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The use of impregnated curtains does not affect antibody responses against *Plasmodium falciparum* and complexity of infecting parasite populations in children from Burkina Faso

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Abstract

In Burkina Faso, where malaria is hyper-endemic and transmission intensity is very high, the majority of malaria-related morbidity and mortality occurs in children less than 5 years of age. A control measure such as the use of insecticide-treated curtains (ITC) significantly reduces transmission of malaria infection. Concerns remain whether reduced transmission intensity may lead to a delay in the development of immunity in younger children and even to a partial loss of already acquired immunity. In this study, the levels of *P. falciparum*-specific IgG subclasses, the number of infecting parasite clones determined by PCR-based genotyping of the *msp2* gene and the parasite density were analysed in 154 asymptomatic children (3–6 years) living in 16 villages (8 with and 8 without ITC) in the vicinity of Ouagadougou, the capital of Burkina Faso. In addition, the parasite inhibitory effects of Ig fractions, prepared from selected children, in co-operation with normal human monocytes were studied. Blood samples from asymptomatic ITC-users showed a significant decrease in *P. falciparum* prevalence as well as in parasite density. However, no significant difference was observed in *P. falciparum*-specific antibodies or in parasite multiplicity of infection between the two groups. Furthermore, Ig fractions from children of both groups showed similar levels of inhibitory activity against autologous parasite growth both on their own and in co-operation with monocytes.

Keywords: Wild isolate of *P. falciparum*; Insecticide-treated curtains; Immunity

1. Introduction

Malaria is a major public health problem in the world. According to recent data, there are 1.5–2.7 million deaths due to malaria each year, the bulk of which occur in sub-Saharan Africa, where about 360 million people live in areas of stable, endemic *Plasmodium falciparum* transmission (Snow et al., 1999). Malaria-related death rates are rising again in Africa (WHO, 2000), reflecting the emergence of drug resistant strains of the lethal *P. falciparum* parasite and highly efficient *Anopheles gambiae sensu lato* and

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Anopheles funestus vectors. This worsening situation emphasises the need for efficient and effective control measures (Snow and Marsh, 1998).

Protection of individuals from mosquitoes can be achieved through a number of methods including repellents, protective clothing, and using insecticide-treated bed net and curtains (ITNs). One of the encouraging developments in malaria control has been the finding that ITNs can significantly reduce mortality and morbidity (Alonso et al., 1991). Consequently, several field trials supported by UNDP/World Bank/WHO, have evaluated their effectiveness as a malaria prevention strategy. These studies in The Gambia (D'Alessandro et al., 1995), Kenya (Nevill et al., 1996), Ghana (Binka et al., 1996) and in Burkina Faso (Habluetzel et al., 1997) demonstrated a positive impact of ITNs on all-cause child mortality over the 2 years following implementation of the intervention and suggest that ITNs seem to work as well in areas of high transmission as in areas of lower endemicity. Studies in Burkina Faso (Habluetzel et al., 1999; Diallo et al., 1999) observed a modest reduction in the overall prevalence of malaria infection, but almost halving of parasite densities among children protected by insecticide-treated curtains (ITCs). The wide surface area of the protected zone and the almost total coverage achieved in the intervention villages appeared to be major determinants of the observed reduction of transmission. The direct effect of ITNs on malaria transmission was demonstrated in Tanzania, with a reduction of the malaria vector populations and the sporozoite rates, leading to a 90% reduction in the entomological inoculation rate (Curtis et al., 1998).

Concerns have been raised that the lower degree of exposure to malaria parasite in individuals using ITNs could delay their acquisition of immunity (Snow and Marsh, 1995). Indeed, lower levels of antibodies to the *P. falciparum* circumsporozoite protein (CSP) as well as various blood stage antigens were seen in children in areas using ITNs as compared with these in unprotected areas (Snow et al., 1996; Askjaer et al., 2001; Nebie et al., 2003). However, in some recent studies, no difference was seen in antibody levels to certain asexual blood stage antigens between ITNs users and non-users (Meraldi et al., 2002; Kariuki et al., 2003a). Thus, there is further need to monitor the long-term effects of ITNs usage on the acquisition of immunity against malaria.

We have in this study further investigated the impact of long-term use of insecticide-treated curtains on children's immune responses for malaria in Burkina Faso, where ITC have contributed to a reduction in intensity of malaria infection. The antibody response to asexual blood stages of *P. falciparum* was analysed with regard to IgG subclasses and capacity to inhibit parasite growth in vitro. Furthermore, the multiplicity of genotypes in the infecting parasite was analysed.

2. Materials and methods

The study received ethical approval from the Ministry of Health of Burkina Faso and the Research Ethical Committee of the Karolinska Institutet (Stockholm, Sweden). Informed consent was obtained from the parents or legal guardians of all children involved in the study after the aims and the protocols were clearly explained in their native language.

2.1. Study area

The study area is situated in the vicinity of Ouagadougou, the capital of Burkina Faso, a typical zone of Sudanese savannah. The rainy season lasts from June to October. Malaria transmission in the region is high, seasonal and peaking around September, approximately 300–500 infective bites/person/year with extremes ranging between 100 and 1000 infective bites/person/year in some villages due to ecological heterogeneity (Cuzin-Ouattara et al., 1999). The main malaria vector is *A. gambiae* with *A. arabiensis* and *A. funestus* contributing to a lesser extent. In villages with ITC, netting was installed at all housing openings (doors, windows, space between wall and roof) during 1994. Thus, the children from these villages had lived all their life in houses with ITC. The introduction of ITC resulted in a drastic reduction in entomological inoculation rate (from 500 to 29 bites/person/year). The majority of the study population belongs to the Mossi ethnic group and lives by livelihood farming.

2.2. P. falciparum isolates

Blood samples were collected in 2001 during the high transmission season (August–October) from 154 randomly selected asymptomatic children (3–6 years)

living in 16 villages with and without ITC. Body temperature was measured twice with an axillary thermometer. A third measuring was undertaken when the difference between the two readings is more than 0.5 °C. The febrile children were treated with chloroquine as recommended by the national policy. Only afebrile children who were not receiving anti-malarial treatment were included in the study.

Five millilitres of venous blood from each child were added to tubes containing EDTA. Plasma was collected after centrifugation at 1200 rpm (350 g) for 10 min. The leukocyte interface was removed after washing the erythrocytes twice in RPMI 1640 medium (Gibco, Paisley, UK). To prepare isolates for in vitro inhibition experiments, each isolate was cultured for 18–22 h in RPMI 1640 medium plus 10% AB human-non immune serum (Wåhlin et al., 1984).

Thick and thin blood films were air dried, thin films were fixed with methanol, and both stained with Giemsa. One-hundred high-power fields were examined on each slide by microscopy, and parasite density was estimated assuming mean leucocyte count of 8000 cells per microlitre of blood.

2.3. Immunoglobulin fractions

The plasma samples were inactivated by incubation at 56 °C for 1 h. The immunoglobulin fractions were prepared from 2 ml plasma by ammonium sulphate precipitation essentially as described by Harboe and Ingild (1973). The immunoglobulin fraction was brought into RPMI by buffer exchange in a PD10 column (Pharmacia, Uppsala, Sweden). The Ig fractions were kept at −30 °C until use.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Determination of the concentration of IgG class and subclasses of anti-malaria antibodies was performed using an extract of mature stages of cultured *P. falciparum* as antigen (Troye-Blomberg et al., 1983). Briefly, plates (Costar, Corning, NY) were coated with 10 μg/ml antigens in sodium carbonate buffer (pH 9.6) with 0.02% (w/v) sodium azide and were then saturated with 1% bovine serum albumin in sodium carbonate buffer. Plasma dilutions were incubated overnight at 4 °C (1:20 for IgG2 and IgG4, 1:100 for IgG1 and 1:400 for IgG3 and 1:1000 for IgG). Total IgG antibodies were detected using alkaline phosphatase conjugated goat anti-human IgG (Fc fragment specific) (Mabtech AB, Nacka, Sweden). Antibodies of IgG1, IgG2, IgG3 and IgG4 subclasses were detected using biotin conjugated mouse anti-human monoclonal antibody (PharMingen, San Diego, CA) and alkaline phosphatase conjugated streptavidine (Mabtech AB) for each subclass. The assay was developed with *p*-nitrophenyl phosphate disodium salt (Sigma, St. Louis, MO) as substrate and the optical densities were read at 405 nm in ELISA plate reader (Vmax^{TM} Kinetic Microplate Reader, Menlo Park, CA).

The concentrations of IgG-subclass of anti-malarial antibodies were calculated from standard curves obtained in a sandwich ELISA with six dilutions of myeloma protein of IgG1-4 isotypes (Serotec, Oxford, UK) or with highly purified IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for total anti-malarial antibodies.

2.5. In vitro invasion inhibition assay

All in vitro invasion inhibition experiments were performed at Laboratoire d'Immuno-Parasitologie, Center National de Recherche et de Formation sur le Paludisme (Ouagadougou, Burkina Faso). Briefly, the ABO blood group of each donor was determined by hemagglutination using Serafol-D bedside cards (Biotest AG, Dreieich, Germany). All tests were performed with compatible blood groups. The invasion inhibition assay was performed as described earlier (Wåhlin et al., 1984). Briefly, the immunoglobulin fraction was added in serial dilutions to autologous cultures of the *P. falciparum* isolates that had developed to schizonts after the initial 18–22 h of incubation. All inhibition experiments were performed in the presence of Swedish human AB serum from a pretested batch. The cultures were set up in duplicate in flat-bottomed, cell culture, 96-well plates (Costar, Corning, NY) and were incubated at 37 °C for 18–22 h in a candle jar.

Monolayers of each culture were prepared in quadruplicate on 8-well multitest slides (ICN Bio-medicals, Inc., Aurora, OH) and fixed in 1% glutaraldehyde, followed by air-drying. Parasites were stained with acridine orange and the percentage of newly infected erythrocytes was determined by

counting 25 microscopic fields per well in a fluorescence microscope. The invasion inhibition was determined by the formula: (% parasitaemia in control wells − % parasitaemia in test wells) × 100/(% parasitaemia in control wells).

2.6. Isolation and preparation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood was diluted with an equal volume of Tris Buffered Hanks solution (SBL, Vaccin AB, Stockholm, Sweden). Blood mononuclear cells (PBMCs) were separated on Ficoll-Paque™ PLUS (Amersham Bioscience AB, Uppsala, Sweden) according to the technique described essentially by Böyum (1976). Briefly, 6 ml diluted blood were layered on 3 ml Ficoll-Paque™ PLUS (at room temperature) and centrifuged at 2800 rpm (1100 × *g*) for 20 min at 20 °C without brake. The upper layer (autologous serum 50%) was removed and kept until use. Interface cells (mononuclear cells) were carefully transferred to a new 10 ml conical centrifuge tubes and washed twice (10 min each) in Tris Hanks solution supplemented with 25–50% autologous serum.

PBMCs were then resuspended in 50% autologous serum (2×10^6 cells/ml) and seeded at 10×10^6 cells into OPTILUX™ petri dish (Becton Dickinson Labware, Becton, Franklin Lakes, NJ, USA). Monocytes were allowed to adhere at 37 °C in 5% CO_2 atmosphere for 1 h. The supernatant and non-adherent cells were washed off and petri dishes were washed three times (10 ml each) with Tris Hanks supplemented with 0.5% human serum albumin (Pharmacia, Stockholm, Sweden) at room temperature to remove the non-adherent cells. A cell lifter removed the adherent cells and their viability was detected by Trypan blue exclusion dye (Sigma). The method permitted recovery of 97% of adherent cells with an average viability of 97%.

2.7. Antibody dependent cell mediated invasion inhibition assay

The effector cell preparations (human monocytes) and Igs were assayed for their capacity to inhibit *P. falciparum* growth in vitro as described essentially by Wåhlin et al. (1984). Prior to the antibody dependent cell mediated inhibition (ADCI) assay we assessed the capacity of the Ig alone to inhibit parasite invasion/growth. In order to detect the ADCI activity, the assay was performed with an antibody concentration giving no or a low inhibition (sub-optimal inhibitory concentration).

Briefly, 100 μl of infected red blood cells at 1% parasitaemia at 4% hematocrit consisting mainly of late stages (trophozoites and schizonts) were distributed in flat bottomed, cell culture 96-well plates (Costar). Adherent cell suspensions in tissue culture medium supplemented with 10% human AB serum were added to the wells at the rate of 1×10^5 to 2×10^5 monocytes. Control wells consisted of (a) parasite culture alone, (b) parasite culture and negative control Ig (Swedish non-immune donor), (c) parasite culture and monocytes. Test wells consisted of (a) parasite culture and test Ig at sub-optimal inhibitory concentrations and (b) parasite culture, test Ig at sub-optimal inhibitory concentrations and monocytes. Wells were carefully mixed and plates incubated at 37 °C in 5% CO_2 atmosphere for 22 h. Microscopic assessment of the degree of parasite growth was then performed in the same way as for the invasion inhibition assay above.

2.8. Parasite DNA preparation and PCR

For assessment of the genotypic composition of *P. falciparum* isolates, parasite DNA was extracted and purified from blood samples as described by Snounou et al. (1993). Briefly, the blood was lysed with saponin, and after centrifugation, the parasite pellet was resuspended in lysis buffer (40 mM Tris, pH 8.0, 80 mM EDTA, 2% SDS) and incubated with proteinase K. The samples were extracted with phenol, followed with phenol–chloroform and lastly chloroform. The DNA was then precipitated in sodium acetate and absolute ethanol and stored frozen for 2–4 h. After centrifugation, washing with 70% ethanol and drying, the DNA was resuspended in TE buffer (10 mM Tris, pH 8.0; 0.1 mM EDTA, pH 8.0).

The polymorphic regions of block 3 of *msp2* were amplified by nested PCR (Snounou et al., 1999). Two classes of *msp2* block 3, denoted Indochina (IC) and FC27, were examined. In the first reaction (PFG-Nest1), Block 3 of the *msp2* was amplified using the oligonucleotide primer pair M2-OF and M2-OR, followed by two separate second amplifications to detect the IC allelic family (M2-ICF and M2-ICR)

or the FC27 allelic family (M2-FCF and M2-FCR), respectively. PCR products were electrophoresed on 1.8% agarose gels, and DNA visualised by ultraviolet trans-illumination after ethidium bromide staining. Bands obtained were compared by size.

2.9. Statistical analysis

Statistical evaluation was done using the unpaired, two-tailed, Student's *t*-test and $P < 0.05$ were considered significant. Parasite densities, number of infecting genotypes and antibody concentrations were log-transformed before the analysis and converted to normal after calculation. We estimated the impact of ITC-use on parasite densities and the multiplicity of infection by villages, age and sex using regression analysis of the StatView software (Cary, NC, USA).

3. Results

This study was designed to investigate the immunological status in children protected by insecticide-treated curtains as compared to that in children freely accessible to mosquito bites. Out of the 154 children aged 3–6 years who were randomly selected for inclusion in the study, 81 were living in villages with ITC whereas, the remaining 73 in villages without ITC.

3.1. Impact of ITC on parasite prevalence and multiplicity

Thick blood film readings were performed on blood samples collected from all children of the two groups. The use of ITC reduced the prevalence of microscopically determined *P. falciparum* markedly, 65.4% of the children being parasite positive as compared to 82% of the children living without ITC. There was also a significant ($P = 0.01$) reduction of the parasite density in ITC-users as compared to non-users, the geometric mean of parasite density ± S.E., being 83 ± 7 and 624 ± 162, respectively (Fig. 1). The parasite density tends to increase with age in ITC-users and non-users (Table 1A).

Isolates from each child were genotyped by a nested PCR method using primers specific for two allelic families of the gene for the merozoite surface protein-2 (MSP-2). Between 1 and 7 *msp2* PCR prod-

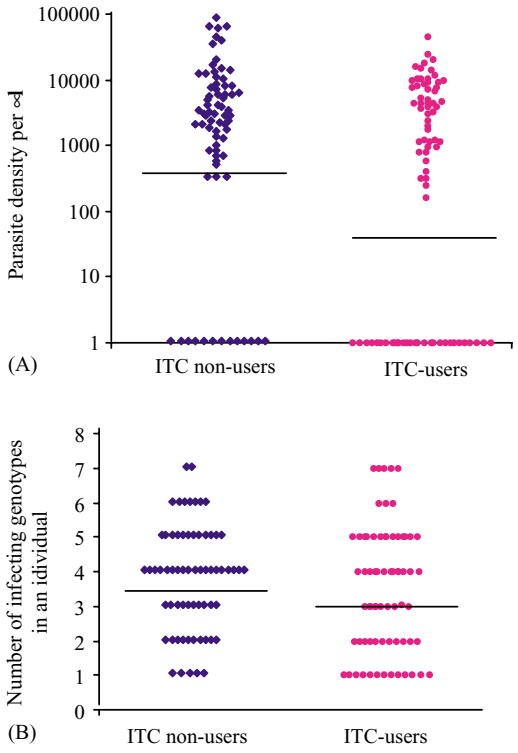

Fig. 1. (A) Parasite densities of blood samples from an asymptomatic children living in villages with and without ITC. The geometric mean parasite density is shown as a horizontal line. (B) Number of infecting genotypes of *msp2* in children living in villages with and without ITC. The geometric mean number of infecting genotypes is shown as a horizontal line.

Table 1
Parasite densities (A) and concentration of *P. falciparum*-specific IgG antibodies (B) in asymptomatic children of different age groups living in villages with and without ITC

Age (years)	No.	Non-user	No.	Users	*P*-value
(A) Geometric mean of parasite densities					
3	19	590 ± 179	14	18 ± 1	0.02
4	14	711 ± 326	22	75 ± 7	0.13
5	12	135 ± 45	18	116 ± 26	0.54
6	28	1116 ± 676	26	164 ± 45	0.64
(B) Geometric mean of antibody concentration					
3	17	50.5 ± 12.5	12	52.7 ± 10.5	0.86
4	12	50.5 ± 9.5	17	48.2 ± 8.1	0.96
5	10	48.6 ± 9.3	16	62.7 ± 8.1	0.36
6	20	59.9 ± 7.5	23	59.1 ± 6.5	0.92

Table 2
Regression analysis of parasite density (A) and multiplicity of infection (B) in relation to age in males and females living in villages with or without ITC

Gender	No.	Mean age (years)	Geometric mean of parasite density ± S.E.	Correlation coefficient	R^2	*P*-value
(A) Parasite density						
Male						
ITC-users	36	4.7	27 ± 10	0.109	0.012	0.52
Non-users	44	4.7	465 ± 25	0.272	0.074	0.07
Female						
ITC-users	45	4.8	246 ± 22	0.060	0.004	0.69
Non-users	29	4.6	974 ± 224	0.006	0.00004	0.97
(B) Multiplicity of infection						
Male						
ITC-users	32	4.8	3.1 ± 1.1	0.032	0.001	0.86
Non-users	36	4.7	3.5 ± 1.0	0.170	0.029	0.20
Female						
ITC-users	36	4.8	2.9 ± 1.0	0.077	0.006	0.65
Non-users	22	4.6	4.2 ± 1.0	0.021	0.0004	0.92

ucts were detected in 70 of ITC-users and 65 in the ITC non-users (Fig. 1B). The geometric mean of *P. falciparum* multiplicity was 3.0 ± 1.1 and 3.5 ± 1.1 in ITC-users and non-users, respectively, and the difference was not statistically significant ($P = 0.14$).

The parasite density and multiplicity of infection were also analysed in relation to age, use of ITC, and child gender (Table 2). Neither parasite density nor multiplicity of infection showed any correlation with age among females or males in any of the groups. Although females tended to show higher parasite density than males among both ITC-users and non-users, the differences were not statistically significant ($P = 0.05$ and 0.46, respectively) (Table 2A). Similarly, no statistically significant differences were seen between males and females regarding the multiplicity of infection ($P = 0.10$ in ITC-users and 0.60 in non-users) (Table 2B). Furthermore, a weak positive correlation was found between parasite density and the number of infecting parasite genotypes in both ITC-users and non-users ($r^2 = 0.01$, $P = 0.48$ and $r^2 = 0.005$, $P = 0.58$, respectively).

3.2. Impact of ITC on levels of IgG antibodies to P. falciparum antigens

In order to analyse the influence of the use of ITC on anti-malarial humoral immune response, the levels of antibodies reactive with *P. falciparum* asexual blood stage antigens were determined with regard to IgG and IgG subclasses. All individuals among ITC-users and non-users showed IgG antibodies and there was no difference in the antibody levels between the two groups (geometric mean concentration ± S.E., 56.0 ± 3.9 and 53.2 ± 3.8 μg/ml, respectively, $P = 0.62$) (Fig. 2A). In neither of the groups age did not have any marked influence on the levels of IgG antibodies (Table 1B).

Antibodies of IgG1 and IgG3 were detected in most of the individuals of both groups (IgG1: 86.4% of ITC-users and 82% of non-users; IgG3: 86.4% of ITC-users and 78% of non-users), while IgG2 and IgG4 antibodies were detected in a few plasma samples (IgG2: 7 ITC-users and 2 non-users; IgG4: 3 ITC-users and 2 non-users). No significant differences in geometric mean concentrations of neither IgG1 antibodies nor IgG3 antibodies from ITC-users as compared with non-users were detected (IgG1: 11.5 ± 1.8 μg/ml in ITC-users and 7.8 ± 1.2 μg/ml in non-users, $P = 0.13$; IgG3: 0.45 ± 0.10 μg/ml in ITC-users and 0.30 ± 0.11 μg/ml in non-users, $P = 0.22$) (Fig. 2B and C).

3.3. Antibody dependent parasite growth inhibition in vitro

In order to study the possible impact of usage of ITC on the development of potential parasite neutralising activities, the capacity of Ig fractions from ITC-users

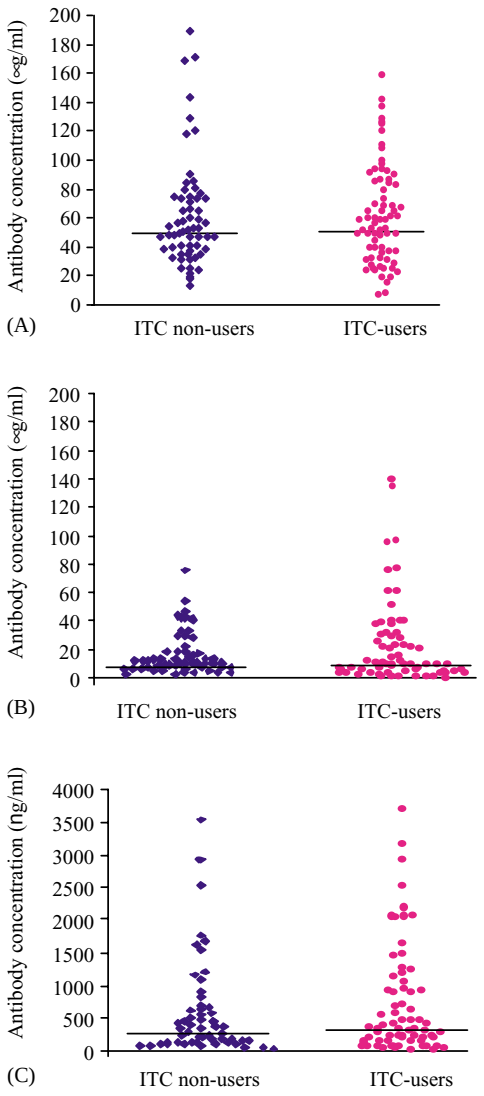

Fig. 2. Levels of parasite-specific IgG antibodies detected in plasma samples from ITC-users ($n = 66$) and non-users ($n = 59$) (A), which were further assessed for antibodies of IgG1 (B) and IgG3 (C) subclass using ELISA method. The geometric mean of antibody levels is shown as a horizontal line.

and ITC non-users to inhibit parasite growth in vitro was analysed. The parasite inhibitory effects mediated by antibodies alone or in co-operation with monocytes were analysed in randomly selected children from both groups.

The Ig fraction from plasma of children living in villages with ITC ($n = 41$) or without ITC ($n = 40$) were tested for their inhibitory effect on the autologous *P. falciparum* isolates. All children in both groups showed high parasite growth inhibitory activity and no significant difference was observed between ITC-users and ITC non-users (mean inhibition±S.E. = 68±20% and 71 ± 21%, respectively, $P = 0.63$).

Using sub-optimal inhibitory concentrations of the Ig and monocytes from parasite-free donors, the antibody dependent cell mediated inhibition on the autologous *P. falciparum* isolates was assayed for selected individuals among ITC-users ($n = 19$) and ITC non-users ($n = 16$). Although monocytes alone gave considerable growth inhibition of some parasite isolates in both groups (mean inhibition ± S.E. in ITC-users = 18 ± 4% and in ITC non-users 14 ± 3%), a marked synergistic inhibitory effect of the co-operation of Ig with monocytes was seen in a majority of cases (Fig. 3). There was no statistically significant difference in inhibitory activity between ITC-users and ITC non-users regarding Ig in combination with monocytes ($P = 0.58$) or monocytes alone ($P = 0.68$).

4. Discussion

The immediate benefits of the use of insecticide-treated bed nets (ITNs) and curtains (ITCs) in reducing morbidity and mortality in children have been well established (D'Alessandro et al., 1995; Habluetzel et al., 1997; Procacci et al., 1991; Pietra et al., 1991; Binka et al., 1996) with 3.8 to 6.9 lives saved per 1000 children protected per year (Lengeler et al., 1998). This emphasises the contribution of malaria to childhood mortality in Africa and the potential benefits from effective control programs. However, concern remains whether reduced transmission intensity may lead to a delay in the development of immunity in younger children and even to loss of already acquired immunity.

This study confirms and extends the results of some previous studies on the impact of the use of ITC on humoral immune responses in children living in a malaria high transmission area (Meraldi et al., 2002; Nebie et al., 2003; Kariuki et al., 2003a). Although children living with ITC protection showed a significantly lower parasite density as compared to children living unprotected, the levels of antibodies to *P. falciparum* asexual blood stage antigens did not differ

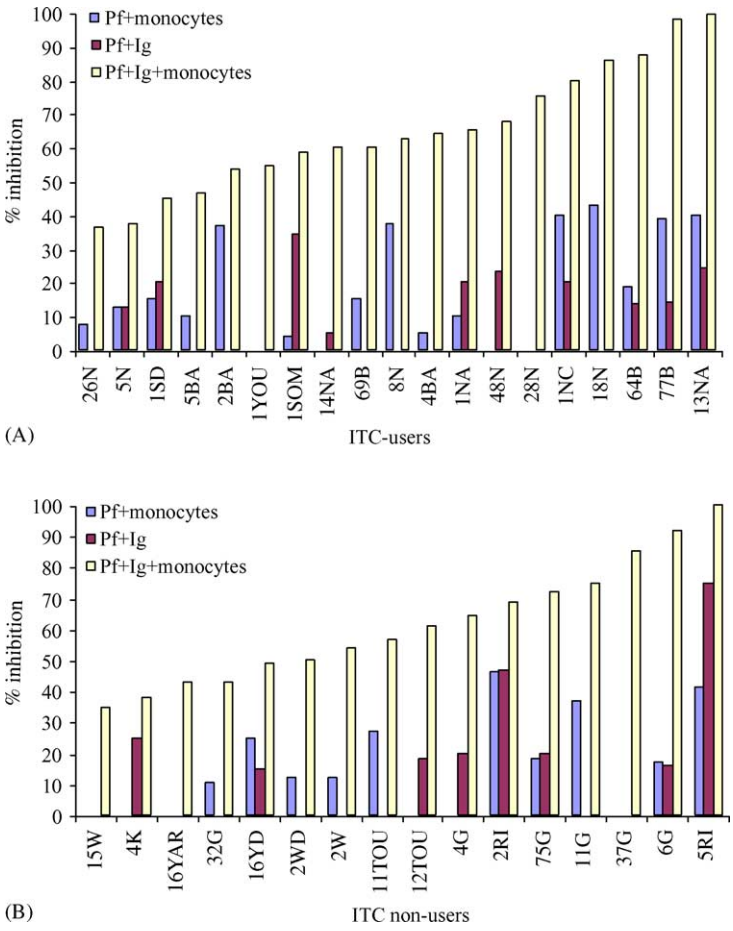

Fig. 3. The percent growth inhibitory activity of Igs from ITC-users (19) (A) and non-users (16) (B) on wild isolates of *P. falciparum* in vitro cultures in the absence or presence of monocytes. Mean parasitemia at time zero was (0.2%) and control well mean parasitemia at 22 h was (1.5%).

significantly between the two groups of children. Neither were there any significant differences between ITC-users and non-users in the levels of in vitro parasite neutralising antibodies, as assayed by the capacity of antibodies, alone or in co-operation with monocytes, to inhibit parasite growth in cultures of *P. falciparum* isolates. This suggests that the reduced exposure to malaria parasites in ITC-users had not compromised the development of potentially protective humoral immune responses.

Several studies on the effect of the use of ITN or ITC on the development of antibody responses to *P. falciparum* antigens have been performed showing considerable inconsistencies, probably depending on differences in endemic settings of the study areas, in the age range of the study populations and in the choice of antibody specificities assayed. The levels of antibodies to the circumsporozoite protein were reduced in children of ITC-users in Burkina Faso (Nebie et al., 2003), while these levels increased in pregnant women using ITN in Kenya (Kariuki et al., 2003b). In contrast, in the latter study, the ITN users showed decreased levels of antibodies to the liver stage antigen LSA-1 and the merozoite surface protein MSP-1 (Kariuki et al., 2003b). In studies among children in Kenya (Snow et al., 1996) and Tanzania (Askjaer et al., 2001), the use of ITN resulted in significantly lower seropositivity of schizont reactive IgM

antibodies or levels of antibodies to variant surface antigens, respectively. Reduced levels of antibodies to the asexual blood stage antigen PfExp-1 were seen in children of ITC-users in Burkina Faso (Nebie et al., 2003), while the levels of these antibodies in adults from the same area did not differ significantly between ITC-users and non-users (Meraldi et al., 2002).

In the present study, we used an extract of late stage *P. falciparum* infected erythrocytes for antibody detection, in an assay assumed to measure antibody reactivities with most of the asexual blood stage antigens. Although many of the target antigens for parasite neutralising antibodies are polymorphic or variant (Bolad and Berzins, 2000), the levels of antibodies detected in this assay have been demonstrated to correlated with protection against malaria (Ahlborg et al., 2002). Antibody dependent cell mediated killing of parasites with monocytes or other leukocytes as effector cells is thought to be a major mechanism for parasite neutralisation in vivo, where cytophilic antibodies of IgG1 and IgG3 subclasses are instrumental (Bouharoun-Tayoun et al., 1990; Groux and Gysin, 1990). In this study, the use of ITC had no significant effect on the levels of IgG1 or IgG3 antibodies to *P. falciparum* antigens, which is also reflected in the similar capacity of IgG from ITC-users and non-users to inhibit parasite growth in vitro in the ADCI assay.

There is some evidence that the presence of multiple concurrent infections with *P. falciparum* of different genotypes is associated with a reduced risk of clinical malaria (al-Yaman et al., 1997; Farnert et al., 1999; Beck et al., 1997), as this might confer cross-protection against newly inoculated parasites (Smith et al., 1999). A positive correlation between complexity of infection and transmission intensity was demonstrated in a study in Tanzania (Bendixen et al., 2001), and a limited genotypic diversity in *P. falciparum* is seen in areas of low transmission (Haddad et al., 1999). ITC-users in Burkina Faso have a reduced exposure to infective mosquito bites, as indicated by their reduced levels of sporozoite reactive antibodies (Nebie et al., 2003) and, in this study, by their lower parasite density as compared to ITC non-users. However, the genotypic multiplicity of the infecting parasites was not significantly different between the two groups of children, which corroborates the results obtained in a trial of insecticide-treated bed nets in Tanzania (Fraser-Hurt et al., 1999). These results indicate that multiple genotypes of the parasite already occur in the mosquito (Taylor, 1999) and thus, that a reduction in transmission has a limited effect on the multiplicity of the infection in the human.

In conclusion, the data reported here indicate that the development of potentially protective humoral immune responses is not compromised in children living in villages equipped with ITC for vector control in a area of high malaria transmission. Although the use of ITC decreases the number of infective mosquito bites, the children appear to receive enough parasite exposure to develop and maintain a state of premunition. However, the presence of conflicting results in this context in different studies, indicates the need for further investigations using larger study populations in areas of different malaria transmission.

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References

Ahlborg, N., Haddad, D., Siddique, A.B., Roussilhon, C., Rogier, C., Trape, J.F., Troye-Blomberg, M., Berzins, K., 2002. Antibody responses to the repetitive *Plasmodium falciparum* antigen Pf332 in humans naturally primed to the parasite. Clin. Exp. Immunol. 129, 318–325.

al-Yaman, F., Genton, B., Reeder, J.C., Anders, R.F., Smith, T., Alpers, M.P., 1997. Reduced risk of clinical malaria in children infected with multiple clones of *Plasmodium falciparum* in a highly endemic area: a prospective community study. Trans. R. Soc. Trop. Med. Hyg. 91, 602–605.

Alonso, P.L., Lindsay, S.W., Armstrong-Scellenberg, J.R.M., Konteh, M., Hill, A.G., David, P.H., Fegan, G., DeFrancisco,

A., Hall, A.J., Shenton, F.C., Cham, K., Greenwood, B.M., 1991. The effect of insecticide-treated bed nets on mortality of Gambian children. Lancet 337, 1499–1502.

Askjaer, N., Maxwell, C., Chambo, W., Staalsoe, T., Nielsen, M., Hviid, L., Curtis, C., Theander, T.G., 2001. Insecticide-treated bed nets reduce plasma antibody levels and limit the repertoire of antibodies to *Plasmodium falciparum* variant surface antigens. Clin. Diagn. Lab. Immunol. 8, 1289–1291.

Beck, H.P., Felger, I., Huber, W., Steiger, S., Smith, T., Weiss, N., Alonso, P., Tanner, M., 1997. Analysis of multiple *Plasmodium falciparum* infections in Tanzanian children during the phase III trial of the malaria vaccine SPf66. J. Infect. Dis. 175, 921–926.

Bendixen, M., Msangeni, H.A., Pedersen, B.V., Shayo, D., Bodker, R., 2001. Diversity of *Plasmodium falciparum* populations and complexity of infections in relation to transmission intensity and host age: a study from the Usambara Mountains, Tanzania. Trans. R. Soc. Trop. Med. Hyg. 95, 143–148.

Binka, F., Kubaje, A., Adjuik, M., Williams, L., Lengeler, C., Maude, G., Armah, G., Kajihara, B., Adiamah, J., Smith, P., 1996. Impact of permethrin impregnated bednets on child mortality in Kassena-Nankana district, Ghana: a randomised controlled trial. Trop. Med. Int. Health 1, 147–154.

Bolad, A., Berzins, K., 2000. Antigenic diversity of *Plasmodium falciparum* and antibody-mediated parasite neutralization. Scand. J. Immunol. 52, 233–239.

Böyum, A.P., 1976. Isolation of lymphocytes, granulocytes and macrophages. Scand. J. Immunol. 5, 9–15.

Bouharoun-Tayoun, H., Attanath, P., Sabchareon, A., Chongsuphajaisiddhi, T., Druilhe, P., 1990. Antibodies that protect humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes. J. Exp. Med. 172, 1633–1641.

Curtis, C.F., Maxwell, C.A., Finch, R.J., Njunwa, K.J., 1998. A comparison of use of a pyrethroid either for house spraying or for bednet treatment against malaria vectors. Trop. Med. Int. Health 3, 619–631.

Cuzin-Ouattara, N., Van den Broek, A.H., Habluetzel, A., Diabate, A., Sanogo-Ilboudo, E., Diallo, D.A., Cousens, S.N., Esposito, F., 1999. Wide-scale installation of insecticide-treated curtains confers high levels of protection against malaria transmission in a hyperendemic area of Burkina Faso. Trans. R. Soc. Trop. Med. Hyg. 93, 473–479.

D'Alessandro, U., Olaleye, B., McGuire, W., Langerock, P., Bennett, S., Aikins, M., Thomson, M., Cham, M., Cham, B., Greenwood, B., 1995. Mortality and morbidity from malaria in Gambian children after introduction of an impregnated bednet programme. Lancet 345, 479–483.

Diallo, D.A., Habluetzel, A., Cuzin-Ouattara, N., Nebie, I., Sanogo, E., Cousens, S.N., Esposito, F., 1999. Widespread distribution of insecticide-impregnated curtains reduces child mortality, prevalence and intensity of malaria infection, and malaria transmission in rural Burkina Faso. Parassitologia 41, 377–381.

Farnert, A., Rooth, I., Svensson, A., Snounou, G., Bjorkman, A., 1999. Complexity of *Plasmodium falciparum* infections is consistent over time and protects against clinical disease in Tanzanian children. J. Infect. Dis. 179, 989–995.

Fraser-Hurt, N., Felger, I., Edoh, D., Steiger, S., Mashaka, M., Masanja, H., Smith, T., Mbena, F., Beck, H.P., 1999. Effect of insecticide-treated bed nets on haemoglobin values, prevalence and multiplicity of infection with *Plasmodium falciparum* in a randomized controlled trial in Tanzania. Trans. R. Soc. Trop. Med. Hyg. 93, 47–51.

Groux, H., Gysin, J., 1990. Opsonization as an effector mechanism in human protection against asexual blood stages of *Plasmodium falciparum*: functional role of IgG subclasses. Res. Immunol. 141, 529–542.

Habluetzel, A., Diallo, D., Esposito, F., Lamizana, L., Pagnoni, F., Lengeler, C., Traore, C., Cousens, S., 1997. Do insecticide-impregnated curtains reduce all-cause child mortality in Burkina Faso? Trop. Med. Int. Health 2, 855–862.

Habluetzel, A., Cuzin, N., Diallo, D.A., Nebie, I., Belem, S., Cousens, S.N., Esposito, F., 1999. Insecticide-treated curtains reduce the prevalence and intensity of malaria infection in Burkina Faso. Trop. Med. Int. Health 4, 557–564.

Haddad, D., Snounou, G., Mattei, D., Enamorado, I.G., Figueroa, J., Stahl, S., Berzins, K., 1999. Limited genetic diversity of *Plasmodium falciparum* in field isolates from Honduras. Am. J. Trop. Med. Hyg. 60, 30–34.

Harboe, N., Ingild, A., 1973. Immunization, isolation of immunoglobulins, estimation of antibody titre. Scand. J. Immunol. 2, 161–164.

Kariuki, S.K., Lal, A.A., Terlouw, D.J., ter Kuile, F.O., Ong'echa, J.M., Phillips-Howard, P.A., Orago, A.S., Kolczak, M.S., Hawley, W.A., Nahlen, B.L., Shi, Y.P., 2003a. Effects of permethrin-treated bed nets on immunity to malaria in western Kenya. II. Antibody responses in young children in an area of intense malaria transmission. Am. J. Trop. Med. Hyg. 68 (Suppl.), 108–114.

Kariuki, S.K., ter Kuile, F.O., Wannemuehler, K., Terlouw, D.J., Kolczak, M.S., Hawley, W.A., Phillips-Howard, P.A., Orago, A.S., Nahlen, B.L., Lal, A.A., Shi, Y.P., 2003b. Effects of permethrin-treated bed nets on immunity to malaria in western Kenya. I. Antibody responses in pregnant women and cord blood in an area of intense malaria transmission. Am. J. Trop. Med. Hyg. 68 (Suppl.), 61–67.

Lengeler, C., Schellenberg, J., D'Alessandro, U., Binka, F., Cattani, J., 1998. Relative versus absolute risk of dying reduction after using insecticide-treated nets for malaria control in Africa. Trop. Med. Int. Health 3, 286–290.

Meraldi, V., Nebie, I., Moret, R., Cuzin-Ouattara, N., Thiocone, A., Doumbo, O., Esposito, F., Traore, A.S., Corradin, G., Terenzi, S., 2002. Recognition of synthetic polypeptides corresponding to the N- and C-terminal fragments of *Plasmodium falciparum* Exp-1 by T-cells and plasma from human donors from African endemic areas. Parasite Immunol. 24, 141–150.

Nebie, I., Cuzin-Ouattara, N., Diallo, D.A., Cousens, S.N., Theisen, M., Corradin, G., Traore, A.S., Esposito, F., 2003. Humoral responses to defined malaria antigens in children living since birth under insecticide-treated curtains in Burkina Faso. Acta Trop. 88, 17–25.

Nevill, C., Some, E., Mung'ala, V., Mutemi, W., New, L., Marsh, K., Lengeler, C., Snow, R., 1996. Insecticide-treated bednets reduce mortality and severe morbidity from malaria among

children on the Kenyan coast. Trop. Med. Int. Health 1, 139–146.

Pietra, Y., Procacci, P.G., Sabatinelli, G., Kumlien, S., Lamizana, L., Rotigliano, G., 1991. Impact of utilization of permethrin impregnated curtains on malaria in a rural zone of high transmission in Burkina Faso. Bull. Soc. Pathol. Exot. 84, 375–385.

Procacci, P.G., Lamizana, L., Kumlien, S., Habluetzel, A., Rotigliano, G., 1991. Permethrin-impregnated curtains in malaria control. Trans. R. Soc. Trop. Med. Hyg. 85 (2), 181–185.

Smith, T., Felger, I., Tanner, M., Beck, H.P., 1999. Premunition in *Plasmodium falciparum* infection: insights from the epidemiology of multiple infections. Trans. R. Soc. Trop. Med. Hyg. 93, 59–64.

Snounou, G., Viriyakosol, S., Zhu, X.P., Jarra, W., Pinheiro, L., do Rosario, V.E., Thaithong, S., Brown, K.N., 1993. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol. Biochem. Parasitol. 61, 315–320.

Snounou, G., Zhu, X.P., Siripoon, N., Jarra, W., Thaithong, S., Brown, K.N., Viriyakosol, S., 1999. Biased distribution of msp1 and msp2 allelic variants in *Plasmodium falciparum* populations in Thailand. Trans. R. Soc. Trop. Med. Hyg. 93, 369–374.

Snow, R., Craig, M., Deichmann, U., le Sueur, D., 1999. A preliminary continental risk map for malaria mortality among African children. Parasitol. Today 15, 99–104.

Snow, R.W., Marsh, K., 1998. New insights into the epidemiology of malaria relevant for disease control. Br. Med. Bull. 54, 293–309.

Snow, R., Marsh, K., 1995. Will reducing *Plasmodium falciparum* transmission alter mortality among African children? Parasitol. Today 11, 188–190.

Snow, R.W., Molyneux, C.S., Warn, P.A., Omumbo, J., Nevill, C.G., Gupta, S., Marsh, K., 1996. Infant parasite rates and immunoglobulin M seroprevalence as a measure of exposure to *Plasmodium falciparum* during a randomized controlled trial of insecticide-treated bed nets on the Kenyan coast. Am. J. Trop. Med. Hyg. 55, 144–149.

Taylor, L.H., 1999. Infection rates in, and the number of *Plasmodium falciparum* genotypes carried by Anopheles mosquitoes in Tanzania. Ann. Trop. Med. Parasitol. 93, 659–662.

Troye-Blomberg, M., Perlmann, H., Patarroyo, M.E., Perlmann, P., 1983. Regulation of the immune response in *Plasmodium falciparum* malaria. II. Antigen specific proliferative responses in vitro. Clin. Exp. Immunol. 53, 345–353.

Wåhlin, B., Wahlgren, M., Perlmann, H., Berzins, K., Björkman, A., Patarroyo, M.E., Perlmann, P., 1984. Human antibodies to a Mr 155,000 *Plasmodium falciparum* antigen efficiently inhibit merozoite invasion. Proc. Natl. Acad. Sci. U.S.A. 81, 7912–7916.

World Health Organisation (WHO), 2000. The Roll Back Malaria Partnership and WHO's RBM Project. In: Proceedings of the Third Meeting of the Global Roll Back Malaria Partnership, Geneva.