

Identification of *Plasmodium falciparum* antigens by antigenic analysis of genomic and proteomic data

Denise L. Doolan^{a,b}, Scott Southwood^c, Daniel A. Freilich^a, John Sidney^{c,d}, Norma L. Graber^{a,e}, Lori Shatney^{a,e}, Lolita Bebris^{a,e}, Laurence Florens^f, Carlota Dobano^{a,e,g}, Adam A. Witney^{a,e,h}, Ettore Appellaⁱ, Stephen L. Hoffman^{a,j}, John R. Yates III^{e,k}, Daniel J. Carucci^a, and Alessandro Sette^{c,d,l}

^aMalaria Program, Naval Medical Research Center, Silver Spring, MD 20910-7500; ^bDepartment of Molecular Microbiology and Immunology, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, MD 21205-2179; ^cEpimmune, Inc., San Diego, CA 92121; ^dHenry M. Jackson Foundation, Rockville, MD 20852; ^eThe Scripps Research Institute, La Jolla, CA 92037; ^fTorrey Mesa Research Institute, San Diego, CA 92121; and ^gLaboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

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The recent explosion in genomic sequencing has made available a wealth of data that can now be analyzed to identify protein antigens, potential targets for vaccine development. Here we present, in the context of *Plasmodium falciparum*, a strategy that rapidly identifies target antigens from large and complex genomes. Sixteen antigenic proteins recognized by volunteers immunized with radiation-attenuated *P. falciparum* sporozoites, but not by mock immunized controls, were identified. Several of these were more antigenic than previously identified and well characterized *P. falciparum*-derived protein antigens. The data suggest that immune responses to *Plasmodium* are dispersed on a relatively large number of parasite antigens. These studies have implications for our understanding of immunodominance and breadth of responses to complex pathogens.

The feasibility of a malaria vaccine is supported by experimental data demonstrating that protective immunity can be induced by exposure to intact parasite (1). In particular, animals or human volunteers immunized with radiation-attenuated *Plasmodium* spp. sporozoites can develop sterile immunity to subsequent challenge with infectious, nonattenuated, sporozoites (2). However, >5,000 proteins are expressed during the life cycle of the *Plasmodium* spp. parasite (3), and the protein antigens mediating the protective immunity induced by whole organism vaccination are largely unknown. Subunit vaccines currently in development are based on a single or few antigens and may therefore elicit too narrow a breadth of response, providing neither optimal protection nor protection on genetically diverse backgrounds. To duplicate the protection induced by whole organism vaccination (1, 2), we envision that a vaccine capable of targeting a large number of parasite-derived proteins by assembling their minimal CD8⁺ and CD4⁺ T cell epitopes into a multiepitope construct might be necessary.

Because of various factors related to antigen abundance and immunodominance, not all possible antigens are recognized by natural immune responses (4). Various approaches have been proposed for antigen identification (5–12). Herein, we report the development of a novel strategy that integrates bioinformatic predictions, HLA-supertype considerations (13), and *in vitro* cellular assays for the purpose of identifying potentially immunogenic protein antigens from the genomic sequences of complex pathogens. Traditionally, the use of these tools has been for the purpose of precisely identifying specific epitope sequences within a protein known to represent the target of cellular immunity. We hypothesized that these same tools could also be used on a broader scale as a means to efficiently identify new proteins that are strongly antigenic in the course of a natural infection, or in vaccinated individuals. The utility of this approach would be especially apparent in the context of a large and complex pathogen. We tested this hypothesis in the context of *Plasmodium falciparum*.

Methods

Study Subjects. Study subjects ($n = 12$; Table 4, which is published as supporting information on the PNAS web site, www.pnas.org)

were healthy Caucasian male volunteers, aged 20–45 years (mean 34.8) recruited under a protocol approved by the Committee for the Protection of Human Subjects of the Naval Medical Research Center, the Office of the Special Assistant for Human Subject Protections at the Naval Bureau of Medicine and Surgery, and the Human Subjects Research Review Board of the Army Surgeon General, in accordance with the U.S. Navy regulation (SEC-NAVINST 3900.39B) governing the use of human subjects in medical research, and described in more detail in *Supporting Text*, which is published as supporting information on the PNAS web site.

Peptide Synthesis. Peptides for assessment of HLA binding antigenicity and immunogenicity were purchased commercially from Chiron Mimotopes (Chiron) initially as crude material. Quality control “spot checks” by mass spectrometry were used. In selected instances, for those antigens selected for additional deconvolution and HLA peptide binding assays, the peptides were resynthesized in-house, purified by reversed-phase HPLC, and the purity assessed by amino acid sequence and/or composition analysis. Purity was >95%.

HLA-Peptide Binding Assays. Assays to quantitatively measure peptide binding to class I and class II MHC molecules, based on the inhibition of binding of a high affinity radiolabeled peptide to purified MHC molecules, have been described in detail elsewhere (14). A brief description is also provided in *Supporting Text*.

Development of Predicted IC₅₀ nM Value (PIC) Algorithms. The method of derivation of specific polynomial algorithm coefficients describing the average relative binding associated with each of the 20 naturally occurring amino acid residues, for each peptide position has been described by Gulukota *et al.* and others (15–17), and is described in more detail in *Supporting Text*.

IFN- γ Enzyme-Linked Immunospot (ELISPOT) Assay. The number of peptide-specific IFN- γ -secreting cells was determined by *ex vivo*

Abbreviations: PBMC, peripheral blood mononuclear cells; ELISPOT, enzyme-linked immunospot; CSP, circumsporozoite protein; EXP-1, exported protein 1; LSA-1, liver-stage antigen 1; MIC, measured IC₅₀ nM value; MudPIT, multidimensional protein identification technology; SFC, spot-forming cells; SSP2, sporozoite surface protein 2; PIC, predicted IC₅₀ nM value.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. PF10.0179, PFL0800c, PFI0165c, PFC0210c, PF14.0372, PF11.0341, mal4T2c4.p1t1, PF13.0278, PFE0765w, PF11.0479, PFC0450w, PF14.0074, PFC0700c, PF14.0291, MAL8P1.78, MAL13P1.218, PFD0425w, PF13.0320, PF11.0435, PFI0260, PF11.0226, PFA0515w, MAL6P1.252, PF14.0236, PF14.0751, PF14.0648, and PF11.0456).

^dPresent address: La Jolla Institute for Allergy and Immunology, San Diego, CA 92121.

^gPresent address: Unidad de Epidemiología y Bioestadística, Hospital Clinic, Universitat de Barcelona, Barcelona E-08036, Spain.

^hPresent address: Department of Medical Microbiology, St. George's Hospital Medical School, London SW17 0RE, United Kingdom.

ⁱPresent address: Sanaria, Gaithersburg, MD 20878.

^lTo whom correspondence should be addressed. E-mail: alex@liai.org.

Table 1. Efficiency of predicting supertype binding by prototype allele algorithms

Source	Refs.	Percent of supertype binders predicted*					
		A2	A11	B7	A1	A24	DR1
HCV	15, 21 [†]	88 (15 of 17) [‡]	71 (10 of 14)	0 (0 of 2)	50 (3 of 6)	40 (2 of 5)	100 (6 of 6)
<i>P. falciparum</i> [§]	15, 22, 23 [†]	82 (9 of 11)	86 (6 of 7)	0 (0 of 1)	67 (2 of 3)	78 (7 of 9)	78 (7 of 9)
Mag2/3	15, 26 [†]	57 (8 of 14)	100 (6 of 6)	100 (4 of 4)	80 (4 of 5)	0 (0 of 1)	100 (3 of 3)
HBV	15, 25 [†]	63 (12 of 19)	50 (6 of 12)	75 (6 of 8)	78 (7 of 9)	65 (11 of 17)	92 (11 of 12)
Total		72 (44 of 61)	72 (28 of 39)	67 (10 of 15)	70 (16 of 23)	63 (20 of 32)	90 (27 of 30)

*A supertype binder is a peptide with the capacity to bind $\geq 50\%$ of the MHC molecules in a given supertype.

[†]J.S. and A.S., unpublished observations.

[‡]The numbers in parentheses indicate the number of supertype binders identified by the respective algorithm versus the total number of source-derived supertype binders.

[§]Supertype peptides derived from the four previously well characterized *P. falciparum* antigens (PfCSP, PfSSP2, PflSA, and PfEXP1).

IFN- γ ELISPOT, as described (18), and summarized in *Supporting Text*.

Statistical Analysis. All assays were performed in triplicate or quadruplicate. The significance of group differences were calculated by using the Student's *t* test, two-tailed (EXCEL version 10.0, Microsoft). $\alpha < 0.05$ was considered significant.

Results

Significant efforts in the last few years have been devoted by several groups toward the generation of procedures and algorithms that allow more effective and accurate prediction of MHC binding capacity. A methodology commonly used is the polynomial matrix, or linear coefficients, method. Polynomial or matrix methods to predict MHC binding (19) are based on the assumption that each of peptide side chains contributes independently to the binding affinity. Based on this assumption, a number of polynomial methods have been described. A common drawback of these methods is the fact that they provide only rank scores, and often limited to predictions for peptides of a specific size. As a result, it is difficult to combine predictions for different peptide sizes, or predictions for different MHC molecules.

The algorithms that we have developed are still based on the polynomial matrix method, but incorporate a number of additional mathematical transformations, including linear polynomial scaling, and further linear corrections based on minimizing the deviation of predicted values, to allow generation of allele-specific PIC. Because all predictions are expressed in terms of predicted nM K_D values, this method, which is described in more detail in *Materials and Methods*, readily allows for combined predictions of ligands of different sizes (for example 9-mer versus 10-mers) or for different HLA molecules. In addition, by expressing algorithm scores in terms of IC₅₀ nM values, scores can be related to affinity thresholds validated to be biologically relevant in the context of class I and class II HLA molecules (15, 20).

An example of a combined simultaneous prediction of HLA-A*0201 binding for 1,735 different 9- and 10-mers, whose binding affinity for A*0201 were also experimentally determined, is shown in Fig. 2, which is published as supporting information on the PNAS web site, where PIC values are plotted as a function of the actual measured IC₅₀ values (MIC). By using 500 nM as an affinity cutoff to define binding to HLA class I molecules (20), an algorithm score threshold of 100 nM allows the prediction of 52% of all peptides binding HLA-A*0201 with an affinity of 500 nM, or better. Notably, 75% of the peptides identified by the algorithm were indeed binders (true positives; conversely, only 25% are false positives). Increasing the threshold score to 200 nM allowed the selection of 78% of the A*0201 binders, and the percent of true positives only dropped to 69%. To date, we developed a panel of algorithms specific for a broad range of HLA class I and class II types (Table 5, which is published as supporting information on the PNAS web site).

We have described previously how a majority of HLA class I and II molecules can be classified in relatively few superotypes characterized by largely overlapping peptide repertoires (13). Peptides that are capable of binding to the majority of the most common molecules of a given supertype can be identified by *in vitro* peptide-MHC binding assays, and are designated as supertype binding peptides. A correlation between immunogenicity and the capacity to bind multiple molecules from a given HLA supertype has been documented in several studies (21–25), and high-affinity binding to the prototype allele of a given supertype has been shown to be highly predictive of supertype binding (17). Herein, we examined whether predictions based on prototype alleles could efficiently identify supertype binders. Accordingly, we analyzed Mag2/3, hepatitis B virus, hepatitis C virus, and *Plasmodium* protein sequences to examine how many of the previously identified supertype binders (15, 21–26) were identified by our predictive algorithms (100 nM was selected as the threshold predictive value; Fig. 2). Between 63% and 72% of known A1-, A2-, A3/A11-, A24-, and B7-supertype degenerate binders, and 90% of known DR-supertype binders, were identified (Table 1).

Next, we proposed to screen potential protein antigens defined by using genomics and/or proteomic information for HLA-supertype peptides. Pools of algorithm-identified peptides could be then screened for reactivity with peripheral blood mononuclear cells (PBMC) of exposed/vaccinated individuals. Each pool is designed to include peptides representative of the six common class I and class II HLA-supertypes, and with ≈ 10 epitopes per supertype. Because the six most common HLA-supertypes afford coverage of $>95\%$ of the individuals in any population (13), both at the level of class I and class II molecules almost any individual should be capable of binding to at least 20 different epitopes (10 epitopes for class I and class II, respectively). Thus, if an antigen is immunodominant and recognized in the process of natural infection (or vaccination or exposure, depending on the study design), this strategy would result in a “hit” even if the accuracy of prediction was as low as 10% (several independent studies have established an actual success rate on the order of 50–100%; refs. 17, 21–25, and 27). Because relatively large pools of peptides can be easily tested in assays such as ELISPOT, the method allows rapid identification of “hot spots,” or immunodominant protein antigens.

The recent completion of the genomic sequence of *P. falciparum* (3) and the elucidation of the *P. falciparum* proteome (28) by using multidimensional protein identification technology (MudPIT) (29) (which combines in-line high resolution liquid chromatography and tandem mass spectrometry) provide a set of ORFs corresponding to potential *P. falciparum* target antigens, and evidence for expression of these genes in different stages of the parasite's life cycle. Therefore, to validate our hypothesis, we analyzed the *P. falciparum* genome sequence (3) and *P. falciparum* proteome (28) data. Tandem mass spectrometry (MS/MS) spectra of peptide sequences generated by MudPIT analysis of *P. falciparum* sporozoite preparations were scanned against the *P. falciparum* genomic sequence

Table 2. Summary of characteristics of the selected panel of 27 putative *P. falciparum* antigens

Antigen	Protein*	Amino acids [†]	Percent sequence		Fraction	SP [§]	TM	Peptides		MudPIT runs
			coverage (PfSpz)	Sporozoite specific [‡]				Stringent criteria	Relaxed criteria	
1	PF10.0179**	1,904	0.6	Yes	Soluble	0	0	1	1	1
2	PFL0800c	208	49.5	Yes	Soluble and insoluble	1	0	14	16	6
3	PFI0165c	2,404	1.3	No	Soluble and insoluble	0	0	8	11	2
4	PFC0210c	396	10.1	Yes	Soluble	1	1	2	2	2
5	PF14.0372	400	3.0	No	Soluble and insoluble	1	2	4	6	4
6	PF11.0341	1,062	5.5	No	Soluble	0	1	0	6	3
7	mal4T2c4.plt1	104	NA	?	Soluble	–	–	?	?	0
8	PF13.0278	954	1.6	No	Soluble	0	0	2	5	1
9	PFE0765w	2,133	0.7	No	Soluble	0	0	1	2	1
10	PF11.0479	3,029	2.4	No	Soluble and insoluble	0	0	4	8	4
11	PFC0450w	109	16.5	Yes	Soluble	1	2	0	1	1
12	PF14.0074	395	26.6	No	Soluble	0	0	8	8	3
13	PFC0700c	307	NA	?	Soluble	0	1	0	0	0
14	PF14.0291	1,234	1.0	Yes	Insoluble	0	0	0	1	1
15	MAL8P1.78	100	48.8	Yes	Soluble and insoluble	0	0	9	9	4
16	MAL13P1.218	253	3.4	Yes	Soluble	0	0	0	1	1
17	PFD0425w	984	25.3	Yes	Soluble	1	0	15	18	4
18	PF13.0320	1,791	1.4	No	Soluble and insoluble	0	21	3	6	3
19	PF11.0435	1,815	0.8	Yes	Soluble	0	6	1	1	1
20	PFI0260	4,544	2.2	No	Soluble and insoluble	0	0	9	13	6
21	PF11.0226	2,024	0.8	No	Soluble and insoluble	1	0	3	5	1
22	PFA0515w	1,471	3.3	No	Soluble	0	0	1	8	2
23	MAL6P1.252	2,647	1.7	No	Soluble	0	1	3	3	1
24	PF14.0236	1,781	3.4	No	Soluble and insoluble	0	0	2	6	3
25	PF14.0751	188	2.1	Yes	Soluble	1	1	0	1	1
26	PF14.0648	96	0.5	No	Soluble	0	0	2	2	1
27	PF11.0456	1,464	4.0	No	Soluble	0	0	0	5	2
CSP	PFC0210c	397	10.1	Yes	Soluble	1	1	2		
SSP2	PF13.0201	574	36.1	Yes	Soluble	1	0	24		
EXP-1	PF11.0224	162	72.9	No	Soluble and insoluble	1	1	6		
LSA-1	X56203	1,909	NA	No	NA	1	1	NA		

NA, not applicable.

*Gene accession number.

[†]The number of amino acids in the translated gene product of initial gene loci predictions, based on an incomplete and heterogeneous *P. falciparum* genomic sequence database (09/05/01 version).

[‡]“Yes” indicates sequences found only in sporozoite preparations, and “No” indicates sequences found in both the sporozoite preparations and preparations from different parasite life stages.

[§]Absence (0) or presence (1) of predicted N-terminal signal peptide (SP), based on the SignalP algorithm (see <http://plasmdb.org>).

^{||}Absence (0) or presence (1–21) of predicted transmembrane domains (TM), based on the TMHMM algorithm (see <http://plasmdb.org>).

^{||}The number of peptides identified by using either the stringent or relaxed criteria, respectively, as described in the text.

**Sequences can be accessed via PlasmoDB, the official database of the malaria parasite genome project, at (<http://plasmdb.org>).

database by using SEQUEST software (30) to identify and prioritize a set of ORFs representing antigens potentially expressed in the sporozoite and intrahepatic stage of the parasite life cycle. Combined parasite/human (31, 32) or parasite/mosquito (*Anopheles*) (33) databases were also searched to account for spectra resulting from contaminating host or vector peptides.

A panel of 27 ORFs representative of putative *P. falciparum* proteins was investigated. These proteins were among the first proteins identified by a *P. falciparum* proteomic analysis presented in more detail elsewhere (28). The panel of selected ORFs was fairly diverse in its characteristics and included a range of expression levels, stage specificity, and membrane association (Table 2). Their size ranged between 96 and 4,544 aa (mean, 1,252), and the percentage of *P. falciparum* sporozoite sequence coverage (i.e., the percentage of the protein sequence covered by identified peptides) ranged from 0.5% to 49.5%. This panel included 10 antigens expressed only in sporozoites, and 17 antigens common to other stages of the parasite life cycle. Seventeen of the ORFs were identified only in soluble MudPIT fractions, one was identified only in insoluble fractions, and nine were identified in both soluble and insoluble fractions. The relevance of this feature relies in the fact that the insoluble fractions are thought to be enriched with membrane or organelle associated proteins (29). Eight of the 27 ORFs

had predicted signal sequences, and 10 had predicted transmembrane domains. However, no correlation between the presence of predicted transmembrane regions and recovery from the insoluble fraction was noted. The frequency of recognition in the *P. falciparum* proteome data set ranged between 16 peptide hits from six different sporozoite runs to single peptide hits. Although mass spectroscopy data cannot be taken as a quantitative measure of protein expression, the number of peptide hits is indicative of the abundance of each protein in the sporozoite preparations (29).

Four known and well characterized *P. falciparum* preerythrocytic-stage antigens [circumsporozoite protein (CSP), sporozoite surface protein 2 (SSP2), exported protein 1 (LSA-1), and exported protein 1 (EXP-1)] were analyzed in parallel by using the same methodology. These antigens vary in molecular mass between 23 kDa (EXP-1) and 230 kDa (LSA-1). CSP and SSP2 are expressed only in the sporozoite stage, LSA-1 is liver stage-specific, and EXP-1 is expressed in both the liver and erythrocytic stages of the parasite life cycle. In the course of proteomic analysis (28), CSP, SSP2, and EXP were identified by between 2 (CSP) and 24 (SSP2) peptide hits, and were associated with between 10.1% (CSP) and 72.9% (EXP-1) sequence coverage (28) (Table 2). LSA-1 was not identified during this analysis because it is expressed only during the liver stage of the *P. falciparum* life cycle (34), and MudPIT studies

were not carried out with *P. falciparum* liver stage parasite preparations (because of limitations in the availability of liver-stage parasite material). Two of the three known antigens (CSP and SSP2) were detected only in the soluble MudPIT fractions, whereas EXP-1 was detected in both soluble and insoluble (membrane protein) fractions (28).

The panel of candidate antigens was originally identified by searching against an incomplete and heterogeneous database. When searched against the final *P. falciparum* genomic sequence database (2) by using refined gene model predictions, and taking into consideration sequence information from the *Anopheles* (vector) (33) and human (host) (31, 32) databases, 19 of the 27 putative antigens could be identified by using stringent selection criteria, six could be identified only if the selection criteria were relaxed (no tryptic end requirement, cross-correlation score for +2 spectra lowered by 0.05) (28), and one (antigen 7) could not be found in the database, indicating that it either is not a gene or the gene modeling algorithm missed it. For another antigen (antigen 13), the peptide used to originally identify the protein is still found in the final *P. falciparum* proteome data set with a good cross-correlation score, meaning that the experimental MS/MS spectrum matched the theoretical spectrum of the identifying peptide fairly well, but its DeltaCn parameter (the normalized difference in cross-correlation scores between top scoring peptides for a given antigen) was very low (0.02). Thus, it cannot be definitively established from the SEQUEST result whether the peptide/protein was in the *P. falciparum* sporozoite preparations analyzed. Overall, the panel of 27 ORFs can be considered representative of a wide range of potential target antigens with different characteristics of expression and subcellular localization. Comparison of the antigenicity of these proteins should therefore allow one to determine those characteristics that may be considered appropriate predictors of antigenicity, and whether proteomic data can be used successfully to identify and prioritize candidate vaccine antigens.

Amino acid sequences from the 27 ORFs were scanned with HLA-A1, -A2, -A3, -B7, and -DR supertype prototype PIC algorithms. A total of 3,241 sequences were identified (range = 14–435; mean = 120 sequences per antigen). A set of 1,142 peptides was synthesized (range = 13–50; mean = 42; see Table 6, which is published as supporting information on the PNAS web site), where only the top 10 scoring candidates per supertype per antigen were selected for larger ORFs. Control sets of peptides from the four known and well characterized *P. falciparum* antigens (CSP, SSP2, LSA1, and EXP1) were predicted according to the same criteria applied to the panel of 27 unknown ORFs, and synthesized. Analysis revealed that the predictive algorithms were highly effective in identifying already known and validated epitopes from those antigens. Specifically, the majority ($\approx 81\%$) of known HLA-degenerate epitopes from those antigens (Table 7, which is published as supporting information on the PNAS web site) were identified by using the ImmunoSense algorithms: 87.1% (27 of 31) of previously identified class I (HLA-A2, -A3/A11, -A1, -A24) epitopes and 63.6% (7 of 11) of class II (DR1) epitopes.

In our previous analysis of known *P. falciparum* antigens, we considered, as a selection criterion, a high degree of conservancy of the predicted epitopes among sequences of different *P. falciparum* isolates. Because only a single *P. falciparum* genomic sequence is available at this time, this criterion could not be used in our analysis; however, it could be considered as additional sequences become available.

Predicted supertype epitopes were tested for their capacity to induce and recall IFN- γ immune responses by using PBMC from volunteers immunized with irradiated *P. falciparum* sporozoites ($n = 8$), or control volunteers mock immunized in parallel ($n = 4$) (Table 4). Sporozoite-immune individuals develop a vigorous and multifaceted immune response directed against multiple antigens, and it is presumed that the entire repertoire of sporozoite-induced T cell specificities is represented in those individuals. Peptides were

tested as pools containing 1 $\mu\text{g}/\text{ml}$ each peptide, with each antigen represented by a separate pool, in IFN- γ ELISPOT assays (18). Positive and negative control epitopes from well characterized non-*P. falciparum* antigens (cytomegalovirus, influenza, Epstein-Barr virus, HIV) were also included.

Considering a stimulation index (ratio test response/control) > 2.0 as positive, 16 of the 27 previously untested proteins were reproducibly recognized as antigens by at least two of the eight irradiated sporozoite immunized volunteers tested in two or more assays, but not by any of the four mock immunized controls (Table 3). Nine of the 27 antigens (nos. 2, 5, 18, 3, 22, 20, 11, 21, and 13) were recognized by at least 50% of irradiated sporozoite volunteers and classified as highly antigenic. Three antigens (nos. 1, 12, and 17) were recognized by 37.5% of volunteers and classified as intermediately antigenic. Four antigens were recognized by two volunteers, and classified as weakly antigenic. Finally, 11 of the 27 unknown antigens induced IFN- γ responses of sufficient magnitude to meet our criteria of positivity. Positive control peptides derived from cytomegalovirus, influenza, and Epstein-Barr virus were recognized by 87.5% of the volunteers tested.

The magnitude of these responses is relatively low, but not substantially different from those observed with the pool of positive control peptides. The specificity of these responses is further demonstrated by the fact that no response was observed for all antigens in mock-immunized controls, and all individuals in the case of 11 of the experimental *P. falciparum* proteins analyzed.

Pools of predicted epitopes from the known antigens (CSP, SSP2, LSA1, and EXP1) were also recognized by irradiated sporozoite volunteers, though the frequency of response to those pools was somewhat lower than that to pools of peptides representing previously validated epitopes derived from the same antigens (22, 23) (Table 3). It is particularly noteworthy that the reactivity against several of the newly identified antigens exceeded the reactivities observed against well-characterized antigens, suggesting that some of the novel antigens identified by using our strategy may represent better candidates for vaccine development. For example, responses to antigen 2 were significantly better than responses to EXP-1, LSA-1 or CSP, as evidenced by the number of responding volunteers (8 of 8 vs. 1 of 8, 2 of 8, and 3 of 8; $P = 0.001, 0.007$, and 0.026), and number of spot-forming cells (SFCs) (122.7 vs. 41.3, 34.2, and 41.6). Responses were also better than to PfSSP2, but those differences were not significant. Similarly, responses to antigen 5 were significantly better than responses to EXP-1 or LSA-1 (number of responding volunteers: 7 of 8 vs. 1 of 8, 2 of 8; $P = 0.010$, and 0.041 ; number of SFCs: 101.8 vs. 41.3 and 34.2), and better than responses to CSP or SSP2 (Table 3).

Overall, there was no significant difference for protected versus not protected volunteers with regard to either rate of response (42 of 128 vs. 32 of 128, $P = 0.215$) or magnitude of response as assessed by stimulation index (all antigens, 2.7 ± 0.9 vs. 2.5 ± 0.6 ; high immune reactivity, 2.9 ± 1.0 vs. 2.6 ± 0.6 ; intermediate, 2.5 ± 0.4 vs. 2.3 ± 0.3 ; low, 2.2 vs. 2.3 ± 0.2 ; $P = 0.259$) or mean SFC (all antigens, 71.4 ± 51.8 vs. 90.5 ± 70.4 ; high immune reactivity, 89.0 ± 50.7 vs. 99.3 ± 80.4 ; intermediate, 29.1 ± 16.8 vs. 68.8 ± 26.9 ; low, 55.3 vs. 65.8 ± 31.8 ; $P = 0.182$). However, at the level of individual antigens, there was a trend for some antigens to be preferentially recognized by protected volunteers: for example, antigens 2 and 13 (Fig. 1). Interestingly, responses to the well characterized antigens, CSP, SSP2, LSA1, and EXP1, were detected predominantly in nonprotected volunteers (Fig. 1). Additional studies with increased sample sizes will be required to more finally establish whether individuals protected against *P. falciparum* sporozoite challenge selectively recognize specific antigens. Such studies will also establish whether protective immune responses in humans vaccinated with irradiated *P. falciparum* sporozoites are narrowly focused on a few immunodominant antigens and epitopes, or alternatively, are dispersed on a relatively large numbers of parasite antigens.

Table 3. Summary of irradiated sporozoite immune reactivities against a subset of the panel of 27 *P. falciparum* proteins and four known antigens

Source	Antigen	No. volunteers responding	Percent volunteers responding	SI*	SFC [†]
Test <i>Plasmodium</i> proteins	2	8	100	3.3 ± 1.3	122.7 ± 90.6
	5	7	87.5	2.8 ± 1.1	101.8 ± 74.0
	18	6	75	2.2 ± 0.2	58.4 ± 24.4
	3	6	75	2.6 ± 0.4	119.1 ± 69.5
	22	5	62.5	2.9 ± 0.9	108.4 ± 78.1
	20	4	50	2.5 ± 0.5	74.8 ± 40.1
	11	4	50	3.1 ± 0.8	81.3 ± 47.9
	21	4	50	2.3 ± 0.3	48.2 ± 16.5
	13	4	50	2.9 ± 1.1	92.2 ± 50.1
	1	3	37.5	2.4 ± 0.2	61.4 ± 42.0
	17	3	37.5	2.4 ± 0.3	57.6 ± 34.5
	12	3	37.5	2.2 ± 0.3	48.2 ± 16.5
	16	2	25	2.2 ± 0.3	27.2 ± 23.3
	15	2	25	2.5 ± 0.7	28.8 ± 20.0
	19	2	25	2.7 ± 0.6	31.3 ± 22.6
	9	2	25	2.5 ± 0.2	32.0 ± 28.3
		Range	2–8	25–100	2.0–5.9
	Mean	4.1	50.8	2.6	80.3
Known <i>Plasmodium</i> antigens	CSP	3	37.5	2.7 ± 0.6	41.6 ± 20.1
	SSP2	5	62.5	2.9 ± 1.0	97.2 ± 82.0
	LSA1	2	25	3.2 ± 1.4	34.2 ± 29.0
	EXP1	1	12.5	3.4	41.3
	Range	1–5	12.5–62.5	2.0–4.2	12.3–230.0
	Mean	2.75	34.4	2.9	65.5
Control epitopes [‡]	CMV, EBV, influenza	7	87.5 [§]	4.0 ± 2.2	59.0 ± 30.5

CMV, cytomegalovirus; EBV, Epstein–Barr virus.

*SI (stimulation index) indicates fold over background response to media and negative control peptide (HLA-A2 restricted HIV gag protein, residues 77–85), mean ± SD.

[†]Net SFC indicates counts above background counts to media and negative control peptide (HLA-A2 restricted HIV gag, residues 77–85), mean ± SD.

[‡]Positive control peptides derived from CMV phosphoprotein, residues 495–503 (HLA-A2); influenza virus matrix protein, residues 58–66 (HLA-A2); influenza virus nucleoprotein, residues 265–273 (HLA-A3); and EBV EBNA3, residues 339–347 (HLA-B8).

[§]Positive responses to control epitopes were detected in all four mock-immunized volunteers.

HLA-A2 peptide pools from antigens 2, 5, and 13, and HLA-A1 and HLA-DR peptide pools from antigens 2 and 5, are recognized by irradiated sporozoite volunteers who express the respective HLA alleles, but not by mock immunized controls.

Additionally, a comprehensive analysis of HLA binding against the HLA-A1, -A2, -A3/11, -A24, and -DR supertypes

has been completed for selected antigens (antigens 2, 5, 12, and 13). Several degenerate binders have been identified for each supertype/antigen combination, and 50–70% of the predicted supertype peptides have been shown to have the capacity to bind to 50% or more of the HLA molecules from a given supertype (Tables 8 and 9, which are published as supporting information on the PNAS web site). These data represent an important validation of the predictive strategy used, and demonstrate that our predictive algorithms can be used to effectively predict pools of supertype binding peptides.

Further analysis revealed the extent to which the antigenicity results correlate with the proteomic data. Of the nine antigens associated with highest immune reactivity, six were identified by multiple peptide hits in multiple MudPIT runs (see Tables 2 and 3). However, the converse was not true in that some proteins identified by multiple hits in multiple runs were either weakly or nonantigenic (e.g., antigens 10, 15, and 24). Clearly, some antigens are preferentially recognized by the immune system for reasons that may not simply reflect the relative abundance of antigen. Furthermore, four of the six antigens identified by only a single peptide hit were moderately to highly antigenic (e.g., antigens 1, 11, 16, and 19). Finally, some but not all antigens that were identified as candidates according to the relaxed MudPIT criteria, but not according to the stringent criteria, were highly antigenic (e.g., antigens 6 and 22). When relative antigenicity (Table 3) was directly compared with relative abundance of expression in the *P. falciparum* life cycle (Table 2), no significant association was detected ($r^2 = 0.088$; simple linear regression analysis). These data indicate that although genomics, proteomics, and *in silico* approaches can be used to

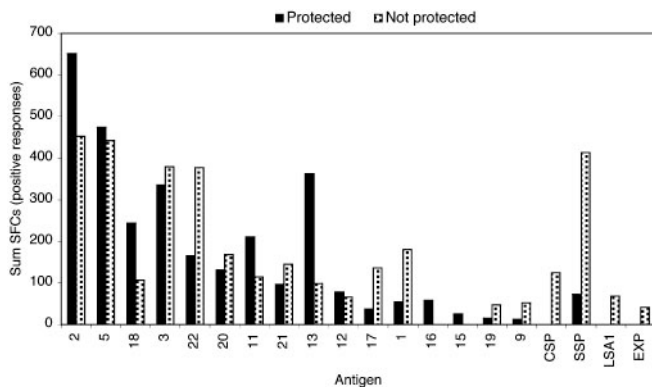


Fig. 1. Antigen-specific reactivity in irradiated sporozoite immunized volunteers protected or not protected against sporozoite challenge. PBMCs derived from volunteers immunized with irradiated *P. falciparum* sporozoites and either protected or not protected against challenge with infectious sporozoites were tested by IFN- γ ELISPOT assay against peptide pools from putative *P. falciparum* proteins, as described in the text. Data are presented as summed SFC of all responses that met predefined criteria of positivity.

prioritize ORFs or antigens for characterization, they are not sufficient on their own and must be considered in combination with other strategies, such as immunological screening, which take into account biological activity or function.

Discussion

The data presented herein have several important implications. First, we describe a novel strategy to mine genomic sequence databases for the identification and prioritization of novel target antigens and epitopes recognized by experimentally vaccinated, infected and/or naturally exposed humans, in the context of multiple genetic restrictions. This report provides an example of identifying antigens through epitope predictions; all other methods and reports described in the literature using algorithm predictions start from known antigens to identify epitopes. Our data support the potential of this approach to identify immunodominant antigens and to allow for prioritization among those antigens on the basis of immune reactivity. We have analyzed 27 proteins out of $\approx 1,000$ estimated to be expressed in the sporozoite/liver stage ($\approx 3\%$ of all potential proteins), and identified nine highly antigenic antigens. Eleven of the 27 antigens were not recognized by any volunteer in any assay. In independent studies in our laboratory, seven of the 27 ORFs tested here were assessed in a humoral immunoscreening assay. Of those, four of four antigens classified with high immune reactivity and one of one antigen with intermediate immune reactivity were shown to be recognized by malaria-immune sera but not by malaria-naïve sera, in as many as five of eight experiments (P. Quinones-Casas, personal communication). Even given that the subset screened represents only a small fraction of putative preerythrocytic stage proteins, the number of antigens shown to be recognized by sporozoite-immune T cell responses are 3-fold more than the sum total of all antigens previously known. Extrapolating this data, it is possible that several hundred proteins are targets of the immune response to irradiated sporozoites in the general human population. We are currently undertaking a systematic study of additional protein antigens to expand the observations to a larger number of *P. falciparum* proteins. Of those that are recognized, antigens of the most interest for vaccine development would be those preferentially targeted by immune responses that efficiently kill or inhibit the function of the parasite. In that regard, antigens and epitopes targeted by CD8⁺ T cell response may be considered of the greatest interest, based on evidence in animal models

implicating CD8⁺ T cells as critical mediators of protective immunity.

Second, it was known previously that B cell responses against complex pathogens are directed against a large number of antigens. In the case of malaria, for example, immunoprecipitation with human sera followed by 2D gels revealed a large number of different antigens (35–37). However, it is commonly assumed that T cell responses are directed against a relatively few immunodominant epitopes. Our study represents the first example showing that T cell responses in humans immunized with irradiated sporozoites are dispersed over a relatively large number of parasite antigens, rather than narrowly focused on very few immunodominant proteins and epitopes. Moreover, our results support the concept that a multiantigen vaccine will likely be needed to induce high effective protective immunity against malaria, and that a multiepitope approach should mimic natural responses in individuals experimentally vaccinated or naturally exposed to *P. falciparum*. Although data from murine studies show that immune responses directed against single antigens can be protective in selected inbred strains, genetic restriction of immune responses and protective immunity is well established (38), and it is widely believed that immune responses against multiple target antigens or epitopes will be required to effectively immunize an outbred human population. Indeed, clinical trials conducted to date with vaccines based on single antigens have been disappointing (39).

Our data also suggest that strict immunodominance (4) (i.e., only one or a few antigens or epitopes dominating the responses) does not apply in the context of the complex *Plasmodium* spp. parasite. Rather, a very complex and multispecific response exists and, among those antigens recognized, defined patterns of immunodominance might be more quantitative than qualitative. We are expanding our studies to determine whether this situation extends to naturally occurring infections, and to vaccination and other experimental systems.

Overall our data provide the foundation for development of an antigen map of sporozoite/liver stages (immunosome), and for the development and optimization of multiantigen and multiepitope vaccines designed to mimic the complexity of responses elicited by natural infection.

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