3 antibodies. Generally, most sera containing IgG4 antibodies also contained antibodies of other three IgG subclasses. Sera containing IgG2 antibodies frequently contained antibodies of both IgG1 and IgG3. The subclass distribution of anti-*P. falciparum* specific antibodies in our study was essentially similar to those reported previously (Wahlgren *et al*, 1986; Dubois *et al*, 1993) which indicated that natural antigenic stimulations first trigger B cells to produce IgG1 or IgG3 antibodies. Subsequent stimulation will trigger B cells to produce IgG2 and IgG4, suggesting that isotype expression reflects sequential activation of specific B cells by repeated antigenic stimulations.

The anti-*P. falciparum* specific IgG subclass expression in malaria endemic individuals was age dependent by the finding of gradually increase of specific subclass antibody levels with age. The IgG subclasses detectable were IgG1, IgG2 and IgG3 which were shown to be significantly correlated with age ($r = 0.416$, $p = 0.000$; $r = 0.320$, $p = 0.000$; $r = 0.295$, $p = 0.000$, respectively), whereas no significant correlation between IgG4 level and age. Recently, it has been shown in Burkina Faso that IgG2 and IgG3 but hardly IgG1 and IgG4 directed against the conserved epitopes of RESA, MSP1 and MSP2 dramatically increase with age (Aucan *et al*, 2000). Similar finding

showed that IgM, IgG2, and IgG3 to crude extract of *P. falciparum* infected erythrocytes were found to increase as a function of age (Aribot *et al*, 1996). The different pattern was reported in the study in Western Kenya, which showed that IgG1 and IgG3 responses to MSP1 were equally frequent and the prevalence of both subclasses increased with age (Ya *et al*, 1996). However, in young age group, the levels of IgG1, IgG2, and IgG3 was lower which dramatically shown to increase till the age of more than 25 years. This is consistent with an accumulation of immune responses against poorly immunogenic, conserved determinants, an accumulation that might explain the development of age-dependent protection (Day *et al*, 1991).

In this study, the individuals who developed at least one malaria attack were considered non-protected, whereas individuals who had never developed a malaria attack were considered protected against malaria. Regarding the levels of anti-*P. falciparum* specific IgG subclasses and malaria attacks, the finding of the levels of specific IgG subclasses in individuals who had never developed a malaria attack were higher than those with malaria attacks in almost all age groups, which seems to indicate that these specific antibodies in such individuals protected against subsequent malaria infection. However, children 6-10 years of age with a malaria attack had higher IgG1 levels than those without a malaria attack. The data therefore suggest that the anti-*P. falciparum* IgG1, IgG2, and IgG3 antibodies in adults and adolescents, and anti-*P. falciparum* IgG2, IgG3, but not IgG1 antibodies in children were associated with resistance to malaria infection. Similar study in Gambian children showed that the presence of IgG3 antibodies to MSP2 serogroup A was negatively associated with the risk of clinical malaria whereas IgG1 antibodies to MSP2 serogroup B were associated with an increased risk of clinical infection (Taylor *et al*, 1998). A recent study in Burkina Faso also demonstrated that IgG2 and IgG3 increased with age and parallel with the protection against infection and disease, but specificity of the anti-

bodies was not investigated (Aucan *et al*, 2000). Conversely, a study in Senegal showed a strong correlation between protection against malaria attacks and levels of IgG2 and IgG3 against glutamate-rich protein 94-489 (Oeuvray *et al*, 2000). These antibodies may not act alone as shown by *in vitro* study, but they seem to control parasitemia in cooperation with monocyte (Bouharoun - Tayoun *et al*, 1990). These IgG1, IgG3 are cytophilic which may essential for the control of circulating parasites *in vivo* in cooperation with cells bearing Fc receptors, like monocytes. On the other hand, purified IgG2 from a myeloma cell line could trigger the production of tumor necrosis factor alpha from human blood monocyte (Foreback *et al*, 1997), one of the soluble factor which was shown to mediate parasite killing (Bauharoun-Tayoun *et al*, 1995). Alternatively, there may be more mechanisms that could participate in the control of parasite multiplication.

In conclusion, our data showed that people who live in an malaria endemic area in western Thailand did develop anti-*P. falciparum* blood stage antibodies with the major distribution of IgG subclasses of IgG1 and IgG3. The results seem to indicate that the anti-*P. falciparum* IgG1, IgG2, and IgG3 antibodies in adults and adolescents, as well as anti-*P. falciparum* IgG2, IgG3, but not IgG1 antibodies in children were associated with resistance to malaria infection. Our findings may contribute to a better understanding of the role of IgG subclasses in protective mechanisms and may provide new tools in the development of malaria control strategies, especially vaccine design.

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