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HLA-DR-Promiscuous T Cell Epitopes from Plasmodium falciparum Pre-Erythrocytic-Stage Antigens Restricted by Multiple HLA Class II Alleles\textsuperscript{1,2}

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Previously, we identified and established the antigenicity of 17 CD8\textsuperscript{+} T cell epitopes from five \textit{P. falciparum} Ags that are restricted by multiple common HLA class I alleles. Here, we report the identification of 11 peptides from the same Ags, circumsporozoite protein, sporozoite surface protein 2, exported protein-1, and liver-stage Ag-1, that bind between at least five and up to 11 different HLA-DR molecules representative of the most common HLA-DR Ags worldwide. These peptides recall lymphoproliferative and cytokine responses in immune individuals experimentally immunized with radiation-attenuated \textit{Plasmodium falciparum} sporozoites (irradiated sporozoites) or semi-immune individuals naturally exposed to malaria in Irian Jaya or Kenya. We establish that all peptides are recognized by individuals of each of the three populations, and that the frequency and magnitude of helper T lymphocyte responses to each peptide is influenced by the intensity of exposure to \textit{P. falciparum} sporozoites. Mean frequencies of lymphoproliferative responses are 53.2\% (irradiated sporozoites) vs 22.4\% (Kenyan) vs 5.8\% (Javanese), and mean frequencies of IFN-\gamma responses are 66.3\% (irradiated sporozoites) vs 27.3\% (Kenyan) vs 8.7\% (Javanese). The identification of HLA class II degenerate T cell epitopes from \textit{P. falciparum} validates our predictive strategy in a biologically relevant system and supports the potential for developing a broadly efficacious epitope-based vaccine against malaria focused on a limited number of peptide specificities. \textit{The Journal of Immunology}, 2000, 165: 1123–1137.

Malaria is the most important parasitic disease affecting the human species, exacting an estimated toll of five deaths and 950 clinical cases per minute (1). Development of a vaccine against malaria presents formidable obstacles in terms of parasite biology and host immune responses. Different Ags that may be targeted by protective immune responses are expressed during the different stages of the parasite’s complex life cycle, and only a limited number of epitopes are contained within a relatively small number of Ags. Furthermore, because of HLA polymorphism, immune responses directed against these epitopes are genetically restricted and a given epitope may be recognized by one individual but not by another. Accordingly, an efficacious vaccine against malaria will need to have the potential to induce responses against a number of epitopes that are recognized in the context of many different HLA alleles.

CD8\textsuperscript{+} T cells have been implicated as critical effector cells in protective immunity against pre-erythrocytic-stage malaria (reviewed in Refs. 2 and 3), the developmental stage preceding the manifestation of clinical symptoms and the subsequent transmission of disease. Active immunization and adoptive transfer experiments also provide strong evidence for a critical role for CD4\textsuperscript{+} T cells (4, 5), as well as for Abs (6, 7) and cytokines such as IFN-\gamma (reviewed in Ref. 2). These CD4\textsuperscript{+} T cells could either directly act as immune effector cells per se or indirectly provide help for the development of CD8\textsuperscript{+} T cell- or Ab-mediated protective immunity. Accordingly, using a combination of immunochemical and cellular immunologic analyses based on specific HLA peptide binding motifs, we are designing a subunit vaccine against malaria comprising a number of CD8\textsuperscript{+} and CD4\textsuperscript{+} T cell epitopes from multiple \textit{Plasmodium falciparum} pre-erythrocytic-stage Ags that are restricted by common HLA class I and HLA class II alleles.

However, a potential obstacle to the development of a vaccine designed to induce T cell-mediated protective immunity is the large degree of polymorphism of human HLA molecules. Therefore, we have focused on HLA alleles that would allow coverage of all racial and ethnic populations, and preference has been given to those peptides that bind to more than one MHC. In particular,

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\textsuperscript{2}The studies reported herein were conducted in accordance with U.S. Navy, Kenyan Ministry of Health, and Republic of Indonesia regulations governing the protection of human subjects in medical research. The research protocols employing human subjects in this study were reviewed and approved by the Naval Medical Research Institute’s Committee for the Protection of Human Subjects, the Walter Reed Army Institute of Research Human Use Committee, the Kenya Medical Research Institute/National Ethical Review Committee, and the Indonesian Communicable Diseases Research Center Committee for the Protection of Human Subjects. The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or as reflecting the views of the U.S. Navy or naval services at large, the Department of Defense, or the Indonesian Ministry of Public Health.

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we have capitalized on the fact that different class I HLA mole-
cules overlap in their peptide binding specificities such that a given
epitope may bind with high affinity to multiple HLA alleles. A
large majority of all human HLA-A and -B molecules can be clas-
sified in one of nine different supertypes (8). HLA-A2, -A3, and
-B7 supertypes have been studied in detail (reviewed in Ref. 9),
and CD8+ T cell epitopes that belong to these supertypes have
now been identified and validated (8, 10–13).

However, class II epitope predictions on the basis of MHC bind-
ing motifs have been generally less accurate than those for class I
epitopes. Lower accuracy is presumably due to the fact that the
peptide binding groove of MHC class II molecules is less confor-
mationally restricted as compared with that of MHC class I mole-
cules (14). However, recent studies have revealed that a majority
of HLA-DR alleles are associated with largely overlapping pep-
tide-binding specificities (HLA-DR supertype) (15). Because the
corresponding Ags are represented with a high frequency in dif-
ferent ethnic populations, these data suggest that effective popu-
lation coverage could be achieved, also in the case of class II, by
the use of peptides binding multiple HLA-DR molecules.

We have previously reported the identification of 17 CD8+ T
cell epitopes on P. falciparum Ags that are recognized in the con-
text of multiple HLA-A2, HLA-A3, and HLA-B7 class I supertypes
(10). Here, we extend our earlier studies and exploit the
recently defined HLA-DR supertype to identify 11 peptides from
the same circumsporozoite protein (CSP),2 ssp2 surface protein
2 (SSP2), exported protein-1 (EXP-1), and liver-stage Ag-1
(LSA-1) Ags of P. falciparum that are capable of binding to at
least five and up to 11 of 14 tested DR molecules commonly
expressed in different populations. We further establish that all of
the identified peptides are antigenic, as assessed by their capacity
to recall specific lymphocyte proliferative and cytokine responses
from PBMC of individuals experimentally immunized with radi-
atation-attenuated from PBMC of individuals experimentally immunized with radi-

Materials and Methods

Sequence analysis

Using a customized computer algorithm analysis program (16), complete
sequences of the Ags CSP (17), SSP2 (18), LSA-1 (19), and EXP-1 (20)
from the 3D7 strain of P. falciparum were searched for the presence of
−15 amino acid sequences conforming to the previously described
HLA-DR binding motifs. Specifically, 15-mer sequences were selected that
contained a 9-residue core bearing the DR supermotif described by
O’Sullivan et al. (21) and Southwood et al. (15) with 3-residue N- and
C-terminal flanking regions. Additional peptides were identified on the
basis of an “extended” HLA-DR peptide binding motif that takes into con-
sideration the effects of secondary anchor residues (15). In cases where two
peptides that overlapped >90% were identified, a single longer peptide
incorporating both sequences was synthesized. Multiple isolates were
available for the CSP and SSP2 Ags, and only motif-containing peptides
that are totally conserved in at least 80% or more of all P. falciparum
isolates sequenced to date were considered for synthesis. Only one se-
quence was available for each of the LSA-1 and EXP-1 Ags at the time of
screening.

Peptide synthesis

Peptides were synthesized at Epimmune (San Diego, CA), as previously
described (22), or purchased as crude material from Chiron Mimotopes
(Chiron Corporation, Clayton, Victoria, Australia). Peptides synthesized at
Epimmune were purified to >95% homogeneity by reverse-phase HPLC.
Purity was determined on an analytical reverse-phase column, and their
composition was ascertained by amino acid analysis and/or mass spectrom-
etry analysis.

Abbreviations used in this paper: CSP, circumsporozoite protein; SSP2, ssp2 surface protein 2; EXP-1, exported protein-1; LSA, liver-stage Ag-1; SI, stimulation index; HTL, helper T lymphocyte.

Cells

The EBV-transformed homozygous B lymphoblastoid cell lines LG2 (DRB1*0101 (DR1)), GM107 (DRB5*0101 (DR2w2a)), MAT (DRB1*0301 (DR3)), PREISS (DRB1*0401 (DR4w4)), SWEIG (DRB1*1101 (DR5w11)), PITOUT (DRB1*0701 (DR7)), KT3 (DRB1*0405 (DR4w15)), Herluf (DRB1*1201 (DR5w12)), H301 (DRB1*1302 (DR6w19)), OLL (DRB1*0802 (DR8w2)), and HID (DRB1*0901 (DR9)), or the transfected fibroblasts L466.1 (DRB1*1501 (DR2w2b)), TR81.19 (DRB3*0101 (DR52a)), and L257.6 (DRB4*0101 (DRw53)) were used as sources of HLA class II molecules. Cells were maintained in vitro by culture in RPMI
1640 medium supplemented with 2 mM L-glutamine (Life Technologies,
Grand Island, NY), 50 μM 2-ME, and 10% heat-inactivated FCS (Irvine
Scientific, Santa Ana, CA). Cells were also supplemented with 100 μg/ml
of streptomycin and 100 U/ml of penicillin (Irvine Scientific, Santa Ana, CA).
Large quantities of cells were grown in spinner cultures.

Lysates were lysed for 30 min at 4°C with a lysis buffer of 50 mM Tris–HCl,
PH 8.5, 1% Nonidet P-40 (Fluka Biochemika, Buchs, Switzerland), 150 mM
NaCl, and 2 mM PMSF (Calbiochem, San Diego, CA). Lysates were cleared of
debris and nuclei by centrifugation at 15,000  g for 30 min.

Affinity purification of HLA-DR molecules

Class II molecules were purified by affinity chromatography, essentially as
described previously (23), using the mAb LB3.1 coupled to Sepharose
CL-4B beads. Lysates were filtered twice through two pre-columns of in-
activated Sepharose CL-4B and protein A-Sepharose, and then passed over
the anti-DR column. The anti-DR column was then washed with 10-vol-
tumes of PBS, 10 mM Tris–HCl, pH 8.0, in 1% Nonidet P-40, PBS,
2-column volumes of PBS, and 2-column volumes of PBS containing 0.4%
n-octylglucoside. Finally, DR molecules were eluted with 50 mM dieth-
arylamine in 0.15 M NaCl containing 0.4% n-octylglucoside, pH 11.5. A
1/25 volume of 2.0 M Tris, pH 6.8, was added to the eluate to reduce the
pH to ≤8.0. The eluate was then concentrated by centrifugation in Cen-
triprep 30 concentrators to 2000 rpm (Amicon, Beverly, MA).

Class II peptide binding assays

Peptide binding assays were performed as described previously (15, 23).
Specifically, purified human HLA class II molecules (5–500 nM) were
incubated with various unlabeled peptide inhibitors and 1–10 nM [3H]-
radiolabeled probe peptide for 48 h in PBS containing 0.05% Nonidet P-40
in the presence of a protease inhibitor cocktail. The final concentrations
of protease inhibitors (each from Calbiochem) were: 1 mM PMSF, 1.3 mM
1.10 phenanthroline, 73 μM pepstatin A, 8 mM EDTA, 6 mM N-ethyl-
maleimide, and 200 μM N-acetyl-L-lysine chloromethyl ketone. Final
detergent concentration in the incubation mixture was 0.05% Nonidet P-40.
Assays were performed at pH 7.0, except for DRB1*0301 and
DRB1*1501, which were performed at pH 4.5 and 5.0, respectively (23).
Class II peptide complexes were separated from free peptide by gel filtra-
tion on TSK200 columns (model 16215, Tosohaas, Montgomeryville,
PA), and the fraction of bound peptide was calculated as previously de-
scribed (23).

Radiolabeled peptides were iodinated using the chloramine-T method
(23). The radiolabeled probe peptides used were hemagglutinin Y307–319
(sequence YPKYVKQNTLKLAT; DRB1*0101), tetanus toxoid 830–843
(sequence QYIKANSKFIGITE; DRB5*0101, DRB1*1101, DRB1*0701,
DRB1*0802, DRB1*0901), myelin basic protein Y85–100 (sequence PVVH
FKKNIVPTTPPY; DRB1*1501), MT 65 kDa Y3–13 with Y7 substituted
with F (sequence YKTIAFDEEARR; DRB1*0301), a nonnatural peptide
sequence YPKYVKQNTLKLAT; DRB1*0101), tetanus toxoid 830–843
(sequence QYIKANSKFIGITE) for DRB1*1302.

In preliminary experiments, each DR preparation was titrated in the pres-
ence of a fixed amount of the appropriate radiolabeled peptide to determine
the concentration of class II molecules necessary to bind 10–20% of the
total radioactivity. All subsequent inhibition and direct binding assays were
performed using these class II concentrations.

Inhibitor peptides were typically tested at concentrations ranging from
120 μg/ml to 1.2 ng/ml in two to four completely independent experiments.
Under conditions where [MHC] and IC50

were determined as [MHC] IC50 values are reasonable approximations of true IC50 values. Peps-

were classified as binders for each DR molecule for which its binding

capacity was ≥1000 nM.

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Table I. Phenotypic frequencies of HLA-DR Ags

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<th>Representative Assay(s)</th>
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<th>Japanese</th>
<th>Chinese</th>
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<td>7.3</td>
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<td>20.0</td>
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</table>

Study populations

Four populations were studied. Subjects were aged between 17 and 65 years, and informed consent was obtained in all cases. Parasitemia at the time of blood collection was assessed by examination of Giemsa-stained thick blood films. Smears were considered negative after examination of 200 oil-immersion fields (×1000).

Population 1. Irradiated sporozoite immunized volunteers (n = 7) were exposed to the bites of between 1008 to 1576 gamma-irradiated (1.5 × 10⁶ rad) Anopheles stephensi mosquitoes infected by membrane feeding with either the NF54 strain (26) or the corresponding 3D7 clone (27) of P. falciparum, as previously described (28). Exposure was during 9–14 sessions over a period of 23 mo. All subjects were Caucasian, the mean age was 24 ± 10 years, and all were male. Immunological studies with these volunteers have been reported previously (10, 28–31). Preimmunization samples collected before immunization with radiation-attenuated sporozoites and postimmunization samples were assayed simultaneously.

Population 2. Malaria-exposed Javanese transmigrants (n = 13) were life-long residents of Saradidi, Siaya District, Nyanza Province, in the Asembo Bay area on the north and east shores of Lake Victoria, Western Kenya. In this area, transmission of malaria is very intense; the year-round prevalence of P. falciparum infection among children 6 mo to 6 years of age has been documented as 94.4–97.8% (32–34), the average daily entomological infection rate (number of bites by infectious Anopheline mosquitoes with P. falciparum sporozoites in their saliva glands per individual person per day) has been estimated as 0.75 ± 0.97 infectious bites per person (32), and the average number of episodes of clinical malaria per year has been estimated as 2–2.5 episodes. The average number of infectious bites per person per year is estimated as 100–200, most bites occurring during the peak transmission periods (April to August and November to January), which coincide with seasonal rains. Samples studied here were collected from subjects in April 1987, just before the peak transmission season. Study subjects were estimated to have been bitten by >100 P. falciparum-infected mosquitoes during a 126-day period immediately following sample collection (32, 35). Subjects were almost exclusively of the Luo ethnic group. The mean age was 30 ± 10 years, and all were male. Immunological studies with these volunteers have been reported previously (30, 28–31).

Population 3. Malaria-exposed Javanese transmigrants were residents of Arso Pir IV (Wonorejo), a lowland village located in the Arso District of northeastern Irian Jaya, the eastern-most province of Indonesia, ~60 km northeast of the International border. This site has been described previously (37, 38). In this area, malaria transmission is moderately intense, the incidence rate of P. falciparum infections has been documented (38, 39) as 3.0 cases per year, and the cross-sectional prevalence of P. falciparum parasitemia ranges from 30% to 70%. The village is populated by native Irianese (~20%) and by Javanese transmigrants (80%) who had moved voluntarily from malaria-free areas of West Java 5 years before the study. Study subjects were recruited from the transmigrant population and all were of the Javanese ethnicity. A total of 188 Javanese transmigrants were HLA typed, and a representative subset of 121 was selected for further study. Subjects reported no history of exposure to malaria before transmigration and were recruited from malaria-free areas of West Java 5 years before the study. Study subjects were estimated to have been bitten by >100 P. falciparum-infected mosquitoes during a 126-day period immediately following sample collection (32, 35). Subjects were almost exclusively of the Luo ethnic group. The mean age was 30 ± 10 years, and all were male. Immunological studies with these volunteers have been reported previously (30, 28–31).

Population 4. Malaria-naive Javanese individuals were residents of Blitar, west central Java, and had no history of exposure to malaria. Blitar is one of several villages in West Java where residents of Arso Pir IV lived before transmigration. A total of 188 residents of Blitar were HLA typed, and a subset of 110 was selected for further study. Subjects were of the same Javanese ethnicity as the malaria-exposed Arso transmigrant subjects and were further matched where possible for socioeconomic status, age, and sex. The mean age was 32.4 ± 10 years (range 17–65 years), 94 were male (52.2%), and 86 were female (47.8%).

HLA typing: frequency and projected population coverage analysis

Phenotypic frequencies of HLA class II alleles were established from peripheral blood samples using standard site-specific oligonucleotide PCR typing, in the case of the Javanese population, or compiled from the literature (40) in the case of the major ethnic groups. The gene frequency of each B1, B3, B4, or B5 allele was then either directly tabulated or calculated from the Ag frequency using the binomial distribution formulae:

\[ pf = 1 - (\text{SQRT}(1 - af)) \]

To obtain overall population coverage estimates for each peptide, cumulative gene frequencies (gf) of DR Ags that bind the peptide were calculated. The cumulative Ag frequency (i.e., the fraction of individuals capable of binding the particular epitope) was then derived by the use of the inverse formula:

\[ af = 1 - (\text{SQRT}(1 - gf)) \]

The impact of the two DR5 subtypes DR11 and DR12 were considered separately because DRB1*1101 and DRB1*1201, representative of DR11 and DR12, respectively, were known to have different binding specificities. In the case of DR6, which is divided between DR13 and DR14, subtypes were also used as the binding specificity of DR14 was not known.

The contribution of B3, B4, and B5 gene products to overall population coverage was also considered, based on the known strict linkage disequilibrium of the B3 gene product DR52a with DR3, of the B4 product DR53 with DR4, DR7, and DR9, and of the B5 product DR51 with DR2. A residual fraction (about 15%) of the genes in an average population are unspecified, using currently available HLA typing data (40). Therefore, to arrive at 100% accounting of genes, a fraction of the residual was added for each hit population cluster in proportion to the relative frequency of the cluster within the HLA-specified population. No adjustment was made for DRX.

The redundancy of coverage by the panel of epitopes is defined herein as the total number of different peptide/DR combinations potentially presented by a given individual and thus yielding a potentially immunogenic signal. Hence, because in the present study 11 peptides are considered, and each individual can express up to 4 different DR molecules, the theoretical maximum number of different peptide/DR combinations presented is \( 11 \times 4 = 44 \). The percentages of individuals yielding any given number of peptide/DR combinations known to bind with \( IC50 \) of 1000 nM or less was then calculated using Monte Carlo analyses (42). For these analyses, model populations for each ethnic group identified in Table I were first con-
structured using the gene frequencies of DR Ags in the corresponding ethnic population. These model populations were constructed without considering linkage disequilibrium, and DR Ags were represented in direct proportion to their corresponding gene frequencies. The number of DR epitopes presented by each individual in the model population was then determined by tabulating, for each model individual, the number of DR epitopes/combina-
tions associated with binding with an IC50 \( \leq 1000 \) nM. Finally, a histogram was generated to summarize the fraction of individuals in the pop-
ulation as a function of the number of DR-epitope combinations presented.

A cumulative plot was also generated to determine the minimal number of DR-epitope combinations presented by 85% of the individuals in a given
population. The analysis was performed for each ethnic group individually and for all ethnic groups combined.

An average population is defined herein as a fictional population com-
posed of equal numbers of Caucasian, North American Black, Japanese, Chinese, and Hispanic individuals, representative of the five major ethnic-
ties. In this study, no attempt has been made to adjust population coverage by either 1) prevalence of the different ethnic groups or 2) incidence of malaria within the different ethnic groups.

**PBMC collection and cultures**

Peripheral blood was collected by venipuncture into heparinized vacutain-
ers, and PBMC were isolated by standard Ficoll-Hypaque density gradient
centrifugation. Cell concentration was adjusted as appropriate, and lympho-
proliferative and cytokine assays were set up simultaneously. Cell cul-
ture medium consisted of RPMI 1640 containing 10 mM HEPES (Life Tech-
nologies) supplemented with 2 mM L-glutamine (Irvine Scientific), 0.5 mM sodium pyruvate (Life Technologies), 100 U/ml penicillin, 100 \( \mu \)g/ml streptomycin (Irvine Scientific), and 10% heat-
inactivated pooled human AB serum (Gemini Bioproducts, Calabasas, CA, or ICN Biomedica, Costa Mesa, CA).

**Induction and assay of peptide-specific lymphoproliferative responses**

In vitro induction of recall peptide-specific lymphoproliferative responses was achieved by culturing fresh (Javanese) or frozen (irradiated sporozo-
ites and Kenyan) PBMC at a concentration of 2 \( \times 10^5 \) cells/well in qua-
drumate in a volume of 0.2 ml complete medium in a round-bottom 96-well tissue culture plate in the presence of 200 and 20 \( \mu \)g/ml (Javanese) or 30, 10, and 3 \( \mu \)g/ml (irradiated sporozoites and Kenyan) of each peptide, without peptide (medium control), 8–12 wells/subject, or with mitogen (PHA at 10 \( \mu \)g/ml and Mycobacterium tuberculosis purified protein derivative at 5.0 \( \mu \)g/ml) for 6 days at 37°C in an atmosphere of 5% CO2. Peptide concentrations were selected based on limitations imposed by cell numbers and peptide quantity in the different populations and preliminary studies with other peptides in the same populations. There was no association between peptide concentration and peptide-specific T cell responsiveness for any assay in any of the populations studied here (data not shown). Wells were pulsed with 1.0 \( \mu \)Ci [3H]methyl thymidine (DuPont NEN, Boston, MA) for 16–18 h, and uptake was assessed by liquid scintillation spec-
troscopy (model LS6800, Beckman Coulter, Fullerton, CA). Results were expressed as a stimulation index (SI, cpm test sample/cpm medium control without peptide). Average medium control values were 263.1, 209.1, and 405.2 for irradiated sporozoites, Kenyan, and Javanese samples, respec-
tively. As defined in previous studies (43–45), the response to a peptide was considered positive if the SI (ratio of stimulated to unstimulated cells) was \( >2.0 \).

**Induction and assay of peptide-specific cytokine responses**

Fresh (Javanese) or frozen (irradiated sporozoites and Kenyan) PBMC were cultured at a concentration of 1 \( \times 10^5 \) cells/ml per well in a volume of 1.0 ml complete medium in 24-well tissue culture plates (Costar, Cam-
bridge, MA) in the presence of each peptide (10 \( \mu \)g/ml), without peptide (medium control), or with mitogen (PHA at 10 \( \mu \)g/ml and Mycobacterium tuberculosis purified protein derivative at 5.0 \( \mu \)g/ml) for 6 days at 37°C in an atmosphere of 5% CO2. Peptide concentrations were selected based on limitations imposed by cell numbers and peptide quantity in the different populations and preliminary studies with other peptides in the same populations. There was no association between peptide concentration and peptide-specific T cell responsiveness for any assay in any of the populations studied here (data not shown). Wells were pulsed with 1.0 \( \mu \)Ci [3H]methyl thymidine (DuPont NEN, Boston, MA) for 16–18 h, and uptake was assessed by liquid scintillation spec-
troscopy (model LS6800, Beckman Coulter, Fullerton, CA). Results were expressed as a stimulation index (SI, cpm test sample/cpm medium control without peptide). Average medium control values were 263.1, 209.1, and 405.2 for irradiated sporozoites, Kenyan, and Javanese samples, respec-
tively. As defined in previous studies (43–45), the response to a peptide was considered positive if the SI (ratio of stimulated to unstimulated cells) was \( >2.0 \).

**IFN-γ**

The IFN-γ bioassay was based on a previously described proto-
col (46) using the WISH cell line and EMC virus. WISH cells were cul-
tured in complete RPMI 1640 medium and were maintained by splitting twice weekly. The recombinant human IFN-γ standard was obtained commerc-
ially (Genzyme,Cambridge, MA; sp. act. = 1.0–4.75 \( \times 10^5 \) U/mg at 10 \( \mu \)g/ml, aliquoted into working stocks of 1 \( \times 10^5 \) U/ml that were stored at \( -70^\circ C \) before working aliquots being stored at 4°C and reused for up to 4–6 wk). For the bioassay, WISH cells were washed three times, resuspended at a concentration of 1 \( \times 10^5 \) cells/ml, and aliquoted in 50-\( \mu \)l volumes (5 \( \times 10^4 \) cells/well) in flat-bottom 96-well plates. Fifty microliters of test samples (diluted 1:3, 1:9, and 1:27) or IFN-γ standard dilutions (100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 U/ml) were added in triplicate. Fifty microliters of medium was added to 12 wells to serve as the medium control. Cells were cultured at 37°C in an atmosphere of 5% CO2 for 24 h or until the monolayer was confluent, and the medium was then aspirated. One hundred microliters of EMC virus (multiplicity of in-
fec tion \( -1.0 \) ) was added to all wells excluding the three wells representing the cell control (without virus). Cells were cultured for an additional 24 h at 37°C, washed three times, fixed with 100 \( \mu \)l of 5% formaldehyde for 10 min at room temperature, stained with 100 \( \mu \)l of 0.05% crystal violet in 20% ethanol for 10 min at room temperature, washed with 100 \( \mu \)l of 100% methanol to dislodge dye from fixed cells, and then assessed for cytopathic effect relative to the virus control wells (100% cytopathic effect) and cell control wells (0% cytopathic effect) by reading the OD at 540 nm. The IFN-γ concentration of test samples was calculated by reference to the standard curve. Values were corrected for the appropriate dilution factor and the average of all three corrected values recorded for each subject.

**Statistical analysis**

Nonparametric continuous outcome variables (mean peptide-specific cyto-
kine response) were compared using the Mann-Whitney U rank sum test (two-tailed). Distributions of cytokine concentrations (log-transformed) did not deviate significantly from normality (data not shown). The preva-
ience of dichotomous outcome variables (frequency of peptide-specific pro-
liferative T cell responses or cytokine responses) was assessed using the

**Results**

**Selection of a panel of representative DR molecules**

In the series of experiments described herein, we sought to identify a set of HLA class II-restricted epitopes that would allow redundant
coverage of the world population, irrespective of ethnicity. Accordingly, we focused our attention on a set of the 10 different HLA-DR Ags most prevalent throughout the world. The pheno-
typic frequencies of these alleles in various common ethnic groups are detailed in Table I. A similar table has been published else-
where (15) and is shown here for reference purposes only.

For each Ag, the most common HLA-DRB1 allelic form was studied, as representative of that particular Ag. However, in the case of DR4, both the DRB1*0401 and DRB1*0405 were studied, as one form is prevalent in Caucasians while the other is prevalent among Oriental ethnicities. Similarly, in the case of DR5, both DRB1*1101 and DRB1*1201 were studied, as representative of that particular Ag. However, in the case of DRB1*1201 was studied, as one form is prevalent in Caucasians while the other is prevalent among Oriental ethnicities. DR10 was not studied herein, but the frequency of this allele is relatively low in most ethnicities.

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At the DR locus of humans, in addition to the product of the B1 gene that is expressed in conjunction with a monomorphic α chain, the products of other genes (B3, B4, and B5) are also expressed, in conjunction with the same α-chain. These DR molecules corre-
spond to the serologic DR51, 52, and 53 Ags. Because of linkage disequilibrium, DR2-positive individuals usually express DR51, individuals expressing DR3, 11, 12, and 13 Ags usually express DR52, and individuals expressing DR4, 7, and 9 Ags usually express

**DR3.** In the present study, DRB5*0101, DRB3*0101 and DRB4*0101 have also been studied as representative of the DR51, 52, and 53 Ags, respectively (Table I).
Table II. HLA-DR binding capacity of \textit{P. falciparum}-derived peptides

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>HLA-DR Alleles Bound</th>
<th>IC(_{50}) nM</th>
<th>Population Conservation</th>
<th>DRB1 Gene Products</th>
<th>DRB3/B4/B5 Gene Products</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DR3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSP 2 MrKLAILSVSSFLFV</td>
<td>DRB1<em>0301, DRB1</em>1201</td>
<td>9/10</td>
<td>50</td>
<td>18</td>
<td>DRB3<em>0101, DRB4</em>0101, DRB5<em>0101, DRB7</em>0101, DRB8*0101</td>
</tr>
<tr>
<td><strong>DR4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSP 5 MrNYGKQENWYSLKK</td>
<td>DRB1<em>0301, DRB1</em>0802, DRB1<em>1101, DRB1</em>1201</td>
<td>6/10</td>
<td>60</td>
<td>6.4</td>
<td>DRB4<em>0101, DRB5</em>0101, DRB7<em>0101, DRB8</em>0101</td>
</tr>
<tr>
<td><strong>DR5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSP 37 SSFVNVVNSSIGLIM</td>
<td>DRB1<em>0301, DRB1</em>0802, DRB1<em>1101, DRB1</em>1201, DRB1*0401</td>
<td>7/12</td>
<td>58</td>
<td>42</td>
<td>DRB4<em>0101, DRB5</em>0101, DRB7<em>0101, DRB8</em>0101</td>
</tr>
<tr>
<td><strong>DR52</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSP2 223 VKNVIGPFMKAVCVE</td>
<td>DRB1<em>0301, DRB1</em>0802, DRB1<em>1101, DRB1</em>1201</td>
<td>7/14</td>
<td>50</td>
<td>56</td>
<td>DRB4<em>0101, DRB5</em>0101, DRB7<em>0101, DRB8</em>0101</td>
</tr>
<tr>
<td><strong>DR53</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSP 37 SSFVNVVNSSIGLIM</td>
<td>DRB1<em>0301, DRB1</em>0802, DRB1<em>1101, DRB1</em>1201</td>
<td>7/12</td>
<td>58</td>
<td>–</td>
<td>DRB4<em>0101, DRB5</em>0101, DRB7<em>0101, DRB8</em>0101</td>
</tr>
<tr>
<td><strong>DR7</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSP 2 MrKLAILSVSSFLFV</td>
<td>DRB1<em>0301, DRB1</em>1201</td>
<td>9/10</td>
<td>50</td>
<td>18</td>
<td>DRB3<em>0101, DRB4</em>0101, DRB5<em>0101, DRB7</em>0101, DRB8*0101</td>
</tr>
<tr>
<td><strong>DR8</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EXP-1 71 KSKYKLATSVLAGLL</td>
<td>DRB1<em>0301, DRB1</em>1201</td>
<td>9/14</td>
<td>64</td>
<td>3.6</td>
<td>DRB4<em>0101, DRB5</em>0101, DRB7<em>0101, DRB8</em>0101</td>
</tr>
<tr>
<td><strong>DR9</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSP2 527 GLAYKFVVPGAATPY</td>
<td>DRB1<em>0301, DRB1</em>1201</td>
<td>9/14</td>
<td>64</td>
<td>3.1</td>
<td>DRB4<em>0101, DRB5</em>0101, DRB7<em>0101, DRB8</em>0101</td>
</tr>
<tr>
<td><strong>DR5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSP2 62 HNWVNHAVPLAMKLI</td>
<td>DRB1<em>0301, DRB1</em>1201</td>
<td>10/14</td>
<td>71</td>
<td>14</td>
<td>DRB4<em>0101, DRB5</em>0101, DRB7<em>0101, DRB8</em>0101</td>
</tr>
<tr>
<td><strong>DR5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSP2 509 KYKIAGGIAGGLALL</td>
<td>DRB1<em>0301, DRB1</em>1201</td>
<td>7/11</td>
<td>64</td>
<td>132</td>
<td>DRB4<em>0101, DRB5</em>0101, DRB7<em>0101, DRB8</em>0101</td>
</tr>
<tr>
<td><strong>DR52</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EXP-1 71 KSKYKLATSVLAGLL</td>
<td>DRB1<em>0301, DRB1</em>1201</td>
<td>9/14</td>
<td>64</td>
<td>3.6</td>
<td>DRB4<em>0101, DRB5</em>0101, DRB7<em>0101, DRB8</em>0101</td>
</tr>
</tbody>
</table>

* Bold indicates IC\(_{50}\) > 1 \(\mu\)M. A dash (–) indicates IC\(_{50}\) > 20 \(\mu\)M.

Identification of conserved high-affinity \textit{P. falciparum} peptides containing specific HLA-DR binding motifs

Next, the sequences of the \textit{P. falciparum} CSP, SSP2, LSA-1, and EXP-1 Ags were screened for the presence of specific HLA-DR binding motifs. Specifically, 15-mer sequences were selected that contained a 9-residue core bearing the DR supermotif described by O’ Sullivan et al. (21) and Southwood et al. (15) with 3-residue N- and C-terminal flanking regions. In cases where two peptides that overlapped >90% were identified, a single longer peptide incorporating both sequences was synthesized.

Peptides were also selected on the basis of conservancy. For CSP and SSP2, where sequences of multiple variants of the same Ag were known at the time of analysis, peptides were considered for further study only if conservancy was >80%. However, only one sequence was available for LSA-1 and EXP-1 at the time of screening, so conservance could not be considered for these Ags.

Eighty-five \textit{P. falciparum}-derived, HLA-DR supermotif-containing peptides were initially tested for binding to the DR molecules in a primary panel of assays, encompassing DRB1*0101, DRB1*0401, and DRB1*0701. Peptides binding at least two of these three DR molecules were then tested for binding to a secondary panel comprised of DRB*1501, DRB5*0101, DRB1*1302, and DRB1*0901 molecules. Peptides binding at least two of the four secondary panel DR molecules, and thus cumulatively at least four of seven different DR molecules, were screened for binding to the DRB1*0405, DRB1*1101, and DRB1*0802, molecules comprising a tertiary assay panel. Finally, peptides binding at least seven of the 10 DR molecules comprising the primary, secondary, and tertiary screening assays were further tested for binding to DRB3*0101, DRB4*0101, DRB1*0301, and DRB1*1201.

Accordingly, eight different HLA-DR cross-reactive peptides were identified that bound at least seven of the 14 HLA-DR molecules tested. In the course of these studies, three other peptides were also identified that bound five or six of the DR molecules tested. Together, these 11 peptides (Table II) include at least one from each of the four \textit{P. falciparum} Ags considered: three were derived from CSP, five from SSP2, two from EXP-1 and one from LSA-1. To further address the issue of conservancy, \textit{P. falciparum} DNA regions coding for the 11 selected peptides were also retrospectively sequenced in 10 to 13 \textit{P. falciparum} parasite isolates from Arso (E. Gomez et al., manuscript in preparation) and analyzed in more recent deposits to the GenBank database. As shown in Table II, all of the peptides studied were found to be highly conserved in both cases, with conservancy values in the 83–100% range; in fact, seven of the 11 peptides were totally conserved. All peptides in which polymorphisms have been reported derive from the SSP2 Ag.

In conclusion, these studies identified a set of 11 conserved and highly cross-reactive DR binding epitopes derived from four different \textit{P. falciparum} Ags and projected to bind to HLA Ags expressed by a vast majority of human populations, irrespective of ethnicity.

Projected population coverage by the 11 epitopes in various different ethnicities

Also shown in Table II is the projected population coverage of each peptide, based on its capacity to bind HLA-DR molecules with IC\(_{50}\) \(\leq\) 1000 nM. Calculations of population coverage were based on the assumption that the DR molecules tested are representative of all subtypes of the same Ag and on the basis of known linkage disequilibriums, with the exception of DR6w13 and DR52a, as described in more detail in Material and Methods. As
Hepatic HTL epitopes from P. falciparum

Table III: Peptide-specific lymphoproliferative and cytokine responses in irradiated sporozoite volunteers

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Lymphoproliferation</th>
<th>IFN-γ</th>
<th>IL-5</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Response</td>
<td>Response (SI)</td>
<td>Range</td>
<td>% Response</td>
</tr>
<tr>
<td>CSP-2</td>
<td>28.6</td>
<td>2.8</td>
<td>2.2–4.0</td>
<td>57.1</td>
</tr>
<tr>
<td>LSA-13</td>
<td>28.6</td>
<td>2.6</td>
<td>2.0–3.6</td>
<td>57.1</td>
</tr>
<tr>
<td>CSP-53</td>
<td>28.6</td>
<td>4.8</td>
<td>2.5–7.4</td>
<td>71.4</td>
</tr>
<tr>
<td>SSP2-61</td>
<td>42.9</td>
<td>2.3</td>
<td>2.0–2.7</td>
<td>42.9</td>
</tr>
<tr>
<td>SSP2-223</td>
<td>28.6</td>
<td>2.5</td>
<td>2.3–3.0</td>
<td>85.7</td>
</tr>
<tr>
<td>CSP-375</td>
<td>57.1</td>
<td>3.3</td>
<td>2.2–4.6</td>
<td>57.1</td>
</tr>
<tr>
<td>EXP-82</td>
<td>85.7</td>
<td>7.6</td>
<td>2.7–28.1</td>
<td>85.7</td>
</tr>
<tr>
<td>EXP-71</td>
<td>85.7</td>
<td>5.4</td>
<td>2.0–9.7</td>
<td>71.4</td>
</tr>
<tr>
<td>SSP2-527</td>
<td>85.7</td>
<td>5.1</td>
<td>2.1–11.7</td>
<td>85.7</td>
</tr>
<tr>
<td>SSP2-62</td>
<td>42.9</td>
<td>3.2</td>
<td>2.0–5.0</td>
<td>28.6</td>
</tr>
<tr>
<td>SSP2-509</td>
<td>57.1</td>
<td>4.1</td>
<td>2.1–8.0</td>
<td>85.7</td>
</tr>
</tbody>
</table>

Overall (41/77) 53.2 4.0 2.0–28.1 66.2 128.2 2.2–708.2 11.7 13.1 2.0–61.1 15.6 74.2 26.3–115.7

TABLE III: Peptide-specific lymphoproliferative and cytokine responses in irradiated sporozoite volunteers. Each individual can express up to four different DR molecules; the theoretical maximum number of different peptide/DR combinations presented is 11 X 4 = 44.

The percentage of individuals yielding any given number of peptide/DR combinations known to bind with an IC50 of 1000 nM or less is shown in Fig. 1a. It can be seen that only 3% of individuals are not predicted to bind any peptide. The average number of DR-peptide combinations presented is 14.1. On Fig. 1b cumulative population coverages are also shown, as a function of ethnicity. It can be seen that regardless of ethnicities, >85% of the individuals are predicted to be capable of recognizing four or more epitope/DR combinations.

The apparent lower coverage of the Black population is most likely artifactual and due to the fact that a significant number (37%) of individuals in this ethnic group, as per the published HLA workshop update, was serologically typed DRX, indicating that no DRB1 allele has as yet been associated, and accordingly, for which no representative DR binding assay can be assigned as yet.

In conclusion, the analysis presented herein supports the notion that the selected set of peptides should allow redundant coverage of a large fraction of the human population, regardless of ethnicity.

Antigenicity of HLA-DR-supertype degenerate binding peptides for irradiated sporozoite-immunized individuals: lymphoproliferative responses

In the next series of experiments, the 11 selected HLA-DR degenerate binding peptides (Table II) were tested for their ability to elicit in vitro recall lymphoproliferative responses from frozen PBMC of seven Caucasian volunteers immunized with irradiated sporozoites. HLA allelic frequencies in this population have been established previously using serological typing and oligonucleotide-specific PCR typing. Of the five individuals typed, three expressed HLA-DRB1*0701, two expressed DRB1*0101, and one expressed each of DRB1*0301, DRB1*1101, DRB1*1201, DRB1*1501, and DRB1*1701. All of these molecules are represented in the assay panel described in Tables I and II.

As defined in Materials and Methods, a lymphoproliferative response was considered positive if the SI was >2.0. According to this criteria, all 11 HLA-DR degenerate, high-binding peptides tested were recognized as helper T lymphocyte (HTL) epitopes by PBMC derived from HLA-matched sporozoite-immunized volunteers (Table III).

Overall, recall lymphoproliferative responses were detected in 53.2% (41/77) of assays. The fraction of individuals responding to

FIGURE 1. Projected population coverage in model populations by the panel of 11 epitopes. a, Percent of individuals projected to present the indicated number of HLA-DR/ P. falciparum epitope combinations bound in a model average population, generated by averaging the gene frequencies in Caucasian, North American Black, Japanese, Chinese, and Hispanic populations (b). Also shown is the cumulative plot of percent projected population coverages, defined as the total number of different ethnic populations, we also calculated and tabulated projected population coverages, defined as the total number of different DR/peptide combinations potentially presented in a given individual and thus yielding a potentially immunogenic signal. Hence, because in the present study 11 peptides are considered, and each individual can express up to four different DR molecules; the theoretical maximum number of different peptide/DR combinations presented is 11 X 4 = 44.

The percentage of individuals yielding any given number of peptide/DR combinations known to bind with an IC50 of 1000 nM or less is shown in Fig. 1a. It can be seen that only 3% of individuals are not predicted to bind any peptide. The average number of DR-peptide combinations presented is 14.1. On Fig. 1b cumulative population coverages are also shown, as a function of ethnicity. It can be seen that regardless of ethnicities, >85% of the individuals are predicted to be capable of recognizing four or more epitope/DR combinations.

The apparent lower coverage of the Black population is most likely artifactual and due to the fact that a significant number (37%) of individuals in this ethnic group, as per the published HLA workshop update, was serologically typed DRX, indicating that no DRB1 allele has as yet been associated, and accordingly, for which no representative DR binding assay can be assigned as yet.

In conclusion, the analysis presented herein supports the notion that the selected set of peptides should allow redundant coverage of a large fraction of the human population, regardless of ethnicity.
each peptide varied between 29% (two of seven individuals tested for reactivity to the peptides CSP-2, LSA-13, CSP-53, and SSP2–223) and 86% (six of seven individuals tested for reactivity to peptides EXP-82, EXP-71, and SSP2–527). The magnitude of peptide-specific lymphoproliferative responses also varied, with an overall mean SI for responders of 4.00 (range of 2.0–28.1).

Representative data, derived from samples collected pre- and postimmunization with irradiated sporozoites from one volunteer who expressed the alleles DRB1*0301 and DRB1*1701 is shown in Fig. 2.

The recall lymphoproliferative responses elicited by the \textit{P. falciparum} peptides were Ag specific because no significant proliferation was detected in the absence of peptide. Furthermore, these responses were induced by exposure to \textit{P. falciparum} because peptide-specific lymphoproliferative responses could not be generated from prebleeds of the same volunteers collected before sporozoite immunization (Fig. 2 and data not shown).

Antigenicity of HLA-DR-supertype degenerate binding peptides for irradiated sporozoite-immunized individuals: cytokine responses

In parallel, we also assessed the ability of the HLA-DR degenerate high-binding peptides to induce the production of cytokines (IFN-\(\gamma\), IL-5, and IL-10) by frozen PBMC of the seven volunteers immunized with irradiated sporozoites. IFN-\(\gamma\) was studied because it has been implicated previously in pre-erythrocytic-stage protective immunity (2) and because it may be considered a marker of Th1-type immune responses. IL-5 was studied as a marker of Th2-type immune responses, and IL-10 was studied because it is known to be involved in the regulation of immune responses. Recently, other studies (47, 48) have implicated a role for IL-10 in protective immunity against malaria. An overall summary of the frequency and magnitude of the peptide-specific induction of IFN-\(\gamma\), IL-5, and IL-10 from irradiated sporozoite-immunized volunteers expressing class II molecules of the HLA-DR supertype for each of the 11 peptides tested is shown in Table III.

All 11 of the peptides were able to induce a recall peptide-specific IFN-\(\gamma\) response from PBMC of volunteers immunized with irradiated sporozoites (mean response, 128.2 pg/ml; range, 2.2–708.2 pg/ml). Specifically, peptide-specific IFN-\(\gamma\) responses were detected in a total of 66.2% (51/77) of assays. The frequency of response ranged between 28.6% (two of seven donors tested for reactivity to peptide SSP2–62) and 85.7% (six of seven donors tested for reactivity to peptides SSP2–223, EXP-82, SSP2–527, and SSP2–509).

In contrast, peptide-specific IL-5 responses were detected in only 11.7% (9/77) of assays (mean response, 13.1 pg/ml; range, 2.0–61.1 pg/ml). Of the 11 peptides, four peptides were not able to induce an IL-5 response in any of the volunteers tested, five peptides were able to induce a response in only one of the seven volunteers (the same volunteer for all peptides), and the other two peptides (SSP2–223 and EXP-71) were able to induce a response in only two of the seven volunteers.

Likewise, peptide-specific IL-10 responses were detected in only 15.6% (12/77) of assays (mean response, 74.2 pg/ml; range, 26.3–115.7 pg/ml). Five of the 11 peptides were not able to induce IL-10 production from PBMC of any of the seven volunteers, two peptides were able to induce IL-10 production from only one of the seven volunteers, three of the peptides were able to induce IL-10 from only two of the seven volunteers, and one peptide (CSP-2) was able to induce IL-10 production from four of the seven volunteers.

A general correlation existed between the proliferation and IFN-\(\gamma\) release data, with the EXP-82, EXP-71, and SSP2–527 peptides being most active in both assays.
These data are consistent with a cytokine profile mostly of the Th1 type for the HLA-DR degenerate binding peptides. Furthermore, the responses appeared to be induced by exposure to *P. falciparum* because peptide-specific cytokine responses could not be generated from prebleeds of the same volunteers collected before sporozoite immunization (data not shown). In summary, the data presented above demonstrate that all of the epitopes predicted on the basis of the in vitro analysis are indeed naturally processed and presented in vivo because all peptides induced a peptide-specific lymphoproliferative response and peptide-specific cytokine responses in volunteers immunized with irradiated sporozoites. These data also establish that a T cell repertoire capable of being expanded as a result of deliberate immunization exists for each of them.

### Antigenicity of degenerate peptides for recall HTL responses from individuals naturally exposed to hyperendemic malaria

In the next series of experiments, we examined whether peptide-specific lymphoproliferative and cytokine responses could also be recalled from frozen PBMC of semiimmune individuals with lifelong natural exposure to hyperendemic malaria, with an estimated average daily entomological inoculation rate of 0.75 infectious bites per person and an average number of 2.1–2.5 episodes of clinical malaria per year.

A total of 13 HLA-typed Kenyan individuals were studied, all of whom expressed DR alleles (including DR1, 3, 6, 9, 11, and 13) predicted to bind the 11 selected peptides. These peptides were tested for their ability to elicit in vitro recall lymphoproliferative responses under the same conditions and according to the same criteria established for the irradiated sporozoite-immunized volunteers above. Results are summarized in Table IV.

As noted for the irradiated sporozoite-immunized volunteers, all 11 HLA-DR degenerate binding peptides were recognized as HTL epitopes by PBMC derived from Kenyan subjects naturally exposed to hyperendemic malaria.

Overall, recall lymphoproliferative responses were detected in 22.4% (32/143) of assays. The fraction of individuals responding to each peptide varied between 7.7% (one of 13 individuals tested for reactivity to peptides CSP-53 and SSP2-509) and 46.2% (seven of 13 individuals tested for reactivity to peptide LSA-13). The magnitude of peptide-specific lymphoproliferative responses also varied, with an overall mean SI for responders of 3.0 (range, 2.0–6.7). Representative data for one individual (4Z) who expressed the alleles B1*0901, B1*1302, B3*0301, and B4*0101 is presented in Fig. 3.

The profile of peptide-specific cytokine responses in Kenyan subjects was similar to that noted for irradiated sporozoite-immunized volunteers. Specifically, peptide-specific IFN-γ responses were detected for 11 of the 11 peptides (mean response, 17.1 pg/ml; range, 2.1–74.1 pg/ml) in a total of 27.3% (39/143) of assays, peptide-specific IL-5 responses were detected for only three of the 11 peptides (mean response, 9.0 pg/ml; range, 7.9–8.2 pg/ml) in 2.8% (4/143) of assays, and peptide-specific IL-10 responses were detected for seven of the 11 peptides (mean response, 32.4 pg/ml; range, 5.1–167.6 pg/ml) in 11.9% (17/143) of assays.

### Expression of HLA-DR Ags in a Javanese population naturally exposed to mesoendemic malaria

Next, we planned to examine whether peptide-specific lymphoproliferative and cytokine responses could also be recalled from PBMC of semiimmune Javanese individuals with a more limited exposure (5 years) to a lower intensity of malaria transmission, with an estimated average daily entomological inoculation rate of 0.28 infectious bites per person and an average of 1.00 episodes of clinical malaria per life time.

Individuals in the Javanese study population were HLA typed using oligonucleotide-specific PCR methods. It was noted (Table V) that the most prevalent alleles in this population were HLA-DRB1*1202, DRB1*1500, DRB1*1502, and DRB1*0701, which were expressed in 65.2, 17.0, 25.9, and 19.6% individuals, respectively. As expected, the most prevalent HLA-DR alleles studied (Table I) afforded an actual coverage of the Javanese population of 100%.

Considering the molecules studied as representative of the DR alleles in the Javanese population, the average number of bound peptide/HLA-DR combinations (per individual) was 13.8. As shown in Fig. 4, 100% of the individuals in the study population would be predicted to have the capacity to present four or more peptide-DR combinations, and 65% to present 12 or more. These data validate, in a real population setting, the notion that redundant HLA coverage of a large fraction of individuals can be achieved with relatively few DR cross-reactive binding peptides.

### Table IV. Peptide-specific lymphoproliferative and cytokine responses in Kenyan subjects

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Lymphoproliferation</th>
<th>IFN-γ</th>
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<th>IL-10</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>% Response (SI)</td>
<td>Mean response (pg/ml)</td>
<td>Range response</td>
<td>% Response</td>
</tr>
<tr>
<td>CSP-2</td>
<td>15.4</td>
<td>2.4</td>
<td>2.3–2.5</td>
<td>15.4</td>
</tr>
<tr>
<td>LSA-13</td>
<td>46.2</td>
<td>3.2</td>
<td>2.1–6.6</td>
<td>23.1</td>
</tr>
<tr>
<td>CSP-53</td>
<td>7.7</td>
<td>2.9</td>
<td>2.7–3.1</td>
<td>15.4</td>
</tr>
<tr>
<td>SSP2-61</td>
<td>15.4</td>
<td>2.9</td>
<td>2.8–3.0</td>
<td>15.4</td>
</tr>
<tr>
<td>SSP2-223</td>
<td>23.1</td>
<td>2.4</td>
<td>2.1–3.4</td>
<td>7.7</td>
</tr>
<tr>
<td>CSP-375</td>
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<td>2.3–6.7</td>
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</tr>
<tr>
<td>EXP-82</td>
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<td>2.2–4.3</td>
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</tr>
<tr>
<td>EXP-71</td>
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<td>2.2–3.5</td>
<td>15.4</td>
</tr>
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<td>SSP2-527</td>
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<td>2.4–3.5</td>
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</tr>
<tr>
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<td>2.0–4.1</td>
<td>61.5</td>
</tr>
<tr>
<td>SSP2-509</td>
<td>7.7</td>
<td>2.9</td>
<td>2.7–3.1</td>
<td>53.8</td>
</tr>
<tr>
<td>Overall</td>
<td>22.4</td>
<td>3.0</td>
<td>2.0–6.7</td>
<td>27.3</td>
</tr>
</tbody>
</table>

Note: Table IV includes data for Peptide CSP-2 and LSA-13 only, as these were the only peptides recognized by the majority of Kenyan subjects.
Antigenicity of degenerate peptides for recall HTL responses from individuals naturally exposed to mesoendemic malaria

The ability of the 11 HLA-DR degenerate binding peptides to elicit in vitro recall lymphoproliferative responses from fresh PBMC of naturally exposed Javanese subjects was assessed next. As noted for the immune irradiated sporozoite-immunized volunteers and semiimmune naturally exposed Kenyan subjects, all 11 peptides were recognized as HTL epitopes by PBMC from the Javanese subjects (Table VI). A representative profile for one individual (4031) who expressed the alleles DRB1*1202, DRB1*1500, DRB3*0301, DRB5*0101, DQB1*0301, and DQB1*0600 is presented in Fig. 5. The frequency of the recall lymphoproliferative responses was markedly reduced as compared with the responses of the irradiated sporozoite-immunized and Kenyan populations. Specifically, recall lymphoproliferative responses were detected in only 5.8% (77/1320) of assays, the fraction of individuals responding to each peptide varying between 2.5% (three of 121 individuals tested for reactivity to the peptide CSP-53, SSP2–527, and SSP2–509) and 10.8% (13 of 121 individuals tested for reactivity to the peptide CSP-375). With regard to the magnitude of peptide-specific lymphoproliferative responses, the overall mean SI of responders was 2.9 (range, 2.0 – 8.9).

Table V. Phenotypic frequency of the Javanese population

<table>
<thead>
<tr>
<th>Ag</th>
<th>Allele</th>
<th>No. of Individuals</th>
<th>Phenotypic Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR1</td>
<td>DRB1*0103</td>
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</tr>
<tr>
<td>DR2</td>
<td>DRB1*1500</td>
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</tr>
<tr>
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<td>DRB1*1502</td>
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<td>DRB1*1503</td>
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<td></td>
<td>DRB1*1600</td>
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<tr>
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<td>DRB1*0404</td>
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</tr>
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</tr>
<tr>
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<td>DRB1*1202</td>
<td>73</td>
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</tr>
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<td>DRB1*1300</td>
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<td>3.6</td>
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<tr>
<td></td>
<td>DRB1*1302</td>
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<td>DRB1*0701</td>
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<td>DRB1*0804</td>
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</tr>
<tr>
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<td>DRB1*0806</td>
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<td></td>
<td>DRB1*0807</td>
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</tr>
<tr>
<td>DR9</td>
<td>DRB1*0901</td>
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<tr>
<td></td>
<td>DRB1*10–</td>
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</tbody>
</table>

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FIGURE 3. Recall peptide-specific, lymphoproliferative responses in an individual naturally exposed to hyperendemic malaria to HLA-DR-supertype peptides. PBMC from a semiimmune individual with lifelong exposure to hyperendemic malaria who expressed the HLA-DRB1*0901, B1*1302, B3*0301, and B4*0101 alleles were stimulated in vitro with each of the peptides (at 30, 10, and 3 μg/ml each), or without peptide, as described in Materials and Methods. Data are expressed as a SI, and the response to a peptide was considered positive if the SI was >2.0.

Table VI. Phenotypic frequency of the Kenyan population

<table>
<thead>
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<th>Ag</th>
<th>Allele</th>
<th>No. of Individuals</th>
<th>Phenotypic Frequency (%)</th>
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<tbody>
<tr>
<td>DR1</td>
<td>DRB1*0103</td>
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<td>0.9</td>
</tr>
<tr>
<td>DR2</td>
<td>DRB1*1500</td>
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<td>17.0</td>
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<td>DRB1*1502</td>
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<tr>
<td></td>
<td>DRB1*0404</td>
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<td>DRB1*1101</td>
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<td>0.9</td>
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<tr>
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<td>DRB1*1202</td>
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</tr>
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<td>DR6</td>
<td>DRB1*1300</td>
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<td>3.6</td>
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<td>DRB1*1302</td>
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<tr>
<td>DR7</td>
<td>DRB1*0701</td>
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<td>19.6</td>
</tr>
<tr>
<td>DR8</td>
<td>DRB1*0800</td>
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<td>2.7</td>
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<td>DRB1*0802</td>
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<td>DRB1*0803</td>
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<tr>
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<td>DRB1*0804</td>
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<td>DRB1*0806</td>
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<td>DRB1*10–</td>
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</tr>
</tbody>
</table>

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Antigenicity of degenerate peptides for recall HTL responses from individuals naturally exposed to mesoendemic malaria

The ability of the 11 HLA-DR degenerate binding peptides to elicit in vitro recall lymphoproliferative responses from fresh PBMC of naturally exposed Javanese subjects was assessed next. As noted for the immune irradiated sporozoite-immunized volunteers and semiimmune naturally exposed Kenyan subjects, all 11 peptides were recognized as HTL epitopes by PBMC from the Javanese subjects (Table VI). A representative profile for one individual (4031) who expressed the alleles DRB1*1202, DRB1*1500, DRB3*0301, DRB5*0101, DQB1*0301, and DQB1*0600 is presented in Fig. 5. The frequency of the recall lymphoproliferative responses was markedly reduced as compared with the responses of the irradiated sporozoite-immunized and Kenyan populations. Specifically, recall lymphoproliferative responses were detected in only 5.8% (77/1320) of assays, the fraction of individuals responding to each peptide varying between 2.5% (three of 121 individuals tested for reactivity to the peptide CSP-53, SSP2–527, and SSP2–509) and 10.8% (13 of 121 individuals tested for reactivity to the peptide CSP-375). With regard to the magnitude of peptide-specific lymphoproliferative responses, the overall mean SI of responders was 2.9 (range, 2.0 – 8.9).

Consistent with responses observed in the irradiated sporozoite-immunized and Kenyan populations, peptide-specific IFN-γ responses were also detected for all 11 of the peptides tested (mean response, 8.7 pg/ml; range, 2.0 – 63.4 pg/ml). However, as noted for the lymphoproliferative responses, the frequency of peptide-specific IFN-γ responses by the Javanese population was markedly reduced as compared with the other populations, with responses detected in a total of only 8.7% (84/962) of assays. The fraction of individuals responding to
each peptide varied between 4.5% (4 of 89 individuals tested for reactivity to the peptide SSP2–61) and 16.9% (14 of 83 individuals tested for reactivity to the peptide EXP-82). The frequency and magnitude of peptide-specific cytokine responses detected in the malaria-exposed Javanese population residing in Arso were nevertheless increased above those of a genetically matched control population residing in Blitar, East Java, with no history of malaria exposure (data not shown).

In summary, the data presented above demonstrate that significant recall lymphoproliferative and IFN-γ responses were detected for all 11 peptides in each of the three populations tested. Therefore, the T cell repertoire for each of the epitopes predicted on the basis of the in vitro peptide binding studies and demonstrated to be antigenic in a population experimentally immunized with high-dose sporozoite inoculum can be primed by natural exposure to both hyperendemic and mesoendemic malaria.

**Frequency and magnitude of HTL responses as a factor of transmission intensity**

The data presented above (Tables III, IV, and VI) suggest that the frequency and magnitude of peptide-specific lymphoproliferative and cytokine responses induced by experimental exposure to the bites of hundreds of infected mosquitoes are significantly greater than those induced by natural exposure to malaria and that the responses induced by natural exposure similarly reflect the level of transmission intensity. A comparison of the frequencies of lymphoproliferative and IFN-γ responses on a population basis are presented in Figs. 6 and 7, respectively.

The frequency of peptide-specific lymphoproliferative responses ranged from 28.6 to 85.7% (41/77, 53.2% overall) in irradiated sporozoite-immunized volunteers, from 7.7 to 46.2% (32/143, 22.4% overall) in Kenyans, and from 2.5 to 10.8% (77/1320, 5.8% overall) in Javanese individuals. The magnitude of response also varied, with a mean SI of 4.0 and maximum SI of 28 in irradiated sporozoite-immunized volunteers, a mean SI of 3.0 and maximum SI of 6.7 in Kenyans, and a mean SI of 2.9 and maximum SI of 8.9 in the Javanese. The difference between each of the three populations with regard to frequency of response was highly significant: irradiated sporozoite vs Kenyan, \( p < 10^{-2} \); irradiated sporozoite vs Javanese, \( p < 10^{-2} \); irradiated Kenyan vs Arso, \( p < 10^{-8} \).

The frequency of peptide-specific IFN-γ response ranged from 28.6 to 85.7% (51/77, 66.2% overall) in irradiated sporozoite-immunized volunteers, from 7.7 to 61.5% (39/143, 27.3% overall) in Kenyans, and from 4.5 to 16.9% (84/962, 8.7% overall) in the Javanese population. As with the lymphoproliferative response, the effect of population on frequency of IFN-γ response was highly significant: irradiated sporozoite vs Kenyan, \( p < 10^{-8} \); irradiated sporozoite vs Arso, \( p < 10^{-8} \); Kenyan vs Arso, \( p < 10^{-8} \).

We have previously reported a similar effect of sporozoite exposure on frequency and magnitude of CD8+ CTL and cytokine responses (10). The data in the present study as well as that reported previously

---

**Table VI. Peptide-specific lymphoproliferative and cytokine responses in Javanese subjects**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Lymphoproliferation</th>
<th>IFN-γ</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>% Response</td>
<td>Mean response (SI)</td>
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<td>CSP-2</td>
<td>6.7</td>
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<td>LSA-13</td>
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<td>2.6</td>
</tr>
<tr>
<td>Overall</td>
<td>5.8</td>
<td>2.9</td>
</tr>
</tbody>
</table>

(77/1320) (84/962)
are consistent with observations that all volunteers immunized with radiation-attenuated *P. falciparum* sporozoites are protected against experimental challenge, while individuals naturally exposed to malaria acquire a degree of protective immunity that reflects in part the degree of exposure to malaria. These data are consistent with studies indicating that the induction of protective immunity following immunization with irradiated sporozoites is affected by the number of immunizing bites (with an apparent threshold of

**FIGURE 5.** Recall peptide-specific, lymphoproliferative responses in an individual naturally exposed to mesoendemic malaria to HLA-DR-supertype peptides. PBMC from a semiimmune individual with 5 years exposure to mesoendemic malaria who expressed the HLA-DRB1*1202, DRB1*1500, DRB3*0301, DRB5*0101, DQB1*0301, and DQB1*0600 alleles were stimulated in vitro with each of the peptides (at 200 and 20 μg/ml each), or without peptide, as described in Materials and Methods. Data are expressed as a SI, and the response to a peptide was considered positive if the SI was >2.0.

**FIGURE 6.** Population comparison of HLA-DR-supertype peptide-specific lymphoproliferative responses. PBMC from immune volunteers experimentally immunized with irradiated sporozoites (Irr.Spz.; *n* = 7), from semiimmune individuals with lifelong exposure to high transmission hyperendemic malaria (Kenyan; *n* = 13), or from semiimmune individuals with 5 years exposure to lower transmission mesoendemic malaria (Javanese; *n* = 121) were stimulated in vitro with each of the peptides, or without peptide, and assayed for peptide-specific lymphoproliferative responses as described in Materials and Methods. For each population, the percentage responding to each of the peptides (SI > 2.0) is presented. Mean frequencies of lymphoproliferative responses were 53.2% (Irr.Spz.) vs 22.4% (Kenyan) vs 5.8% (Javanese).
exposure to ~900–1000 bites being required to confer protection), the interval between immunization and challenge, and the number of bites used in the challenge (L. Goh, S. L. Hoffman, et al., unpublished data).

**Discussion**

In this study, we have used a combination of immunochemical and cellular analyses based on HLA-specific peptide binding motifs and in vitro binding assays with purified HLA class II molecules to identify 11 degenerate HTL epitopes from four *P. falciparum* pre-erythrocytic-stage Ags that are restricted by multiple HLA-DR alleles. The sequences of the *P. falciparum* Ags CSP, SSP2, LSA-1, and EXP-1 were screened for the presence of the HLA-DR-supertype binding motif. A total of 85 peptides were identified. Fifty-two of these peptides contained the HLA-DR motif described by O'Sullivan et al. (21) (3× CSP, 26× SSP2, 11× LSA-1, 12× EXP-1) and an additional 33 peptides were identified on the basis of an “extended” HLA-DR peptide binding motif (15) (6× CSP, 7× SSP2, 15× LSA-1, 4× EXP-1). Twenty-one of the 85 peptides (24.7%) bound with high affinity to at least one HLA-DR molecule in vitro. Eleven of the 21 exhibited degenerate binding to between five and 11 of 14 common HLA-DR molecules, illustrating that degenerate HLA-DR binding capacity appears to be relatively frequent.

These 11 HLA-DR binding peptides were selected for further study and tested for their capacity to induce recall lymphoproliferative and cytokine responses from the PBMC of seven volunteers immunized with irradiated sporozoites, 13 volunteers exposed to hyperendemic malaria in western Kenya, and 121 individuals exposed to mesoendemic malaria in Irian Jaya. Each of the 11 peptides was shown to be recognized by T cells from individuals experimentally immunized with radiation-attenuated sporozoites or naturally exposed to malaria. The finding that each of the 11 peptides identified was antigenic for both recall lymphoproliferative and IFN-γ responses in each of three distinct populations is remarkable. This high frequency of success is likely attributed to the fact that all peptides were preselected on the basis of their ability to bind with high affinity to multiple HLA molecules in vitro and parallels previous results that identified 17 HLA class I degenerate CD8+ T cell epitopes (10). In the case of the class I (10), only seven of 49 peptides tested for binding to HLA-A*0201, eight of 203 peptides tested for binding to HLA-A3/A11 and two of 24 peptides tested for binding to HLA-B7 were selected for antigenicity studies and all were shown to be recognized as epitopes for both CTL and cytokine responses. Here, 11 of 85 peptides cross-reactive for binding to HLA-DR molecules in vitro were assessed for their ability to be recognized by peptide-specific lymphoproliferative and IFN-γ responses, and all were found to be antigenic.

Because each individual can express up to four different HLA-DR molecules, and the peptides studied here were selected on the basis of their ability to bind to multiple HLA-DR molecules in vitro, the present data do not address the degeneracy of the recall immune responses to each of the peptides at the level of individual HLA-DR alleles. Nonetheless, the data described above establish that each peptide epitope is capable of inducing a peptide-specific recall immune response in the context of multiple HLA-DR molecules. Likewise, studies of the biological relevance of the MHC binding affinity of peptides indicate that there is a correlation between the affinity of binding of peptides...
to MHC molecules and their ability to be recognized by specific T cells (15, 49). This aspect was not addressed in the present study, because nonbinding peptides were not tested. In fact, all of the peptides studied here (Table II) bound multiple HLA-DR molecules in vitro with an affinity <1000 nM, which is the threshold previously associated with immunogenicity for HLA class II epitopes (15).

Previously, Sinigaglia and colleagues (50) reported a conserved CSP epitope (CSP 378–398, sequence DIEKKIAKMKEASS VFNVVNS) that is recognized by HLA-DR1-, 2-, 4-, 5-, and 7-specific human T cell clones. More recently, another conserved CSP epitope that is presented by multiple HLA class II DR molecules (CSP 326–345, sequence EYLNIKQNSLSTEWSPCSVT) has been identified (51).

Interestingly, a high degree of overlap was noted (Table VII) between the regions of the P. falciparum Ags from which the 11 HLA-DR degenerate HTL epitopes were identified with those of the 17 HLA class I degenerate CD8+ T cell epitopes identified previously. Other studies have demonstrated a similar overlap of CD4+ T cell and CD8+ T cell epitopes (52–54) or have reported that mouse and human T cell epitopes map to similar regions for malaria (55) or other systems (56, 57).

Further validating our predictive strategy, all three CSP epitopes characterized in the present study were independently reported (43, 44) as the most immunodominant, conserved CD4+ T cell epitopes recognized by individuals naturally exposed to malaria in Gambia or Papua New Guinea, as assessed by lymphoproliferation. In those studies, the epitopes were identified by screening a panel of 29 overlapping peptides (20-mers) spanning the complete Ag. With regard to LSA-1, one study (58) reported lymphoproliferative and CD8+ T cell-mediated IFN-γ responses to three epitopes from LSA-1 (residues 84–107, 1813–1835, and 1888–1909) not studied here in individuals with lifelong exposure to holoendemic malaria in the Wosera region of Papua New Guinea. Another study (45) reported lymphoproliferative responses to four other peptides (residues 1613–1636, 1633–1659, and 1686–1719 as well as an epitope in the repeat region) in 6–20% of individuals residing in a low transmission area of Madagascar but not in individuals in a moderately high transmission area of West Africa or a seasonal low transmission area of northern Senegal. The only study of HTL responses to SSP2 (59) assessed IFN-γ and IL-4 responses by enzyme-linked immunospot assay, but not lymphoproliferative responses, to pools of overlapping peptides (20-mers); 21 epitopes were identified, but in all cases frequencies of responses were low (2%), and 81% of the identified epitope sequences were polymorphic. No studies of HTL responses to EXP-1 have been reported.

Together with the HLA class I degenerate CD8+ T cell epitopes identified previously (10), the class II epitopes identified herein may be incorporated in a vaccine designed to protect humans against P. falciparum malaria via T cell-mediated immune responses. Such a multivalent vaccine incorporating a wide repertoire of specificities at the epitope level would be expected to circumvent the problem of genetic restriction of the host immune response that has thus far been a major obstacle for malaria vaccine (60). On the basis of the frequencies of HLA-DR Ags in the general population, it was expected that 85% of the study population could be covered by at least one epitope. In the present study, 100% coverage was demonstrated in a real setting. Indeed, 100% of the Javanese study population was determined to have the capacity to present four or more DR-peptide epitopes. These results further validate the DR supermotif approach as a method to identify vaccine candidate epitopes.

In conclusion, herein we have analyzed the CSP, SSP2, EXP-1, and LSA-1 Ags of P. falciparum and identified 11 peptide epitopes capable of binding with high affinity (IC50 ≤ 10,000 nM) to between five and 11 of the 14 common HLA-DR alleles expressed by a high proportion of different ethnicities. From our data, it is inferred that all epitopes are naturally processed and an active T cell repertoire exists for each one of them, because all peptides recall specific lymphoproliferative and IFN-γ responses in individuals experimentally immunized with radiation-attenuated sporozoites. Further, this T cell repertoire could be primed by natural exposure because all peptides induced specific recall lymphoproliferative and IFN-γ responses in individuals naturally exposed to malaria. Finally, the frequency and magnitude of the recall immune response was influenced by the intensity of exposure to P. falciparum sporozoites.

These data provide considerable experimental support for the development of a subunit malaria vaccine comprising a small number peptide binding specificities that would be predicted to be broadly efficacious in the majority of all racial and ethnic populations.

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