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Acta Tropica 98 (2006) 261–269
ACTA TROPICA
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Development of cellular immune responses to *Plasmodium falciparum* blood stage antigens from birth to 36 months of age in Cameroon

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Received 18 May 2006; accepted 19 May 2006
Available online 3 July 2006

Abstract

Naturally acquired immunity to *Plasmodium falciparum* is related to immune system that changes during normal development and ageing. The effects of repeated infections during the early life on the maturation of the immune system are still unknown. Elucidation of these effects is of considerable interest given that malaria originates high mortality, especially during the first years of life.

We conducted a cohort study to identify naturally acquired immune responses to *P. falciparum*. Cellular responses of Cameroonian neonates from birth to 36 months of age were evaluated every 6 months by cell proliferation and cytokines (IFN-γ, IL-2 and IL-4) production after *in vitro* culture in the presence of schizont extract and Pf155/RESA peptides.

Data were analyzed by a multiple correspondence analysis (MCA) exhibiting three main findings. Firstly, the lack of time-dependant evolution of specific immune pathways recruitment in the response to a given antigen, no antigen inducing a specific mode of response at a given time-point. Secondly, most of the data variability was expressed by IFN-γ and IL-4 productions, and the major variation of the immune response with age involved this change in IFN-γ production. Thirdly, the age-related immune response evolution is characterized by the acquisition of the capacity to mount a IFN-γ response, a transient phase during which children produce a high IL-4 response, and the fast vanishing of the dominance of the IL-2 response. These results suggest that *P. falciparum* specific immune responses are first oriented towards a Th2-type of response, and later switch to Th1-type of response.

Keywords: *Plasmodium falciparum*; Children; Cytokines; Immunity

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doi:10.1016/j.actatropica.2006.05.010

1. Introduction

Plasmodium falciparum malaria remains a major cause of death among children in Africa. In malaria endemic areas, host age is a determinant of naturally acquired immunity, as exposure is uniform among age groups, but adults are resistant to infection while children remain susceptible (Baird, 1998). Naturally acquired immunity to *P. falciparum* may be related to key features of the immune system that change during normal development and ageing.

The newborn immune system differs quantitatively and functionally from that of adults. Neonates mount poor immune responses, and it has been assumed that neonatal T cells differ qualitatively from adult T cells. The ratio of naive to memory T cells gradually diminishes during ageing, as a result of the cumulative effect of exposure to the myriad antigens encountered throughout the normal course of life. For some time, it was believed that there was an intrinsic inability of newborns to mount developmentally immature Th1 responses. However, more recent data indicate that T cells in neonates are developmentally mature in their capacity to mount protective Th1-type, but that they still develop Th2-dominant responses (Adkins, 1999). At birth, the functional predominance of Th2 is suggested by the immaturity of the dendritic antigen-presenting cells to generate Th1 response (Langrish et al., 2002). Several studies showed a progressive increase of circulating Th1-type, IFN-γ and/or IL-2-producing T cells along with ageing and with a strong correlation to CD45RO surface antigen expression. Meanwhile populations of cells capable of producing IL-4 are comparably minimal and stable across all age groups although higher than in cord blood. Collectively, these results may reflect the maturation and expansion of Th1 cell populations from the neonatal period to adulthood, most probably dependent on antigen exposure (Chipeta et al., 1998; Krampera et al., 1999).

After birth, neonates and infants suffer infections by malaria parasites as often as several hundreds times a year, in areas of intense malaria transmission. During his first years of life, the young child will progressively gain reduced susceptibility to severe malaria; this protection is believed to be related to the acquisition of a *P. falciparum* specific immunity. The precise effects of such repeated infections during the early life on the maturation of the immune system are still unknown. However, elucidation of these effects might be of considerable interest given that malaria gives rise to mortality mainly during the first years of life, and that Th1 mechanisms are involved in protection against malaria parasites in mice, and probably in humans.

Pf155/RESA has long been considered as a major candidate for a vaccine against blood stages of *P. falciparum* (Perlmann et al., 1989). Although additional *P. falciparum* antigens are now considered as vaccine candidates, Pf155/RESA is still considered for inclusion in a putative multiantigenic vaccine, and still remains an invaluable model to study the immune response to asexual blood stages of *P. falciparum*, because numerous works led to extensive knowledge of the protein, including the identification of epitopes (Perlmann et al., 1989; Troye-Blomberg et al., 1989), and characterization of antibodies (Berzins et al., 1986).

We conducted a prospective, longitudinal field-based study to identify naturally acquired immune responses to *P. falciparum*. To investigate the age-related evolution of Th1 and Th2 cell-mediated immunity during the initial maturation of the immune response, we measured the cytokine production after *in vitro* malaria-specific stimulation in a cohort of Cameroonian neonates from birth to 36 months of age.

2. Materials and methods

2.1. Study area

The study was conducted from January 1993 to December 1995 in Ebolowa, a town of 35,000 inhabitants in South Cameroon, 160 km South of Yaoundé. The region is characterized by an equatorial climate. Rain falls all year round (1700 mm) with two rainy seasons from September to December and April to June, and two short dry seasons. This allows the persistence of anopheline breeding sites all year round, and perennial transmission of malaria parasites, as confirmed by entomological studies. The yearly inoculation rate is 62 (Legoff, unpublished data).

2.2. Clinical, parasitological and biological monitoring

During 6 months, from January to June 1993, pregnant women living in or within 10 km around Ebolowa delivering in one of the two maternities of Ebolowa were proposed their children to participate in the protocol. Newborn babies were enrolled at birth in the cohort and were followed-up for 30 (for the last enrolled) to 36 (for the first enrolled) months. Informed consent was given by the two parents.

At inclusion into the cohort, a questionnaire was filled in for each child with information related to the pregnancy history and the newborn baby. Thick and thin smears of blood from both the maternal side of the pla-

centa and peripheral venous blood were examined for the presence of parasites, pigment or both and to determine parasite density. Blood samples were also collected from cord and mother peripheral bloods. Each 6 months, a venous blood sample was collected to study the cellular response. A thick blood smear was done at each blood sampling to assess parasite presence and density after Giemsa-staining and counting the number of asexual parasites against 200 leucocytes, assuming a mean count of 8000 leucocytes per microlitre of blood. This study was approved by the Ministry of Public Health. The protocol was approved by the ethic and scientific committees of the Cameroon Ministry of Public Health.

2.3. Antigens

Leucoagglutinine (LA; Sigma, St Louis, MO) was used at a final concentration of 10 μg/ml. The recall antigen tuberculin purified protein derivative (PPD; Statens Serum Institut, Copenhagen, Denmark) was used at a final concentration of 12.5 μg/ml. A crude preparation of asexual falciparum components was obtained by sonication of an *in vitro* culture of the Palo Alto strain of *P. falciparum* (35% parasite density, 55% late stages). This preparation, referred to as schizont antigen, was used at a final concentration of 5 μg/ml.

Five synthetic peptides, P1–P6, reproducing T cell epitopes from the Pf155/RESA molecule (Favaloro et al., 1986; Troye-Blomberg et al., 1989), were also used at a final concentration of 1 μM. These peptides were obtained from the Emory University Protein Structure Facility (USA) and the Bachem Company (Bubendorf, Switzerland). Their structure and localization are shown in Table 1.

2.4. Isolation of peripheral blood mononuclear cells and lymphocyte proliferative assay

Within 16 h after bleeding, peripheral blood mononuclear cells (PBMC) were isolated on Ficoll-Paque® (Pharmacia, Uppsala, Sweden), and cell viability (>90% in all cases) was confirmed by trypan blue staining. Purified PBMC were suspended at 10^6 cells/ml in buffered RPMI (Roswell Park Memorial Institute) 1640 containing 10% human serum and 50 μg/ml gentamycin, and 100 μl aliquots plated in triplicate in round bottom 96-well plates. Mitogen, antigens or RPMI alone were added in 100 μl amounts. Plates were incubated at 37 °C in a humidified chamber with 5% CO_2. After 6 days, 110 μl culture supernatants were removed and 50 μl fresh medium containing 0.5 μCi methyl-^{3}H-thymidine (specific activity 2 Ci/mmole, Amersham, Les Ulis, France) were added to each well. After 16 additional hours, cells were collected on glass-fibre filter paper and radioactivity was counted. Stimulation indices (SI) were calculated by dividing the geometric mean cpm of antigen-stimulated cultures by the geometric mean cpm of unstimulated cultures. According to the response of non-immune individuals, the threshold of positivity for all antigens was a SI ≥ 2.5 (Chougnet et al., 1990).

Table 1
List of synthetic peptides

No.	Residues[a]	Sequence[b]	Epitope
P1	181–195	LGRSGGDIIKKMQTL	T
P3	533–551	NADMNEITERYFKLAENYY	T + B
P4	700–714	SLRWIFKHVAKTHLK	T + B
P5	862–878	IVGYIMHGISTINTEMK	T + B
P6	944–959	$(EENVEHDA)_4$	T + B

[a] Position according to Favaloro et al. (1986).
[b] The sequences are given in one letter code.

2.5. In vitro production of IFN-γ, IL-2 and IL-4

The 6-day culture supernatants from each triplicate were pooled and stored at −80 °C. IFN-γ was assayed by a commercial 2-site ELISA according to the manufacturer's instructions (Genzyme, Cambridge, USA). The absorbance was read at 405 nm. IFN-γ concentrations were determined by reference to human IFN-γ standard (Gg 23-901-530; National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, MD). The threshold of sensitivity was 2.5 U/ml. For statistical purposes, values under this threshold were assigned a concentration of 1.25 U/ml. Mitogen or antigen induced IFN-γ production was derived from the difference between the IFN-γ content in stimulated cultures and the spontaneous IFN-γ content in unstimulated cultures. Similar cultures were incubated for 72 h, and triplicate supernatants were pooled and stored at −80 °C for assaying the IL-2 and IL-4 contents. Cells from an IL-2-dependent mouse cytotoxic T cell line (CTLL2) were used as responder cells. Briefly, 10^4 CTLL2 cells/well were plated in 96-well round bottom plates in 100 μl volume and 100 μl of undiluted supernatants were added. After 24 h, cells were pulsed for 12 h with 0.5 μCi ^{3}H-thymidine/well, harvested and processed as previously. Data were expressed as SI as for lymphoproliferation. According to the response of non-immune individuals, the threshold of positivity for all antigens was set to a SI ≥ 1.77 (Migot et al., 1993).

IL-4 was assayed by a 2-site immuno-assay as described (Fievet et al., 1995). A mouse monoclonal antibody (IL4-38) was used as capture anti-

body while a second monoclonal antibody (IL4-3; Fab$^+$-acetylcholinesterase conjugate; both gifts from Dr. J. Grassi, CEA) was used as tracer antibody. Bound enzyme was detected with Ellman's reagent and absorbance read at 412 nm. IL-4 concentrations were determined by reference to a standard (recombinant IL-4 produced in CHO cells, a gift from Dr. J. Banchereau, Schering-Plough). The threshold of sensitivity was 12 pg/ml. As for IFN-γ, values under this threshold were assigned a concentration of half this value (6 pg/ml). Mitogen and antigen induced IL-4 production was calculated as for IFN-γ (IL-4 content in stimulated cultures minus IL-4 content in unstimulated cultures).

2.6. Statistical analyses and descriptive multivariate analysis

We first performed a description of data by age, by antigen and by immunological assay. Then, we used descriptive multivariate analysis methods to facilitate the interpretation of this large data set. These multivariate analysis methods consider the initial data table as two clouds of points in two multidimensional vector spaces: one cloud for the columns (immunological data) and one cloud for the rows (children). The objective is to find subspaces on which the projection of these clouds will give the best picture of the original cloud. The "best picture" here means that the explained variance is the highest possible.

We started from a rectangular data table, containing all data, with children in rows and immunological data in columns. The cloud of points of the children is in a *P*-dimensional vector space (*P* being the number of biological variables) and the cloud of points of the assays is in a *N*-dimensional vector space (*N* being the number of children). In order to perform multiple correspondence analysis (MCA) (Tenenhaus and Young, 1985), the results of all the tests were considered as positive or negative, using the above thresholds. Indeed, MCA is more suited to categorical data than principal components analysis, a more common multivariate analysis method.

All multivariate analysis methods work by computing successive axes, called principal axes, of decreasing importance. These axes define the subspaces onto which the clouds of points are projected (generally two-dimensional planes). Each axis is characterized by an eigenvalue that gives axis importance (i.e., its percentage of explained variance). Computations and graphical displays of multivariate analyses were done with the ADE–4 freeware (Université de Lyon 1 [http://pbil.univ-lyon1.fr/ADE-4/]) (Thioulouse et al., 1997).

Table 2
Number of children present at each of the two-yearly bleeding dates and malaria infection prevalence

Age	*n*	Parasite prevalence
Cord	123	0
6 months	99	15.6
12 months	100	20.2
18 months	86	27.6
24 months	81	29.6
30 months	79	37.9
36 months	56	34.5

Enrolment lasted for 6 months, while follow-up duration was 36 months, explaining that last enrolled children were followed up for only 30 months.

3. Results

One hundred and twenty-three children were enrolled for immunological studies. The Ebolowa population includes workers originating from the North and West parts of the country that are susceptible to move, most often due to transfer, progressively reducing the size of our cohort (Table 2). A venous blood sample was obtained from each child every 6 months, from birth to 36-month of age, for the first enrolled.

Placental blood smear carried out at delivery showed an infection rate of 12% among multiparae and 57% among primiparae. All thick blood smears from umbilical cord were negative. Prevalence of malaria infection increased progressively with age ($p = 0.0001$), to reach 29.6% at 24 months and 34.5% at 36 months (Table 2).

3.1. Immunological data

LA induced lymphoproliferative, IFN-γ and IL-2 responses in 70–100% of children at all sampling dates, except at birth, when the prevalence rates of responses were 45, 15 and 70%, respectively. An IL-4 response to LA stimulation was observed in 25–30% of children at birth and at 6 months of age, then the prevalence rate reached 55–80% in 12–36 months old children, and was higher than 90% afterwards. Following culture in the presence of PPD, similar patterns of lymphoproliferative response (LPA) as well as IFN-γ and IL-2 responses were observed, but the prevalence rates at birth were 75, 10 and 55%, respectively. As opposed, IL-4 responses were consistently low at all ages, being between 8 and 25%.

Fig. 1 shows the lymphoproliferative and cytokine production responses in the presence of malaria antigens. Lymphoproliferative responses, as well as IL-2 and IL-4 response prevalence rates were in the same range for all

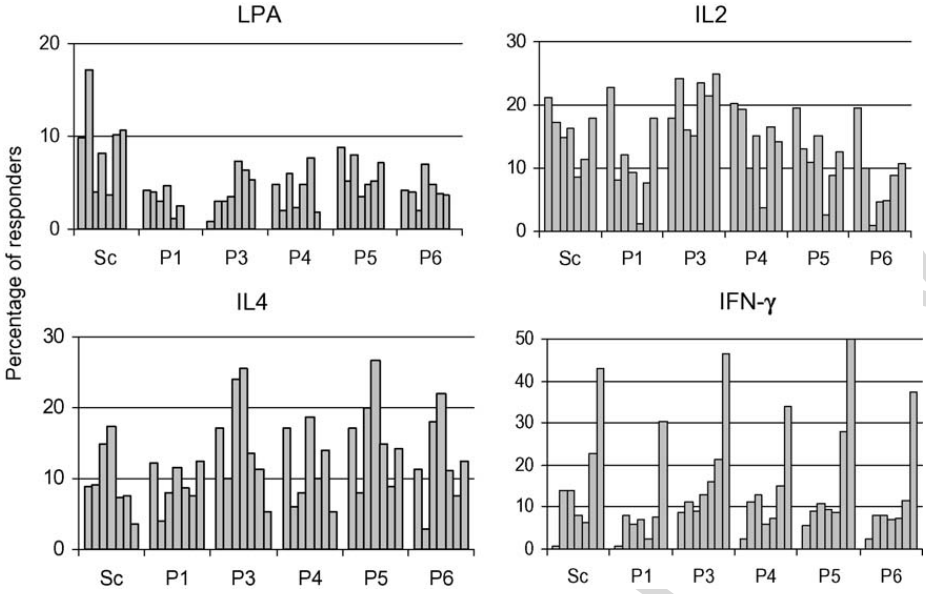

Fig. 1. Evolution of immune responses following PBMC culture in the presence of malaria antigens, from birth to 36 months. Immune responses include lymphoproliferation (LPA), and *in vitro* production of IL2, IL4 and IFN-γ. Antigens used are Sc: crude *P. falciparum* schizont extract; P1–P6: synthetic peptides from Pf155/RESA, see Table 1. Each bar represent data obtained from cells collected at birth (first bar on the left of each group) and every 6 months up to 36 months of age (right bars).

malaria antigens tested, and there was no clear tendency of an age-related increase. Lymphoproliferative response was generally observed in less than 10% of children, IL-2 response in 10–25% and IL-4 responses in 5–25% of children. Conversely, the prevalence rate of IFN-γ response increased with age. IFN-γ production was very weak in cord blood, then prevalence rate increased to around 8–15% in the 6–24 month-old children, and to 30–50% in 36-month old children, this evolution being observed with all antigens.

3.2. MCA Results

The first five axes have a high percentage of explained variance, totalling 44% of the total inertia. The remaining axes show no structured variance. However, examination of all the projection planes ($F1 \times F2$, $F1 \times F3$, $F1 \times F4$, etc.) shows that the more interesting graphics are obtained by projecting the clouds of points on planes $F1 \times F2$ and $F4 \times F5$. Indeed, axis 3 is an inversion and a slight modification of axis 1, and its interpretation is poorly informative.

MCA revealed on factorial axis 1 ($F1$; horizontal), which corresponds to 12.6% of total inertia, a strong association with variables related to an IFN-γ production (Fig. 2). Variables related to IL-4 production are projected on $F2$ (vertical; 10.4% of total inertia). None of the other immune responses (including those related to IL-2 production) could be distinguished as their projections on both axes were too near to the centre of gravity.

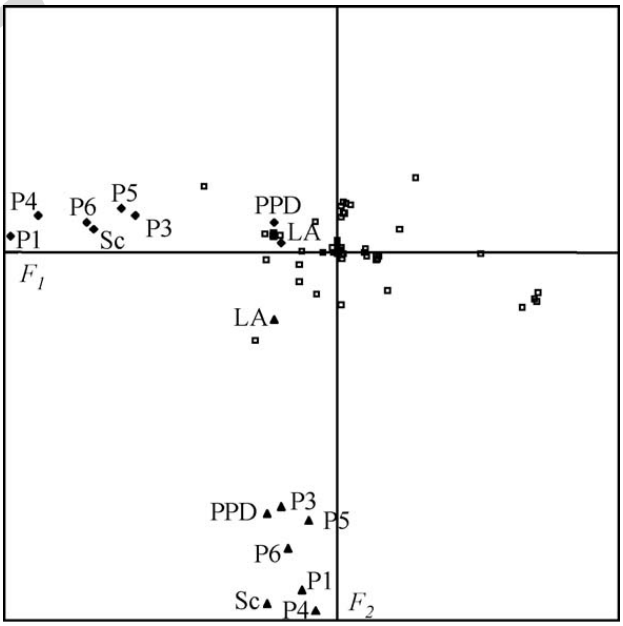

Fig. 2. Graphical representation on the first two factorial axes of IFN-γ and IL-4 responses following PBMC culture in the presence of malaria antigens, LA or PPD. Variables used in the MCA analysis with a significant weight are identified by a closed diamond (♦), representing IFN-γ production) or by a closed triangle (▲) representing IL4 production); other variables are shown as a small open square (□). The stimulating agent used during *in vitro* culture is indicated next to the corresponding circle: LA, leucoagglutinine; PPD: tuberculin purified protein derivative; Sc: crude *P. falciparum* schizont extract; P1–P6: synthetic peptides representing epitopes from Pf155/RESA (see Table 1).

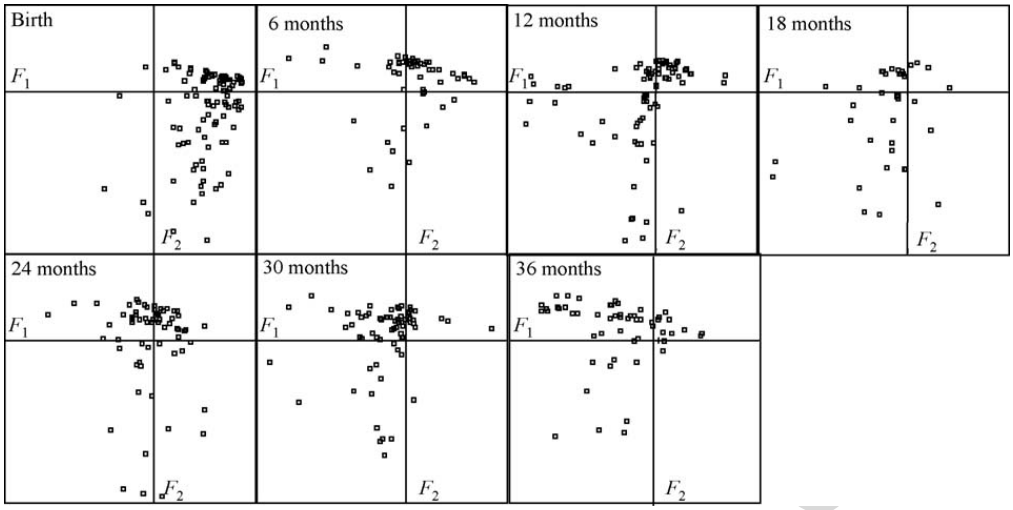

Fig. 3. Graphical representation of children immune response, by age, on the plane (defined by factorial axes F_1 and F_2) representing IFN-γ and IL-4 responses. Subjects were studied at birth, and at 6, 12, 18, 24, 30 and 36 months of age.

This shows that a high proportion of the information variability is included in IFN-γ production, as well as to a lesser extent in IL-4 production. This graph also shows that for each read-out, responses to all various malaria antigens are grouped, suggesting that the ability to respond is not antigen-dependant. The position of the two types of responses (IFN-γ and IL-4) along the two axes demonstrates an exclusive response, being dedicated to either IFN-γ production, or IL-4 production.

Fig. 3 shows the projections of subjects, according to the age of sampling, on the plane defined by factorial axes *F*1 and *F*2. It demonstrates a clear 90° migration of the cloud of subjects, being spread along *F*2 at birth, and along *F*1 starting at 6 months of age. The evolution with age along *F*1, from birth at the right, to 36 months of age at the left, illustrates the evolution of the IFN-γ production with age. Conversely, no evolution along *F*2 was observed. This clearly explains an overall age-related evolution of children along *F*1, as previously defined by immunological variables, demonstrating that IFN-γ production capacity increases with age. However, the projection of each individual shows highly variable figures, reflecting the inter-individual variability, but the age-related progression from right to left on *F*1 is generally observed. In several children, to this progression along *F*1, is superimposed a progression on *F*2 done by transient jumps to the bottom, indicating a transitory production of IL-4.

Fig. 4 shows the projection of variables on the plane defined by factorial axes *F*4 and *F*5. *F*4 and *F*5 axes correspond to 6.9 and 6.2% of the total inertia, respectively. This graph shows again that for each read-out, all responses to all malaria antigens are grouped. It demonstrates the projection of variables related to IL-2 and LPA responses. LPA-related variables participate to both *F*4 and *F*5 axes, while IL-2-related variables only participate in *F*5, being opposed to LPA responses along this axis. This graph suggests that IL-2 production and LPA response vary among subjects, although this variation is of lower weight than that of IL-4 and IFN-γ

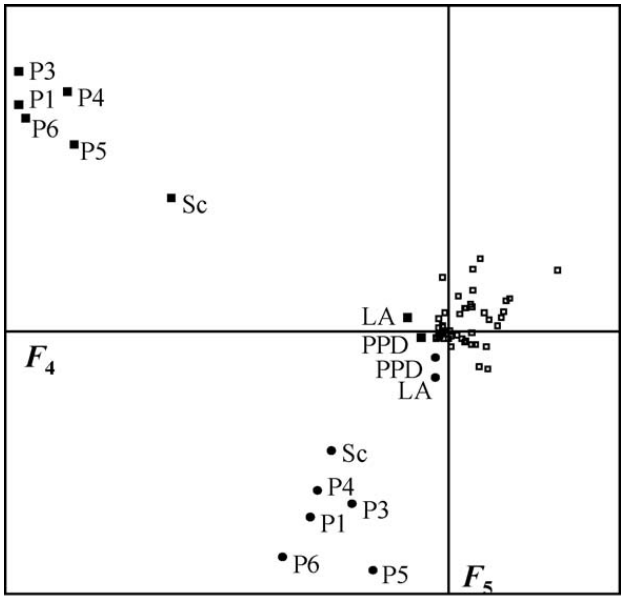

Fig. 4. Graphical representation on factorial axes F_4 and F_5 of IFN-γ and IL-4 responses following PBMC culture in the presence of malaria antigens, LA or PPD. Variables used in the MCA analysis with a significant weight are identified by a closed square (■, representing LPA production) or by a closed circle (●, representing IL2 response); other variables are shown as a small open square (□). The stimulating agent used during *in vitro* culture is indicated next to the corresponding circle: LA, leucoagglutinine; PPD: tuberculin purified protein derivative; Sc: crude *P. falciparum* schizont extract; P1–P6: synthetic peptides representing epitopes from Pf155/RESA (see Table 1).

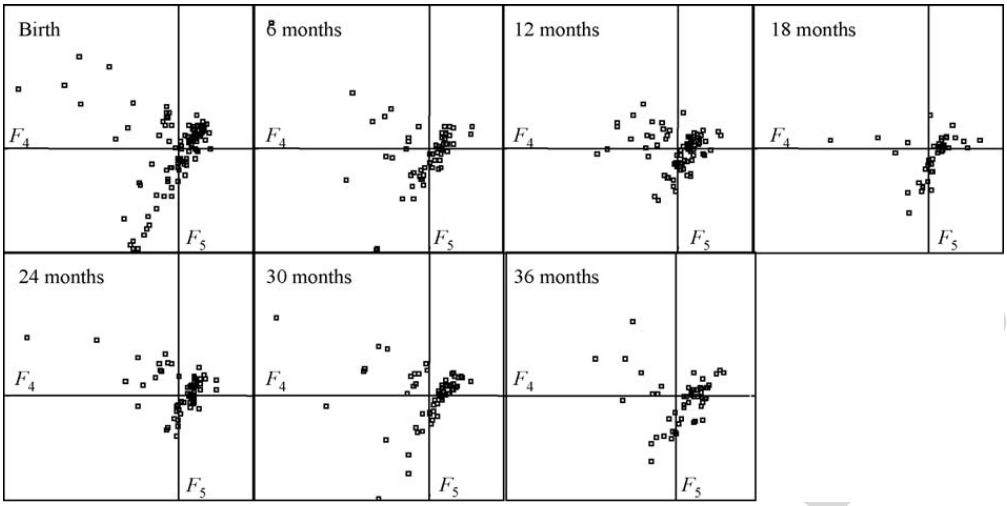

Fig. 5. Graphical representation of children immune response, by age, on the plane (defined by factorial axes F_4 and F_5) representing LPA and IL-2 responses. Subjects were studied at birth, and at 6, 12, 18, 24, 30 and 36 months of age.

productions. The projection of subjects (Fig. 5) allows to understand the extent and the meaning of these variations of responses. Indeed, the projection of subjects according to the age of sampling, on the $F4 \times F5$ plane, shows a clear diminution with age of the rate of IL-2 and LPA responders. The rearrangement of the cloud along $F5$ around the centre is quite clear from birth to 6 months of age, thereafter no additional evolution is observed.

4. Discussion

In newborns, the ability to mount an immune response to a given antigen, as well as the mode of response, vary with age, and with the antigen studied. The numbers of immunological assays, of antigens, and of sampling dates investigated in this study make difficult the overall interpretation of the data. Descriptive multivariate analysis methods are particularly well adapted to this type of data. The multiple correspondence analysis of the results allowed to identify linear summaries and to project variables (or subjects) on factorial planes representing most of the information from the raw data. While univariate statistics allow to study immunological variables consecutively, descriptive multivariate methods are able to take into account all variables simultaneously. This allows much more power, and to get an overall picture of the data structures, that cannot be obtained by univariate statistics. For example, general trends mixed among a high level of overall variability can be detected, even if univariate statistical tests are not significant.

This MCA analysis allowed exhibiting three main findings. Firstly, in the factorial plan $F1 \times F2$, the variables related to responses to a given antigen in all investigated read-outs are grouped. This demonstrates the lack of time-dependant evolution of the recruitment of specific immune pathways in the response to a given antigen, and that no antigen was able to induce a specific mode of response at a given time-point. However, with the exception of the crude schizont antigen, all of the antigens used for *in vitro* stimulation were peptides from the same *P. falciparum* protein, Pf155/RESA. Therefore, one cannot predict whether similar results would have been found with peptides or proteins from another *P. falciparum* antigen. Secondly, the projection of variables in the factorial plan defined by $F1 \times F2$ clearly shows that most of the data variability was expressed by IFN-γ and IL-4 productions. Factorial axis 1, $F1$, only represented IFN-γ production, suggesting that most of the variation of the immune response with age is represented by this change in IFN-γ production. Thirdly, the projection of subjects on the factorial planes $F1 \times F2$ and $F4 \times F5$ clarifies the age-related immune response evolution in these young children, characterized by the acquisition of the capacity to mount an IFN-γ response, a transient phase during which children produce a high IL-4 response, and the fast vanishing of the dominance of the IL-2 response. These results suggest that *P. falciparum* specific immune responses are first oriented towards a Th2-type of response, and later switch to Th1-type of response. Our results reflect the maturation and expansion of T cell populations from the neonatal period, most probably dependent on antigen exposure.

The preponderance of Th2 response of cells from the cord blood (Krampera et al., 2000; Perez-Cruz et al., 2000) is not really surprising, as neonatal T cells are usually considered to be naive, and to be unable to pro-

duce any cytokine, but IL-2 (Lewis et al., 1991). We previously demonstrated in the same area of Cameroon that mononuclear cells from cord blood produced only very small amounts of IFN-γ, when cultured in the presence of both malaria and non-malarial antigens (Fievet et al., 1996). However, cord cells from offspring born from a malaria infected mother have been shown to contain malaria-specific immune cells able to exhibit a full range of responses, including cytokine and antibody productions (Fievet et al., 1996; King et al., 2002; Malhotra et al., 2005). Recent studies have shown that neonatal T cells develop Th2-dominant responses (Adkins, 1999), but can produce a full range of Th1 and Th2 lymphokines, when adequately driven (Adkins, 2000; Chipeta et al., 2000). *In vitro* analysis of cord blood T cell responses indicated that any Th2 bias of neonatal responses was not related to defective intrinsic T cell function (Delespesse et al., 1998), but more probably resulted from the immaturity of neonatal APCs. The antigen presentation by HLA-DR molecules of monocytes represents an important link connecting the innate and the acquired immune responses, the low HLA-DR expression on neonate monocytes contributing to impair the neonatal defences (Birle et al., 2003). The immature neonatal APCs function is also evidenced by the impairment of IL-12 production (Upham et al., 2002) that persists throughout the first year of life (Chougnet et al., 2000).

Similarly, the age-related evolution of the cellular responses, including the gradual increase in the production IFN-γ, as well as the variations in IL-4 production, and to both crude schizont extract and Pf155/RESA peptides, is in line with the fact that IFN-γ and IL-4 responses to MSP2 antigens increased significantly with age (Al-Yaman et al., 1997b), such as IFN-γ response to Pf155/RESA (Al-Yaman et al., 1997a). A recent study established that frequencies and levels of IFN-γ responses to LSA-1 and MSP-1 were low in the first 2 years of life but increased to close to adult levels by the age of 4 years (Chelimo et al., 2003). Moreover, intracellular cytokine staining and flow cytometry demonstrated that IFN-γ producing $CD4^+$ (Th1) and $CD8^+$ cell populations progressively increase with age, being strongly correlated to CD45RO surface antigen expression. Meanwhile, populations of cells capable of producing IL-4 (Th2) are comparably minimal across all age groups (Chipeta et al., 1998). A slow maturation of Th1 cell-mediated immunity was also suggested by responses to measles (Rowe et al., 2000) or tetanus (Gans et al., 1999) immunisation, suggesting that the phenomena evidenced in our study may not be malaria-specific.

5. Conclusions

We shown the preponderance of Th2-like response of cells from the cord blood and the age-related gradual increase of a Th1-like response, including the gradual increase of the production of IL-4 and IFN-γ to both crude *P. falciparum* schizont extract and Pf155/RESA peptides. These changes in the immune response that we observed from birth to the age of 3 years are in line with the changes in the clinical presentation of malaria during the same period of time. The orientation of the immune response, from a Th2-like type towards a Th1-like type, may explain the decrease of susceptibility to severe malaria that occurs during the first years of life in areas of intense transmission of *P. falciparum*.

Acknowledgements

This work was supported by the French Ministry of Research and Space (grant 92S0034) and AUPELF/UREF. BM was the recipient of a fellowship from the French Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche.

We acknowledge the strong support of the staff of the maternity clinics of Ebolowa. We are very grateful to the mothers.

References

Adkins, B., 1999. T-cell function in newborn mice and humans. Immunol. Today 20, 330–335.

Adkins, B., 2000. Development of neonatal Th1/Th2 function. Int. Rev. Immunol. 19, 157–171.

Al-Yaman, F., Genton, B., Taraika, J., Alpers, M.P., 1997b. Naturally acquired cellular immune responses to the synthetic malarial peptide SPf66 in children in Papua New Guinea. Trans. R. Soc. Trop. Med. Hyg. 91, 709–712.

Al-Yaman, F., Genton, B., Taraika, J., Anders, R., Alpers, M.P., 1997a. Cellular immunity to merozoite surface protein 2 (FC27 and 3D7) in Papua New Guinean children. Temporal variation and relation to clinical and parasitological status. Parasite Immunol. 19, 207–214.

Baird, J.K., 1998. Age-dependent characteristics of protection. V. Susceptibility to *Plasmodium falciparum*. Ann. Trop. Med. Parasitol. 92, 367–390.

Berzins, K., Perlmann, H., Wåhlin, B., Carlsson, J., Wahlgren, M., Udomsangpetch, R., Björkman, A., Patarroyo, M.E., Perlmann, P., 1986. Rabbit and human antibodies to a repeated amino acid sequence of a *Plasmodium falciparum* antigen, Pf155, react with the native protein and inhibit merozoite invasion. Proc. Natl. Acad. Sci. U.S.A. 83, 1065–1069.

Birle, A., Nebe, C.T., Gessler, P., 2003. Age-related low expression of HLA-DR molecules on monocytes of term and preterm newborns with and without signs of infection. J. Perinatol. 23, 294–299.

Chelimo, K., Sumba, P.O., Kazura, J.W., Ofula, A.V., John, C.C., 2003. Interferon-gamma responses to Plasmodium falciparum liver-stage antigen-1 and merozoite-surface protein-1 increase with age in

children in a malaria holoendemic area of western Kenya. Malariol. J. 2, 37 [Online http://www.malariajournal.com/content/2/1/37].

Chipeta, J., Komada, Y., Zhang, X.L., Azuma, E., Yamamoto, H., Sakurai, M., 2000. Neonatal (cord blood) T cells can competently raise type 1 and 2 immune responses upon polyclonal activation. Cell. Immunol. 205, 110–119.

Chipeta, J., Komada, Y., Zhang, X.L., Deguchi, T., Sugiyama, K., Azuma, E., Sakurai, M., 1998. $CD4^+$ and $CD8^+$ cell cytokine profiles in neonates, older children, and adults: increasing T helper type 1 and T cytotoxic type 1 cell populations with age. Cell. Immunol. 183, 149–156.

Chougnet, C., Deloron, P., Lepers, J.P., Tallet, S., Rason, M.D., Astagneau, P., Savel, J., Coulanges, P., 1990. Humoral and cell-mediated immune responses to the *Plasmodium falciparum* antigens PF155/RESA and CS protein: seasonal variations in a population recently reexposed to endemic malaria. Am. J. Trop. Med. Hyg. 43, 234–242.

Chougnet, C., Kovacs, A., Baker, R., Mueller, B.U., Luban, N.L., Liewehr, D.J., Steinberg, S.M., Thomas, E.K., Shearer, G.M., 2000. Influence of human immunodeficiency virus-infected maternal environment on development of infant interleukin-12 production. J. Infect. Dis. 181, 1590–1597.

Delespesse, G., Yang, L.P., Ohshima, Y., Demeure, C., Shu, U., Byun, D.G., Sarfati, M., 1998. Maturation of human neonatal CD4 + and CD8+ T lymphocytes into Th1/Th2 effectors. Vaccine 16, 1415–1419.

Favaloro, J.M., Coppel, R.L., Corcoran, L.M., Foote, S.J., Brown, G.V., Anders, R.F., Kemp, D.J., 1986. Structure of the RESA gene of *Plasmodium falciparum*. Nucl. Acids Res. 14, 8265–8277.

Fievet, N., Cot, M., Chougnet, C., Maubert, B., Bickii, J., Dubois, B., Le Hesran, J.Y., Frobert, Y., Migot, F., Romain, F., Verhave, J.P., Louis, F., Deloron, P., 1995. Malaria and pregnancy in Cameroonian primigravidae: humoral and cellular immune responses to *Plasmodium falciparum* blood stages antigens. Am. J. Trop. Med. Hyg. 53, 612–617.

Fievet, N., Ringwald, P., Bickii, J., Dubois, B., Maubert, B., Le Hesran, J.Y., Cot, M., Deloron, P., 1996. Malaria cellular immune responses in neonates from Cameroon. Parasite Immunol. 18, 483–490.

Gans, H.A., Maldonado, Y., Yasukawa, L.L., Beeler, J., Audet, S., Rinki, M.M., DeHovitz, R., Arvin, A.M., 1999. IL-12, IFN-gamma, and T cell proliferation to measles in immunized infants. J. Immunol. 162, 5569–5575.

King, C.L., Malhotra, I., Wamachi, A., Kioko, J., Mungai, P., Wahab, S.A., Koech, D., Zimmerman, P., Ouma, J.H., Kazura, J.W., 2002. Acquired immune responses to *Plasmodium falciparum* merozoite surface protein-1 in the human fetus. J. Immunol. 168, 356–364.

Krampera, M., Tavecchia, L., Benedetti, F., Nadali, G., Pizzolo, G., 2000. Intracellular cytokine profile of cord blood T-, and NK- cells and monocytes. Haematologica 85, 675–679.

Krampera, M., Vinante, F., Tavecchia, L., Morosato, L., Chilosi, M., Romagnani, S., Zanolin, M.E., Pizzolo, G., 1999. Progressive polarization towards a T helper/cytotoxic type-1 cytokine pattern during age-dependent maturation of the immune response inversely correlates with CD30 cell expression and serum concentration. Clin. Exp. Immunol. 117, 291–297.

Langrish, C.L., Buddle, J.C., Thrasher, A.J., Goldblatt, D., 2002. Neonatal dendritic cells are intrinsically biased against Th-1 immune responses. Clin. Exp. Immunol. 128, 118–123.

Lewis, D.B., Yu, C.C., Meyer, J., English, B.K., Kahn, S.J., Wilson, C.B., 1991. Cellular and molecular mechanisms for reduced interleukin 4 and interferon-gamma production by neonatal T cells. J. Clin. Invest. 87, 194–202.

Malhotra, I., Mungai, P., Muchiri, E., Ouma, J., Sharma, S., Kazura, J.W., King, C.L., 2005. Distinct Th1- and Th2-type prenatal cytokine responses to *Plasmodium falciparum* erythrocyte invasion ligands. Infect. Immun. 73, 3462–3470.

Migot, F., Chougnet, C., Raharimalala, L., Astagneau, P., Lepers, J.P., Deloron, P., 1993. Human immune responses to the *Plasmodium falciparum* ring-infected erythrocyte surface antigen (Pf155/RESA) after a decrease in malaria transmission in Madagascar. Am. J. Trop. Med. Hyg. 48, 432–439.

Perez-Cruz, I., Fallen, P., Madrigal, J.A., Cohen, S.B., 2000. Naive T cells from cord blood have the capacity to make types 1 and 2 cytokines. Immunol. Lett. 75, 85–88.

Perlmann, H., Perlmann, P., Berzins, K., Wåhlin, B., Troye-Blomberg, M., Hagstedt, M., Andersson, I., Hogh, B., Petersen, E., Björkman, A., 1989. Dissection of the human response to the malaria antigen Pf155/RESA into epitope specific components. Immunol. Rev. 112, 115–132.

Rowe, J., Macaubas, C., Monger, T.M., Holt, B.J., Harvey, J., Poolman, J.T., Sly, P.D., Holt, P.G., 2000. Antigen-specific responses to diphtheria-tetanus-acellular pertussis vaccine in human infants are initially Th2 polarized. Infect. Immun. 68, 3873–3877.

Tenenhaus, M., Young, F.W., 1985. An analysis and synthesis of multiple correspondence analysis, optimal scaling, dual scaling, homogeneity analysis and other methods for quantifying categorical multivariate data. Psychometrika. 50, 91–119.

Thioulouse, J., Chessel, D., Dolédec, S., Olivier, J.M., 1997. ADE-4: a multivariate analysis and graphical display software. Statistics and Computing 7, 75–83.

Troye-Blomberg, M., Riley, E.M., Perlmann, H., Andersson, G., Larsson, A., Snow, R.W., Allen, S.J., Houghten, R.A., Olerup, O., Greenwood, B.M., Perlmann, P., 1989. T and B cell responses of *Plasmodium falciparum* malaria-immune individuals to synthetic peptides corresponding to sequences in different regions of the *P. falciparum* antigen Pf155/RESA. J. Immunol. 143, 3043–3048.

Upham, J.W., Lee, P.T., Holt, B.J., Heaton, T., Prescott, S.L., Sharp, M.J., Sly, P.D., Holt, P.G., 2002. Development of interleukin-12-producing capacity throughout childhood. Infect. Immun. 70, 6583–6588.