

Profiling the Antibody Immune Response against Blood Stage Malaria Vaccine Candidates

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Background: The complexity and diversity of the antibody immune response to the antigen repertoire of a pathogen has long been appreciated. Although it has been recognized that the detection of antibodies against multiple antigens dramatically improves the clinical sensitivity and specificity of diagnostic assays, the prognostic value of serum reactivity profiles against multiple microbial antigens in protection has not been investigated.

Methods: Using malaria as a model we investigated whether antigen reactivity profiles in serum of children with different levels of clinical immunity to *Plasmodium falciparum* malaria correlated with protection. We developed a microarray immunoassay of 18 recombinant antigens derived from 4 leading blood-stage vaccine candidates for *P. falciparum* [merozoite surface protein 1 (MSP1), MSP2, MSP3, and apical membrane antigen (AMA)-1]. Associations between observed reactivity profiles and clinical status were sought using *k*-means clustering and phylogenetic networks.

Results: The antibody immune response was unexpectedly complex, with different combinations of antigens recognized in different children. Serum reactivity to

individual antigens did not correlate with immune status. By contrast, combined recognition of AMA-1 and allelic variants of MSP2 was significantly associated with protection against clinical malaria. This finding was confirmed independently by *k*-means clustering and phylogenetic networking.

Conclusions: The analysis of reactivity profiles provides a wealth of novel information about the immune response against microbial organisms that would pass unnoticed in analysis of reactivity to antigens individually. Extension of this approach to a large fraction of the proteome may expedite the identification of correlates of protection and vaccine development against microbial diseases.

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To date studies of vaccine development and immune response analysis have focused on analyzing either one or a few immunodominant antigens at a time (1). Bacterial- and parasite-encoded molecules have been proposed as vaccine candidates with a priority that has been largely dominated by the order in which the corresponding genes have been discovered (2). Completed genome sequences of bacteria and parasites have disclosed the presence of ~1000–5000 candidate proteins (and variants thereof) for each microorganism. The disproportion between the antigen repertoire of microbial organisms and the number of antigens that have been investigated may well explain the difficulties encountered in developing vaccines, particularly for more complex organisms, such as parasites.

In the case of malaria, epidemiological and experimental evidence has revealed the crucial importance of antibody-mediated effector mechanisms directed predominantly at antigens of blood stage parasites (3–5). In spite of decades of effort, we still lack immunological markers that correlate with protective immunity, and little is known about the identity of the blood stage parasite antigens that function as targets of naturally acquired

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immunity (6). In humans none of the immune responses studied against *Plasmodium falciparum* vaccine candidates correlate unequivocally with protection from infection, decreased morbidity, and/or a specific effector mechanism. Most seroepidemiological studies have looked for associations between immunity to malaria and simple measurements of antibody quantity (prevalence of antibodies among the population, antibody titer, or ELISA absorbance) to individual antigens (7–13). The target antigens that have been evaluated are too few, too polymorphic, and expressed for only brief periods of the parasite life cycle (14). Despite the frequent assertion that antimalarial immunity is likely to be multifactorial and require effector mechanisms directed at multiple antigens, studies have failed to determine the protective contribution of antibodies to more than 1 malaria protein. This is, in part, because available immunoassays (such as ELISA) are laborious, require relatively large amounts of serum, and determine only responses to individual antigens.

Recently, reports have shown that antigen microarray immunoassays are suitable for detecting serum antibodies directed against a vast repertoire of microbial antigens (15–17), thus offering the opportunity to correlate protective immunity with seroreactivity profiles (or patterns) rather than with antibody responses to single antigens (18). We arrayed several recombinant blood stage malaria proteins (19–25), all of which are prime vaccine candidates, to form the substrate of an antibody-capture assay that has been used to investigate the antibody reactivity profiles among children with different levels of clinical immunity to malaria. We then searched for associations between reactivity to different combinations of antigens and resistance to malaria.

Materials and Methods

SERUM SAMPLES

For validation and optimization of the microarray immunoassay (as compared with ELISA), we used 30 sera from heavily malaria-exposed adults, obtained during a longitudinal survey of antimalarial immunity in an area of seasonal malaria transmission in The Gambia (26). Subsequently, we screened 189 serum samples from children, collected at the end of the long dry season before the initiation of a prospective epidemiological study (Table 1) around the town of Farafenni in The Gambia (27). Individual serum donors would have received, on average, between 1 and 5 infectious mosquito bites per year (28). After being monitored throughout the subsequent malaria transmission season, children (age 3–9 years) were classified into 1 of 3 groups: no malaria (group A) included children having no evidence of infection throughout the study period; clinical malaria (group B), children having at least 1 episode of fever (temperature $\geq 37.5^\circ\text{C}$) and parasitemia $\geq 5000/\mu\text{L}$; and asymptomatic (group C), children with parasitemia or acquired splenomegaly but with no evidence of fever or other symptoms of clinical malaria. Both studies were approved by the

Table 1. Epidemiological and clinical characteristics of the 189 children analyzed in the study.^a

Sex	
Male	105 (55.6)
Female	84 (44.4)
Age (years)	
3	25 (13.2)
4	34 (18.0)
5	34 (18.0)
6	33 (17.5)
7	20 (10.6)
8	32 (16.9)
9	11 (5.8)
Ethnic group	
Fulani	52 (27.5)
Mandinko	70 (37.0)
Wolof	67 (35.5)
Clinical status	
No malaria (group A)	55 (29.1)
Clinical malaria (group B)	81 (42.9)
Asymptomatic (group C)	53 (28.0)

^a Percentages are shown in parentheses. Children in group B experienced at least 1 confirmed clinical episode of malaria; the total number of episodes is unknown since children were censored after the first confirmed episode. Of the 53 children in group C, 35 (66%) were asymptotically parasitemic at the end of the study, 4 (8%) had splenomegaly without parasitemia, and 14 (26%) had splenomegaly plus parasitemia; the number of asymptomatic episodes is unknown because during follow-up blood samples were not obtained from children unless they had fever.

MRC/Gambia Government Ethical Review Committee. All participants (or their parents or guardians in the case of children) gave informed consent to participate in the study. Control sera were obtained from European donors who had never been exposed to malaria.

P. FALCIPARUM ANTIGENS

The recombinant antigens used in this study are detailed in Table 1 in the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol53/issue7>. Antigens were produced in *Escherichia coli* with hexahistidine, glutathione S-transferase, or maltose-binding protein tags.

ELISA IMMUNOASSAYS

ELISA was carried out as previously described (29), except that plates were blocked using skimmed milk powder, 20 g/L in PBS (containing, per liter, 137 mmol NaCl, 2.7 mmol KCl, 10 mmol Na_2HPO_4 , 1.8 mmol KH_2PO_4 , pH 7.4) with Tween 20, 0.5 mL/L; antigens were coated at 0.5 mg/L, and sera were diluted 1:1000.

MICROARRAY IMMUNOASSAYS

For array construction, purified antigens were resuspended in PBS containing Tween 20, 0.2 mL/L, to a concentration of 50 mg/L (15). Purified human IgG (Sigma) was diluted in PBS containing polyvinylpyrrolidone, 10 g/L, and SDS, 0.2 g/L, to generate an internal

calibration curve. Proteins were printed to aldehyde-derivatized glass microscope slides (CEL Associates) by use of high-speed robotics (Microgrid Compact; Biorobotics). Arrays were printed at 23 °C/60% humidity and stored overnight inside the printing cabinet before removal for storage with desiccant. For processing, printed slides were blocked with PBS containing Tween 20, 0.1 mL/L, and BSA, 20 g/L, for 1 h at room temperature. Slides were then rinsed with PBS containing Tween 20, 0.1 mL/L (PBS/Tween), and incubated for 15 min at room temperature with human serum diluted 1:200 in 2× PBS (containing, per liter, 274 mmol NaCl, 5.4 mmol KCl, 20 mmol Na₂HP0₄, and 3.6 mmol KH₂PO₄) with Tween 20, 0.1 mL/L, and BSA, 20g/L. To reveal bound human IgG, slides were washed with PBS/Tween and incubated for 10 min with Alexa 546-labeled goat antihuman IgG secondary antibody (Invitrogen). Unbound secondary antibody was removed with PBS/Tween, and slides were dried at 37 °C before fluorescence was measured at 543 nm by confocal scanning (PerkinElmer S5000). Images were generated with ScanArray™ software (PerkinElmer) and quantified using TotalLab™ software (NonLinear).

TREATMENT OF MICROARRAY DATA FOR PROFILE ANALYSIS

Quantified array signals were analyzed using Excel (Microsoft). Measurements for each spot were corrected against the internal PBS negative control to identify signals above background. The mean of duplicate measurements was then found, and the reactivity signal of the corresponding affinity tag was subtracted before concentrations of antigen-bound IgG were determined by interpolation from an internal calibration curve. The mean plus 2 SD of antigen-bound IgG recorded from the 20 European control sera was used as a positive reactivity cutoff. Digital profiles (positive/negative) were then generated using either positive or negative reaction.

CLUSTERING, PHYLOGENETIC NETWORKING, AND STATISTICAL ANALYSIS

Digital reactivity profiles derived from array data were pooled into a data matrix that defined each serum as an 18-dimensional vector (1 dimension for each antigen). We then used *k*-means clustering to group sera into distinct clusters on the basis of similarities in their reactivity profiles. The *k*-means algorithm partitions objects into a user-defined number (*k*) of clusters such that the clusters are internally similar but externally dissimilar (30). To define distances between sera, the Euclidean distance similarity metric was used, which, for *n*-dimensional vectors, is explained by the equation:

$$d = \sqrt{\sum_{a=1}^n (x_a - y_a)^2}$$

where x_a and y_a are the measured reactivity values for sera *x* and *y*, respectively, against antigen *a*, with the summation running over the *n* antigens under analysis. With iteration, the algorithm minimizes the sum of distances within each cluster to provide the optimal clustering solution. Analysis showed that convergence to an optimal solution was achieved within 2000 iterations of the algorithm. In this way, on discrete repetition of the clustering process, an identical output was returned irrespective of the initial random assignment. We performed *k*-means clustering using Cluster 3.0 (31, 32) and visualization with TreeView (Java) 1.0.12 (33). Phylogenetic networks were constructed using SplitsTree4.0 beta version (34) using *p*-distances under a NeighborNet network. Other statistical analyses were carried out using STATA8 (StataCorp).

Results

DEVELOPMENT OF MALARIA MICROARRAY IMMUNOASSAYS

The protein microarray immunoassay contained 18 recombinant proteins [derived from 4 leading malaria vaccine candidates (see Table 1 in the online Data Supplement)] spotted in duplicate alongside specificity and sensitivity controls, including (a) a calibration curve to quantify IgG bound to arrayed antigens, (b) fusion tag controls (hexahistidine, glutathione *S*-transferase, and maltose-binding protein) to determine levels of antibody directed against fusion sequences, (c) a series of blank prints arrayed after each individual antigen print-wash cycle to rule out carryover of antigens during the printing process, and (d) negative controls (PBS and 2% BSA) to confirm the specificity of observed signal (Fig. 1). Sensitivity and specificity of the microarray compared with ELISA were assessed using sera from 30 African adults immune to malaria. The ELISA absorbance readings for *P. falciparum* antigens merozoite surface protein (MSP)⁷ 1₁₉ (Wellcome) (22, 23) and MSP2 (FC27) (24) were compared with the fluorescence measured for the corresponding antigens in the microarray assay (Fig. 2). ELISA and microarray data correlated well, with $r^2 > 0.7$ (Fig. 2). To validate the integrity of the remaining antigens in the printed panel, arrays were interrogated with a small number of sera with characterized reactivities for each antigen. For all antigens, the signals observed within arrays were in broad agreement with those anticipated. Interslide and interbatch comparisons for the microarray assay showed $r^2 > 0.9$, confirming the robustness of the microarray assay and its suitability for studying the antibody response against malaria antigens in large groups of individuals (see Fig. 1 in the online Data Supplement). Sera from European volunteers never showed significant reactivity against the arrayed malaria

⁷ Nonstandard abbreviations: MSP, merozoite surface protein; AMA, apical membrane antigen.

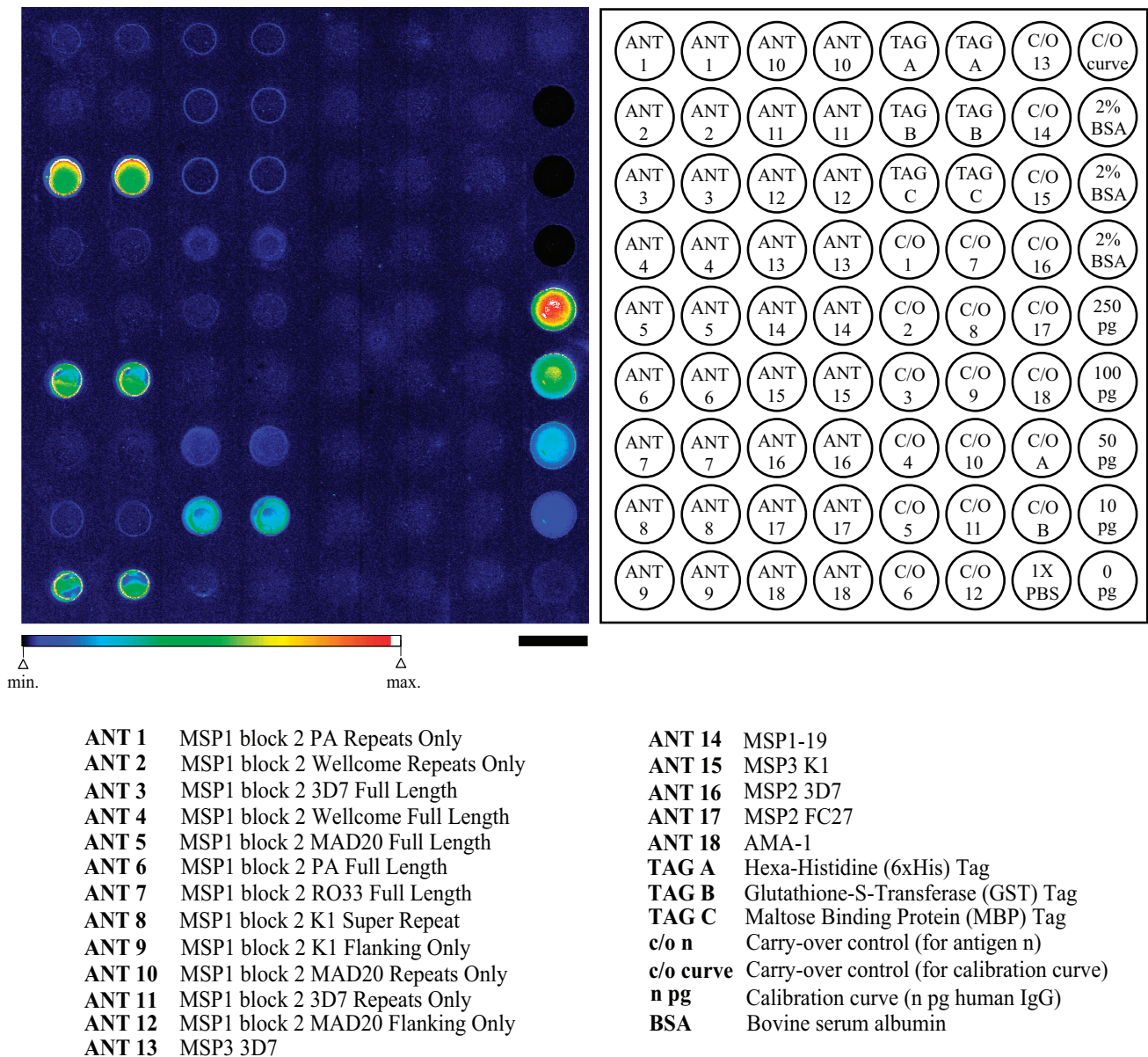


Fig. 1. A microarray immunoassay for the simultaneous detection of antibodies to malaria antigens.

An image of a representative antigen array exposed to an adult African serum is shown (left panel) together with the corresponding printing scheme (right panel). Fluorescence was visualized in a pseudocolor scale (dark blue–white) corresponding to increasing fluorescence; scale bar, 500 μ m.

antigens, results that confirm the high specificity of the assay.

ANTIGEN REACTIVITY PROFILES

Selected sera from African and European adults were screened for an initial assessment of individual-to-individual variation of malaria antigen reactivity profiles (Fig. 3). Despite being collected from individuals of similar age living in the same community, the African sera presented a high degree of complexity in terms of profile diversity (i.e., different combinations of antigens recognized by different sera) and antibody concentrations (Fig. 3). Although some antigens, such as apical membrane antigen

(AMA)-1, MSP2, and MSP3, were recognized more frequently than others, each serum differed in its antigen reactivity profile.

To investigate the significance of antibody diversity patterns in protection against malaria, we next used the microarray immunoassay to analyze sera from 189 children, recruited during a cross-sectional, prospective survey of malaria morbidity carried out in The Gambia, where malaria is seasonally endemic (27). Sera collected from 20 European donors were used as negative controls to determine the positive/negative cutoff value for each antigen. This approach generated 189 distinct reactivity profiles and a total of ~15 000 antibody-antigen determi-

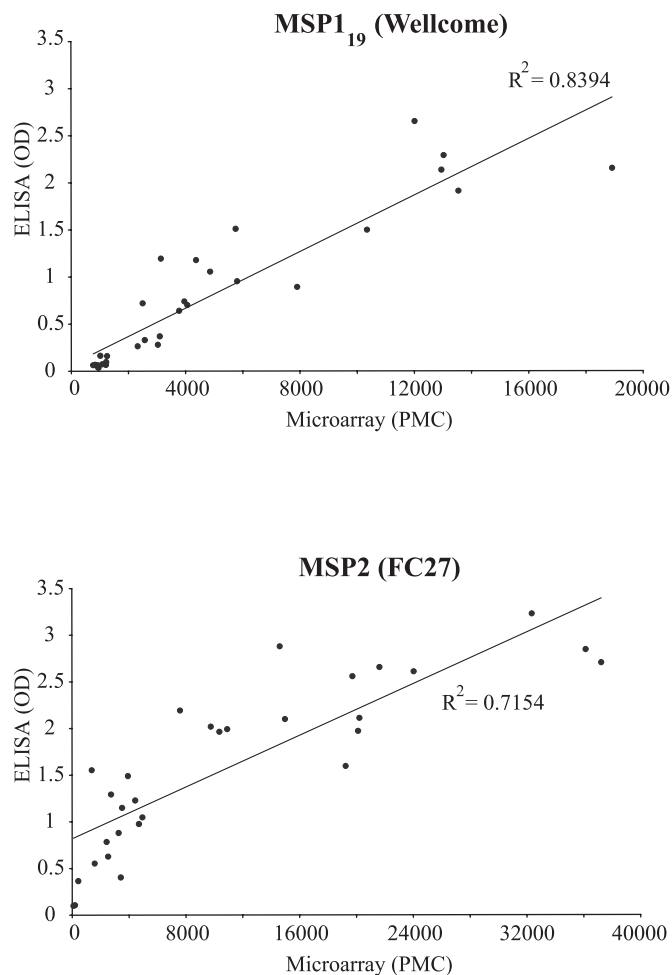


Fig. 2. Correlation of serum reactivities generated by microarray and ELISA immunoassays against MSP1₁₉ and MSP2.

Sera from 30 individuals living in a malaria-endemic region were analyzed to detect the presence of IgG against MSP1₁₉ (Wellcome; *top panel*) and MSP2 (FC27; *bottom panel*) antigens. For each serum, photomultiplier counts (PMC) collected on the microarray slides were plotted against A values in ELISA.

nations. A color-coded (red/black) digital profile matching antibody reactivity (positive vs negative) against the arrayed antigens was generated for each serum (Fig. 4). This analysis took into account only the presence or absence of specific antibody to individual antigens as a marker for immune priming and the ability to produce antibodies of given specificities. Antibody titers were not included when generating profiles, because (a) their inclusion dramatically increases individual differences, thus impairing subsequent clustering analysis, and (b) antibody levels vary greatly with time and are largely influenced by the amount of time since parasite exposure. These samples were collected at the end of the low transmission season, when it is known that absolute antibody levels are poorly representative of the titers that are present later, during the peak malaria transmission season, particularly in children (35, 36). Seropositivity rates, on the other hand, are stable over much longer periods of time (23).

SEARCH FOR CORRELATES OF PROTECTION IN SERUM REACTIVITY PROFILES

Children's sera also showed remarkable variation in antibody profiles to the arrayed antigens (Fig. 4), but some of the profiles shared a similar pattern of combinations of antigen-antibody reactivity. To look for associations between such patterns and protection against malaria, we used 2 independent statistical approaches. The first used *k*-means clustering (30, 37), a partitioning method commonly used to identify group structure within microarray data. To test the null hypothesis of the underlying epidemiological study (that sera presenting with distinct clinical outcomes possess distinct reactivity profiles), sera were arranged into 3 clusters, reflecting the number of clinical groups (Fig. 5).

The clustering algorithm was also run with different values of *k* to accommodate the possibility of subprofiles existing within clinical groups. As *k* varied, however, the algorithm was forced to either split 1 cluster (or more) into 2, producing additional similar clusters (as *k* increased), or amalgamate dissimilar clusters into a single heterogeneous cluster (as *k* decreased). This observation confirmed that assortment into 3 clusters provides the optimal solution for describing the patterns of immunity existing within the population.

Sera falling into cluster 1 typically recognized AMA-1, the 2 MSP2 variants (FC27 and 3D7), and various combinations of the MSP1 block 2 proteins. Sera in cluster 2 showed very little reactivity (if any) against the arrayed malaria antigens, and sera in cluster 3 recognized the majority of the arrayed antigens. For each of the 3 clusters, the characteristics of the included children were compared to identify differences in age, sex, ethnic origin, and clinical immunity to malaria. The mean age of the children differed significantly among clusters (Kruskal-Wallis nonparametric rank sum test: $\chi^2 = 15$; 2 *df*; $P < 0.001$); children in clusters 1 and 3 were older than children in cluster 2, with mean (SD) ages of 6.3 (1.7) and 6.0 (1.6) vs 5.2 (1.8) years, respectively, but there was no significant difference in sex distribution or ethnic origin. Importantly, however, cluster 1 contained a statistically significantly higher proportion of children from clinical group C (asymptomatic infection) than did either of the other clusters ($\chi^2 = 12.0$; 4 *df*; $P = 0.017$; Fig. 5). After adjustment for the potential confounding effects of age, sex, and ethnic group by logistic regression, the risk of developing clinical rather than asymptomatic malaria was significantly lower for children in cluster 1 than for children in either cluster 2 (odds ratio 0.34, 95% CI 0.15–0.78; $P = 0.011$) or cluster 3 (odds ratio = 0.26, 95% CI 0.08–0.82; $P = 0.021$). Comparing children in cluster 1 to all other children, the odds ratio was 0.32 (95% CI 0.14–0.69; $P = 0.004$).

Reactivity profiles and associations were also investigated using phylogenetic networking (34), which examined the interrelationships between antibody reactivity to different antigens for each of the 3 clinical groups. Net-

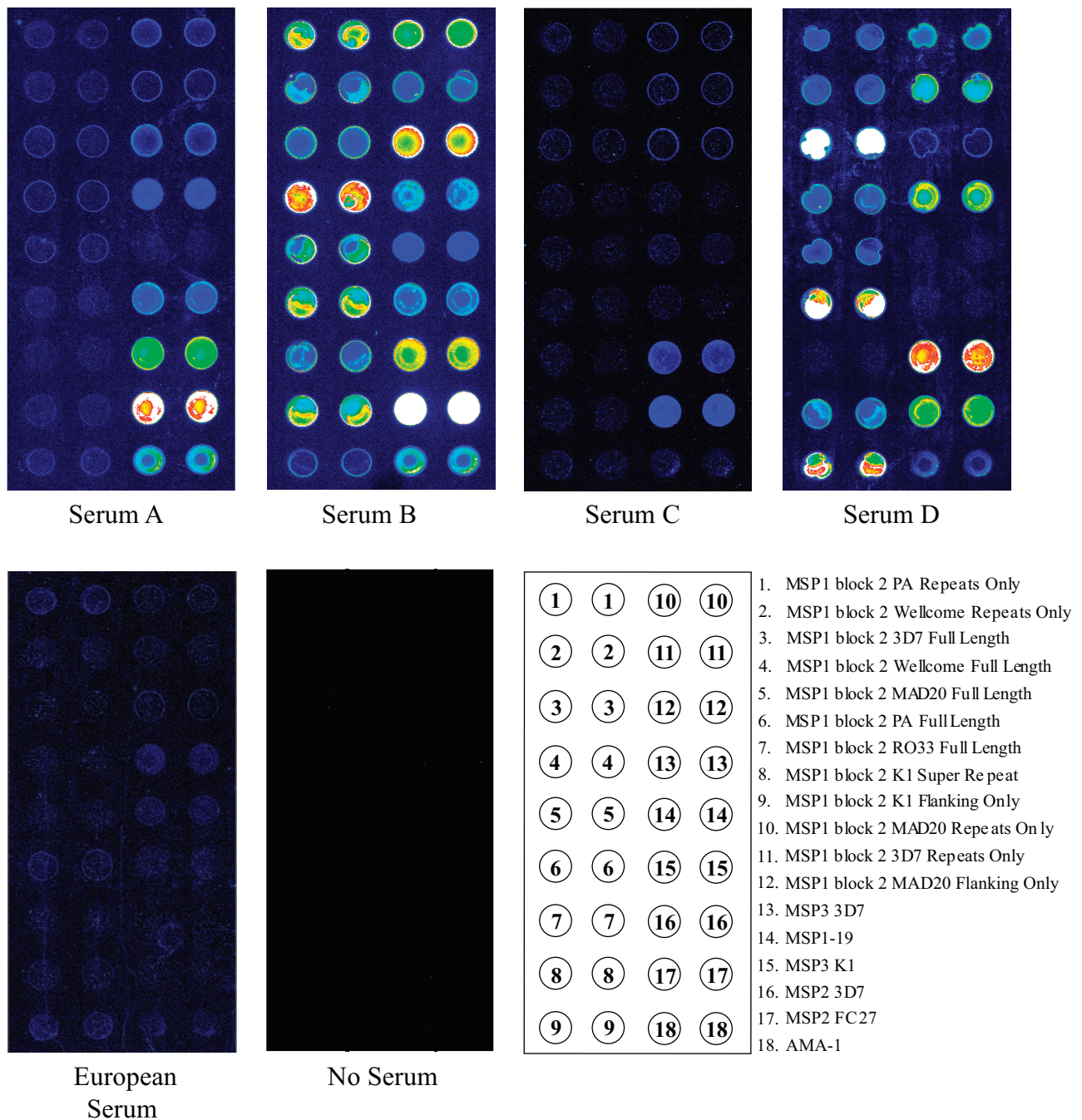


Fig. 3. Serum reactivities of individuals naturally exposed to *P. falciparum* parasites.

Sera from 4 adult individuals (A, male, age 55 years; B, male, 50 years; C, male, 49 years; and D, male, 38 years) living in the same village (Brefet) in The Gambia were exposed to arrays containing the arrayed malaria antigens. As controls, European sera (1 shown here) and a blank slide were used to confirm the specificity of the assay and to enable background signal from nonspecific binding to be determined for each antigen.

works were constructed for the children in groups A, B, and C (see Fig. 2 in the online Data Supplement). The group C phylogeny demonstrates that antibody reactivities to the 2 MSP2 variants (3D7 and FC27) are uniquely linked with statistical significance (bootstrap >95%), and reactivity to both MSP2 variants is tightly linked to reactivity against AMA-1 (bootstrap 100%). The network analysis also revealed an increase in the proportion of the

children showing reactivity against AMA-1 and/or both MSP2 variants (3D7 and FC27) in clinical group C (asymptomatic infection) compared with groups A and B (>45% increase in phylogenetic "antigen reactivity per person" distances).

We then searched for associations between microarray antibody reactivity to all 18 individual antigens and protection from clinical malaria, to rule out the possibility

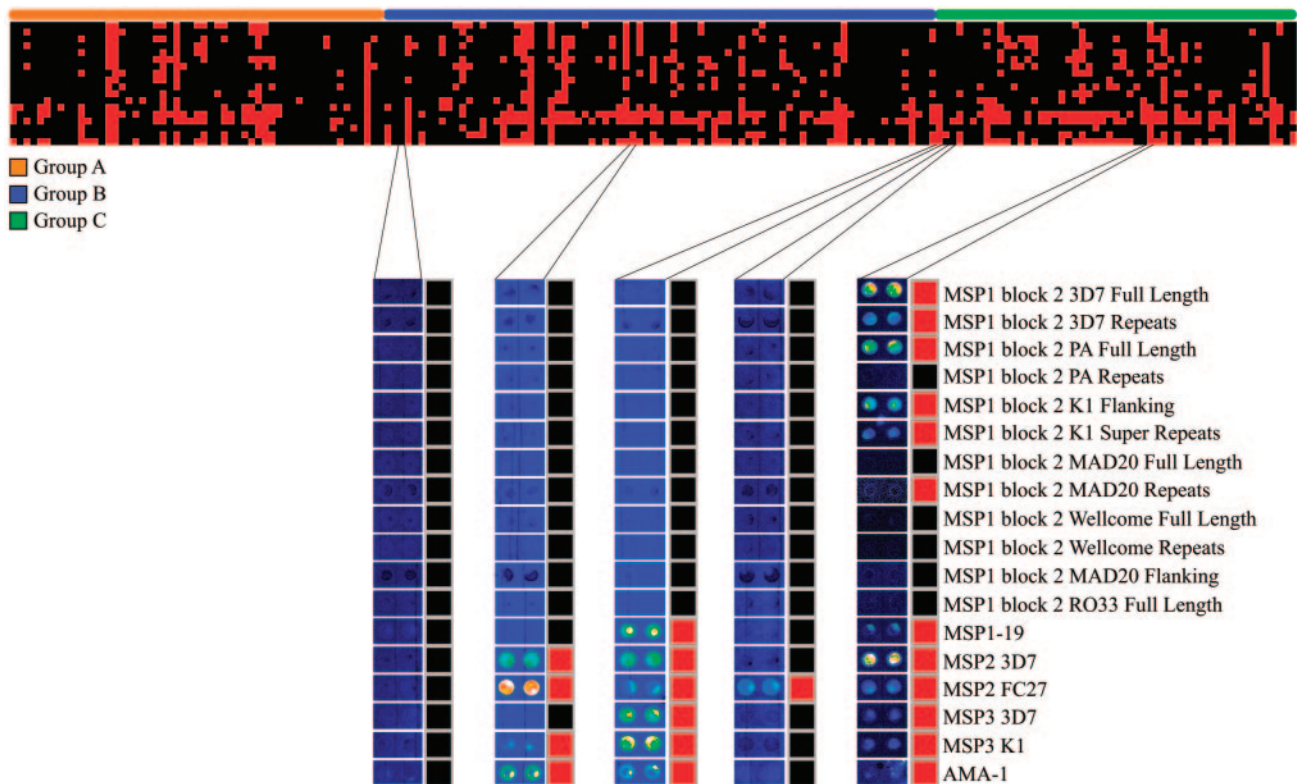


Fig. 4. Antibody reactivity profiles of children's sera (columns) analyzed against the arrayed malaria antigens (rows).

The graphic combines information derived from ~15 000 individual data points to provide a complete set of antibody profiles collated across the entire children cohort. For the purpose of antigen-antibody recognition profile analysis, reactivities to individual antigens were classified as either positive (red boxes) or negative (black boxes). For each antigen, the negative cutoff value was defined as the mean plus 2 SD signal obtained using 20 negative control European sera. In the figure, examples are shown for 5 sera to demonstrate the correlation between fluorescent image data and digital coding.

that antibodies to a single antigen had biased clustering and phylogenetic networking analysis (see Table 2 in the online Data Supplement). Three significant or marginally significant associations were observed between clinical outcome and recognition of an individual antigen (MSP2 FC27, $P = 0.023$; MSP2 3D7, $P = 0.07$; and MAD20 flanking sequence of MSP 1 block 2, $P = 0.06$). Only the 1st of these remained significant after correction for age, however, and none of the associations remained significant after Bonferroni correction for multiple comparisons (38) ($P > 0.012$ in all cases), suggesting that the frequency of serum antibodies to each antigen across the 3 clinical groups did not differ significantly from a random distribution. Furthermore, antibody responses to MSP2 FC27 differed only between clinical groups A and C (and not between B and C) and may therefore reflect differences in exposure to malaria rather than differences in immunity.

Discussion

We have analyzed, in individuals showing different degrees of clinical immunity to malaria, the antibody reactivity profile against different segments and allelic variants of the major *P. falciparum* blood-stage vaccine candidates MSP1, MSP2, MSP3, and AMA-1. When we looked for association between protection against malaria and serum reactivity to individual antigens, we found

only weak correlations that did not stand up to robust statistical evaluation. However, when antibody profiles were clustered into 3 groups on the basis of their similarity, we found that cluster 1 contained a statistically significantly higher number than expected by chance of children classified as being clinically immune to malaria. This finding indicates that the combinations of antigen-antibody reactivities that define such a cluster may have some association with the development of clinical immunity in children, most likely forming part of a larger protective pattern of antibody specificity that may include antigens not assessed in this study. Although the profiles in cluster 1 show some differences, the majority of sera in the cluster are characterized by reactivity against the antigens AMA-1 and the 2 MSP2 variants (3D7 and FC27). The association between clinical immunity and antibody reactivity to both strains of MSP2 is particularly interesting because vaccination studies have suggested that induction of antibody to both 3D7 and FC27 variants may enhance protection by preventing strain selection (39, 40).

The cluster analysis was in agreement with an independent analysis carried out using phylogenetic networks that also showed an association between recognition of these antigens and protection against malaria. The close agreement of these 2 analytical approaches, which derive from very different starting points (one arranged by

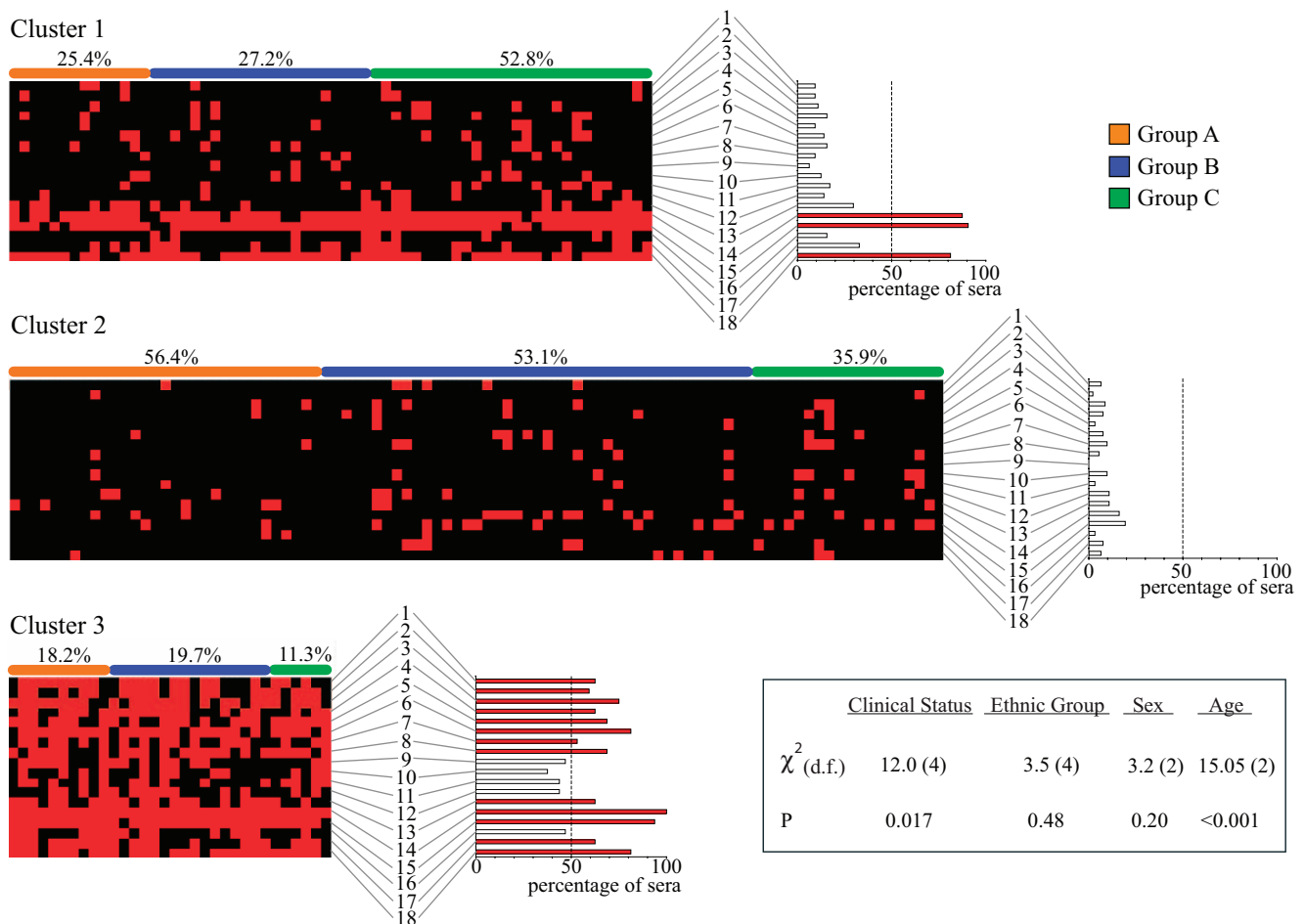


Fig. 5. *k*-means clustering of the digitalized profiles.

Sera were clustered into 3 distinct groups on the basis of their similarity in reactivity against combinations of malaria antigens. Percentages above each cluster describe the proportion of children from each of the 3 clinical groups present within clusters. Associations between cluster and other variables were assessed using the χ^2 test. In the case of the association with age, the Kruskal–Wallis rank sum test of equality was used. Significant associations are shown in **boldface**. Bar charts for each cluster show the percentage of sera with antibody toward each antigen. Antigens against which more than half the sera possess antibody are depicted in red. Antigens: 1 = MSP1 block 2 3D7 full length; 2 = MSP1 block 2 3D7 repeats; 3 = MSP1 block 2 PA full length; 4 = MSP1 block 2 PA repeats; 5 = MSP1 block 2 K1 flanking; 6 = MSP1 block 2 K1 super repeats; 7 = MSP1 block 2 MAD20 full length; 8 = MSP1 block 2 MAD20 repeats; 9 = MSP1 block 2 Wellcome full length; 10 = MSP1 block 2 Wellcome repeats; 11 = MSP1 block 2 Wellcome flanking; 12 = MSP1 block 2 RO33 full length; 13 = MSP1-19; 14 = MSP2 3D7; 15 = MSP2 FC27; 16 = MSP3 3D7; 17 = MSP3 K1; 18 = AMA-1.

serum profile, the other by clinical outcome), suggests that these associations are robust.

Our data demonstrate that the information obtained by profiling serum reactivity against multiple antigens is both quantitatively and qualitatively different from that obtained with single antigen-antibody reactivity determinations. Whereas single antigen-antibody determinations permit arrangement of individuals into 2 groups, either positive or negative, profiling of sera revealed how the immune response to the parasite differs on an individual basis, in terms of antibody amounts and combinations of antigens recognized, to the point that very few profiles are identical. Such diversity likely reflects the complexity of the parasite proteome, the combined genetic diversity of the human and parasite populations, and stochastic events associated with the induction of specific antibody responses. Together these elements may provide an in-

triguing explanation of the difficulties that have been encountered in vaccine development. Our data demonstrate how, in an outbred human population exposed to malaria infection, the immune response will vary between individuals, thus making associations between antibody responses and immunity hard to identify. These findings suggest that categorizing individuals on the basis of serum reactivity to single antigens dramatically underestimates underlying similarities and differences in individual reactivity to the parasite antigen repertoire that may be relevant for understanding how immunity develops.

The accumulation of antibody specificities may be partially related to age and exposure (younger children tended to have less complex antibody profiles), but the development of “appropriate” antibody specificities is not (children in clusters 1 and 3 did not differ significantly in age). Moreover, antibodies directed against a large frac-

tion of parasite antigens may not be protective (children in cluster 3, with the broadest antibody repertoire, were no more protected against clinical malaria than children in cluster 2, with the most limited antibody repertoires) and may indeed impair the function of protective antibody specificities (children in cluster 3 were significantly less protected than children in cluster 1). The simultaneous assessment of hundreds of distinct antigen-antibody reactions, and the ability to monitor temporal stability—or lack thereof—of these patterns in individual children will dramatically facilitate the identification of the parasite antigens that, in combination, function as targets of the protective immune response and hence facilitate both the development and the evaluation of antimalarial vaccines.

To date the identification of more than 320 prokaryotic and eukaryotic genomes, including those of numerous pathogenic organisms, has been completed or approaches completion. The approach and the technology used here for *P. falciparum* might also be applied to identify correlates of protection for many of these other microorganisms.

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