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Degenerate Cytotoxic T Cell Epitopes from P. falciparum Restricted by Multiple HLA-A and HLA-B Supertype Alleles

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Summary

We recently described human leukocyte antigen (HLA) A2, A3 and B7 supertypes, characterized by largely overlapping peptide-binding specificities and represented in a high percentage of different populations. Here, we identified 17 Plasmodium falciparum peptides capable of binding these supertypes and assessed antigenicity in both vaccinated and naturally exposed populations. Positive cytotoxic T lymphocyte recall and cytokine (interferon-γ and tumor necrosis factor α) responses were detected for all peptides; all were recognized in the context of more than one HLA class I molecule; and at least 12 of the 17 were recognized in the context of all HLA alleles studied. These data validate the concept of HLA supertypes at the biological level, show that highly degenerate peptides are almost always recognized as epitopes, and demonstrate the feasibility of developing a universally effective vaccine by focusing on a limited number of peptide specificities.

Introduction

An estimated 300–500 million new Plasmodium infections and 1.5–2.7 million deaths occur each year due to malaria (World Health Organization, 1994). Fatal outcomes are not confined to first infections, and constant exposure is apparently a prerequisite for maintaining immunity. Naturally acquired sterile immunity is rare if it exists at all. Accordingly, major efforts to develop an efficacious malaria vaccine have been undertaken. The finding that sterile protective immunity against malaria can be induced by immunization with radiation-attenuated sporozoites of different Plasmodium spp. in mice, monkeys, and humans (reviewed by Hoffman et al., 1996) has focused much research on the preerythrocytic (sporozoite/liver) stage. A vaccine targeted at the preerythrocytic stage could prevent both sporozoites from invading hepatocytes and liver-stage parasites from developing to maturity. Such a vaccine would therefore prevent both clinical disease, which is manifested only during the erythrocytic stage, and the transmission of malaria, since no gametocytes would develop.

Considerable data implicate $CD8^+$ T cells in protection against preerythrocytic-stage malaria. $CD8^+$ cytotoxic T lymphocytes (CTLs) can eliminate Plasmodium berghei– or Plasmodium yoelii–infected hepatocytes from in vitro culture in a major histocompatibility complex (MHC)–restricted and antigen-specific manner (Hoffman et al., 1989, 1990; Weiss et al., 1990). Furthermore, adoptive transfer of such CTLs can also protect against P. yoelii and P. berghei sporozoite-induced malaria in the absence of other parasite-specific immune responses (Romero et al., 1989; Rodrigues et al., 1991; Weiss et al., 1992; Khusmith et al., 1994).

Indirect evidence that CTLs may be important in protective immunity against Plasmodium falciparum in humans has also accumulated. First, humans immunized with irradiated sporozoites or naturally exposed to malaria can generate a CTL response to the preerythrocytic-stage antigens, circumsporozoite protein (CSP), sporozoite surface protein 2 (SSP2), liver-stage antigen-1 (LSA-1), and exported protein-1 (Exp-1) (Malik et al., 1991; Doolan et al., 1991, 1993; Hill et al., 1992; Sedegah et al., 1992; Aidoo et al., 1995; Wizel et al., 1995a, 1995b; Lalvani et al., 1996). Second, there is evidence that the polymorphism within the CSP may be the result of selection by CTLs of parasites that express variant forms (McCutchan and Waters, 1990), since all variation is nonsynonymous at the nucleotide level, indicative of selective pressure at the protein level; the polymorphism primarily maps to identified CTL and T helper epitopes (Doolan et al., 1992); and CTL responses to some of the parasite variants do not cross-react (Hill et al., 1992). Finally, the MHC class I human leukocyte antigen (HLA)–Bw53 has been associated with resistance to severe malaria in The Gambia, and CTLs to a conserved epitope restricted by the HLA-Bw53 allele have been identified on P. falciparum LSA-1 (Hill et al., 1991, 1992). Since HLA-Bw53 is found in 15%–40% of the population of sub-Saharan Africa but in less than 1% of Caucasians and Asians, these data suggest evolutionary selection on the basis of protection against severe malaria.

Based on these data, our group has undertaken the task of developing a vaccine designed to elicit $CD8^+$, class I–restricted CTL responses. Specifically, we have chosen an epitope-based approach because a multiepitope vaccine would deal with the problems of genetic

restriction and parasite polymorphism (Doolan and Hoffman, 1997), and lipopeptide constructs have been shown to be highly immunogenic for CTL induction in humans (Vitiello et al., 1995) and efficacious in the prophylactic vaccination mode in the murine lymphocytic choriomeningitis virus model (van der Most et al., 1996).

The stringent specificity of the $CD8^+$ T cell–HLA interactions and the high degree of genetic polymorphism of HLA molecules could represent a major obstacle to the development of such epitope-based vaccines. However, it has recently been shown that many different HLA alleles significantly overlap in their peptide-binding specificity (reviewed by Sidney et al., 1996a), leading to the definition of HLA class I supertypes. Specifically, an A2-binding supertype including at least HLA-A*0201, *0202, *0203, *0205, *0206, *6802, and *6901 has recently been defined on the basis of a common peptide-binding motif (del Guercio et al., 1995). Peptide-binding motifs associated with two other supertypes, the A3 supertype (including but not restricted to A*0301, A*1101, A*3101, A*3301, A*3401, A*6601, A*6801, and A*7401) (Sidney et al., 1996b) and the B7 supertype (including B*0702–5, B*3501–3, B*5101–5, B*5301, B*5401, B*5501–2, B*5601, B*6701, and B*7801) (Sidney et al., 1995, 1996c), have also been identified. Since each supertype is represented in high frequencies (40%– 60%) in all major ethnicities, these data suggest that it should be possible to represent approximately 80%–90% of the world population by focusing on only three peptide-binding specificities.

In this study we screened the sequences of P. falciparum preerythrocytic-stage antigens for the presence of the A2-, A3- and B7 supertype binding motifs. Seven A2 supertype, eight A3 supertype, and two B7 supertype highly degenerate binding peptides were identified and tested for their capacity to induce recall CTL and cytokine responses from peripheral blood mononuclear cells (PBMCs) of 6 volunteers immunized with irradiated sporozoites and 85 individuals naturally exposed to malaria.

Results

Identification of Conserved, High-Affinity P. falciparum Peptides Containing Specific HLA Class I Binding Motifs

Sequences of the target antigens were scanned for the presence of HLA-A*0201, A3, A11, and B7 binding motifs as described in Experimental Procedures. Following conservancy determination and algorithm analysis to take into account the influence of secondary anchors, 274 peptide–HLA combinations of potential interest were identified. Of these, 50 peptides contained the HLA-A*0201 binding motif, 203 the HLA-A3/A11 motif, and 26 the HLA-B7 motif. Five peptides contained both HLA-A*0201 and A3/A11 binding motifs. When the affinity of peptide–HLA interaction was quantitated for each of the peptide–HLA combinations, good-affinity binding (half-maximal inhibitory concentration [IC_{50}] $\leqslant$500 nM) was detected in 42 of the 274 cases, 14 peptides binding to HLA-A*0201, 25 to HLA-A3/A11, and 4 to HLA-B7 (data not shown). One peptide (Exp-1_2) bound with high affinity to both HLA-A*0201 and HLA-A3/A11. Overall, 5 of the high-affinity–binding peptides were derived from CSP, 16 from SSP2, 10 from LSA-1, and 11 from Exp-1. Previous studies have indicated that an affinity threshold of 500 nM or less is associated with immunogenicity in the context of HLA class I molecules (Sette et al., 1994). Accordingly, these 42 peptide–HLA combinations were selected for further study.

Selection of Potential HLAA2-Supertype P. falciparum Epitopes

A total of 14 peptides of 50 tested bound HLA-A*0201 with good affinity ($IC_{50} \leqslant$500 nM) (data not shown). These peptides were tested for immunogenicity using primary CTL cultures with PBMCs from at least four malaria-naive human donors and immunization of at least three HLA-A*0201/K^b transgenic mice, as described previously (Wentworth et al., 1996). The induction of primary CTL responses in vitro with PBMCs from normal naive humans requires a brief treatment of the antigen-presenting cells with acidic buffer and subsequent neutralization in the presence of excess β_2-microglobulin and exogenous peptide (Wentworth et al., 1996). By ensuring that the majority of the HLA class I molecules are occupied by exogenous peptide, these steps are essential for the induction of primary CTL responses. Such responses cannot be induced using methods developed for the induction of recall CTL responses (see below). Here, a peptide was considered positive if yielding more than 2 $LU_{30}/10^6$ cells (lytic units 30% per 10^6 cells, where one lytic unit corresponds to the number of effector cells required to induce 30% ^{51}Cr release from 10,000 target cells during a 6 hr assay) or 15% peptide-specific lysis, respectively, in at least two different transgenic mice and two different primary CTL cultures. Of the 14 peptides tested, 5 scored positive both in primary in vitro CTL responses and in HLA transgenic mice (data not shown).

Next, these 5 peptides were tested for their capacity to bind other common A2 supertype alleles (HLA-A*0202, *0203,*0206, and *6802). The peptide $SSP2_{14–23}$, which was immunogenic in primary human CTL cultures and contains the $SSP2_{14–22}$ epitope (rather then $SSP2_{14–22}$ itself), was included in this analysis. In addition, we also arbitrarily included the peptide Exp-1_{83} (positive in the murine CTL assays) and the peptide CSP_{394} (the fourth–highest-affinity A*0201 binder of the 50 A2-motif peptides tested).

Seven of seven peptides tested bound ($IC_{50} \leqslant$500 nM) to two or more of the A2 supertype alleles tested (Table 1A). In particular, $SSP2_{14}$ bound all five molecules, while Exp-1_{80} and CSP_{394} bound four of the five alleles tested. In summary, the results presented in this section identified a set of seven potential CTL epitopes, characterized by degenerate binding capacity to multiple HLA-A2 supertype molecules.

Selection of Potential A3 Supertype and B7 Supertype P. falciparum Epitopes

Next, the binding capacity of A3 and B7 supertype P. falciparum–derived peptides was examined. Of 203 peptide–HLA combinations tested for binding, 25 peptides bound with high affinity to either A3 or A11 (Table 1B).

Table 1. Potential Epitopes

(A) A2-Like Supertype Potential Epitopes

Protein	First Position	Sequence	Binding (IC_{50}, nM) A*0201	A*0202	A*0203	A*0206	A*6802	Alleles Bound
SSP2	14	FLIFFDLFLV	12	10	5.9	11	333	5
Exp-1	80	VLAGLLGNV	19	24	0.67	31	606	4
CSP	394	GLIMVLSFL	22	19	3.6	74	4396	4
Exp-1	2	KILSVFFLA	5.0	172	3448	8.0	9524	3
Exp-1	83	GLLGNVSTV	24	1194	1.2	25	—	3
Exp-1	91	VLLGGVGLVL	94	—	2500	420	—	2
CSP	7	ILSVSSFLFV	208	3583	19	587	2105	2

(B) A3-Like Supertype Potential Epitopes

Protein	First Position	Sequence	Binding (IC_{50}, nM) A*0301	A*1101	A*3101	A*3301	A*6801	Alleles Bound
LSA-1	94	QTNFKSLLR	50	14	180	617	4.4	4
SSP2	523	LACAGLAYK	423	143	5294	64	32	4
CSP	344	VTCGNGIQVR	6875	11	15	64	444	4
Exp-1	10	ALFFIIFNK	9.2	2.2	720	1261	73	3
SSP2	522	LLACAGLAYK	22	73	692	1526	24	3
LSA-1	105	GVSENIFLK	151	5.0	2250	8286	10	3
LSA-1	59	HVLSHNSYEK	407	200	—	—	114	3
LSA-1	11	FILVNLLIFH	733	1333	1957	397	154	2
LSA-1	49	RINEEKHEK	786	40	486	—	—	2
Exp-1	2	KILSVFFLA	18	3.2	7826	5918	8889	2
LSA-1	1855	SLYDEHIKK	28	8.6	—	—	615	2
LSA-1	60	VLSHNSYEK	79	19	—	—	2857	2
Exp-1	28	GTGSGVSSK	126	40	—	—	3478	2
LSA-1	111	FLKENKLNK	145	3750	—	2636	444	2
SSP2	522	LLACAGLAY	190	5000	—	6591	2500	1
CSP	85	KLRKPKHKK	250	—	2250	—	—	1
SSP2	102	RLHSDASKNK	256	600	—	—	—	1
Exp-1	99	VLYNTEKGR	282	3158	8571	4203	1231	1
SSP2	100	IIRLHSDASK	324	—	—	—	—	1
SSP2	521	ALLACAGLAY	355	779	5455	5088	—	1
LSA-1	16	LLIFHINGK	478	612	—	—	167	1
LSA-1	1854	KSLYDEHIKK	1000	29	—	—	—	1
Exp-1	28	GTGSGVSSKK	1100	353	—	—	—	1
SSP2	120	SLLSTNLPY	1146	353	—	—	—	1
LSA-1	1854	KSLYDEHIK	—	231	—	—	—	1
SSP2	307	RGDNFAVEK	—	105	—	—	—	1

(C) B7-Like Supertype Potential Epitopes

Protein	First Position	Sequence	Binding (IC_{50}, nM) B*0701	B*3501	B*5101	B*5301	B*5401	Alleles Bound
Pfs16	77	MPLETQLAI	86	31	8.1	1.5	1.7	5
SSP2	539	TPYAGEPAPF	31	14	15	158	—	4
SSP2	305	RPRGDNFAV	5.0	—	—	—	526	1
SSP2	206	HPSDGKCNL	275	—	—	3321	—	1
SSP2	303	QPRPRGDNF	500	—	—	—	—	1

Dash indicates binding affinity ⩾10,000 nM.

These peptides were further tested for their capacity to bind other common members of the A3 supertype (A31, A33, and A*6801). Seven of them exhibited degenerate binding to three or more of the five alleles and were selected for further study. We also arbitrarily considered for further study the $LSA\text{-}1_{11}$ peptide, which bound strongly to A33 and A*6801 and weakly to A3 and A11 (Table 1B).

Likewise, 4 of 24 peptides tested for binding to HLA-B7 bound with high affinity. Of these, 1 peptide ($SSP2_{539}$) bound four of five B7 supertype alleles (Table 1C). The previously reported HLA-B–restricted peptide $Pfs16_{77}$ (Hill et al., 1992) was also tested and found to bind five of the five alleles (Table 1C).

In summary, the data presented here identified a total of eight HLA-A3 and two HLA-B7 peptides that bound

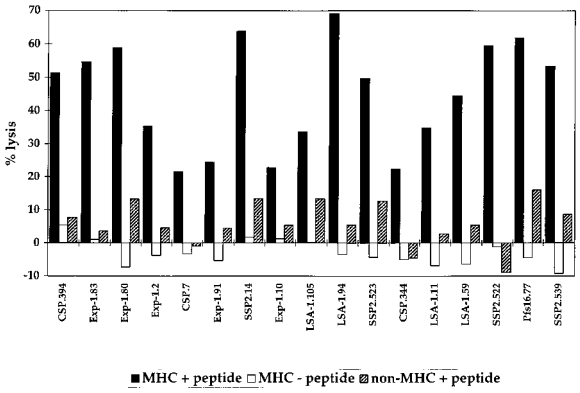

Figure 1. Induction of Peptide-Specific, MHC-Restricted, Sporozoite-Induced Recall CTL Response to A2, A3, and B7 Supertype Peptides

PBMCs from a volunteer immunized with irradiated sporozoites who expressed the HLA A*0201, A33, and B7 antigens were stimulated in vitro with each of the peptides as described in Experimental Procedures. Cultures were assessed for cytotoxicity at an effector:target ratio of 30:1 against PHA blast targets: either appropriate peptide-sensitized autologous PHA blasts (MHC + peptide), unsensitized autologous PHA blasts (MHC − peptide), or appropriate peptide-sensitized MHC-mismatched PHA blasts (non-MHC + peptide).

multiple members of the respective supertype with high affinity.

Antigenicity of HLA Supertype Degenerate Binding Peptides for Irradiated Sporozoite–Immunized Individuals

In the next series of experiments, the HLA class I degenerate binding peptides selected as described in the previous sections were tested for their ability to elicit in vitro recall responses from frozen PBMCs of six volunteers immunized with irradiated sporozoites.

Representative data, derived from one donor who expressed the HLA-A*0201, A33, and B7 alleles, are shown in Figure 1. The recall CTL responses elicited by the P. falciparum peptides were antigen specific since no lysis of MHC-matched target cells was detected in the absence of peptide. Furthermore, these responses were MHC restricted since no specific lysis was detected in the presence of appropriate peptide in association with MHC-mismatched target cells. These responses were indeed induced by exposure to P. falciparum since no specific CTL responses could be generated from prebleeds of the same volunteers collected before sporozoite immunization (data not shown).

Finally, the detected CTL activity was reproducible, since most donors were tested multiple times and responses were detected in up to 87.5% of assays. Specifically, the reproducibility of CTL responses for the A2 supertype peptides ranged from 16.7% (1/6 assays) with CSP_7 and $SSP2_{14}$ to 66.7% (4/6 assays) with CSP_{394} and Exp-1_{83}. For the A3 supertype epitopes, reproducibility ranged from 50.0% (4/8 assays) with Exp-1_{10} to 87.5% (7/8 assays) with CSP_{344}; activity was detected in 100% (5/5) of assays with peptides LSA-1_{94}, LSA-1_{11}, and CSP_{344} when tested in HLA-A3$^+$ individuals. For the B7 supertype peptides, responses to both $Pfs16_{77}$ and $SSP2_{539}$ were detected in 85.7% (6/7) assays; 100% reproducibility (2/2 assays) was observed for both peptides in an HLA-B57$^+$ individual. The overall reproducibility of response was 42.9% (18/42 assays) for the A2 supertype peptides, 67.2% (43/64 assays) for the A3 supertype peptides, and 85.7% (12/14 assays) for the B7 supertype epitopes.

A summary of the frequency and magnitude of the CTL responses detected in the sporozoite-immunized volunteers is presented in Table 2. As defined in Experimental Procedures and in previous studies (Aidoo et al., 1995; Wizel et al., 1995a, 1995b; Lalvani et al., 1996), a CTL culture was considered positive if yielding greater than 10% peptide-specific lysis (percentage peptide lysis − percentage control lysis). According to this criteria, all HLA-A2, HLA-A3, and HLA-B7 degenerate, high-binding peptides tested were recognized as CTL epitopes by PBMCs derived from HLA-matched sporozoite-immunized volunteers.

Specifically, it was established that all of the A2-binding peptides were recognized in association with HLA-A*0201 and that all of the A3-binding peptides were recognized in association with both A3 and A33, while the B7-binding peptides were presented in association with B7, B35, and B57 (Table 2). None of sporozoite-immunized volunteers expressed the alleles HLA-A*0202, A*0205, A11, A31, A68, B51, or B53, and antigenicity of the peptide panel in the context of these alleles therefore could not be addressed.

The fraction of individuals responding to each peptide varied between 33.3% (for one of three A3$^+$ individuals tested for reactivity to the peptide Exp-1_{10}) and 100% (for five of seven A2 peptides, three of eight A3 peptides, and two of two B7 peptides) (Table 2). The magnitude of responses also varied, with average specific lysis values in the 15%–39% range for A2 supertype–restricted responses, in the 16%–55% range for A3 supertype peptides, and 15%–27% in the case of B7 supertype epitopes (Table 2).

In summary, these data demonstrate that all of the epitopes predicted on the basis of the in vitro analysis are indeed processed and presented in vivo and recognized following immunization with irradiated sporozoites. Furthermore, they also show that a T cell repertoire susceptible of being expanded as a result of deliberate immunization exists for each of them.

Antigenicity of Degenerate Peptides for Recall CTLs from Naturally Exposed Individuals

In the next series of experiments, we examined whether specific CTL responses could also be recalled from frozen PBMCs of semi-immune or nonimmune individuals naturally exposed to malaria, with an estimated mean of 22 bites per 28 day period (Beier et al., 1994; McElroy et al., 1994).

Initially, a total of 197 Kenyan individuals were HLA typed. Of these, 39.0% expressed class I alleles of the HLA-A2 supertype (subtyped as A*0201, 55.6%; A*0202, 30.6%; and A*0205, 13.9%); 23.4% expressed HLA-A3 supertype antigens (A3, 7.1%; A31, 10.2%; A33, 6.1%; and A*6801, 11.7%); and 34.5% expressed HLA-B7 supertype molecules (B7, 8.6%; B35, 7.1%; B51, 4.6%;

Table 2. Frequency and Magnitude of CTL Responses in Irradiated Sporozoite–Immunized Volunteers

(A) A2 Supertype Peptides

HLA		CSP_{394}	Exp-1_{83}	Exp-1_{80}	Exp-1_{2}	CSP_{7}	Exp-1_{91}	$SSP2_{14}$
A201	Frequency of response[a]	100% (2/2)	100% (2/2)	100% (2/2)	100% (2/2)	50% (1/2)	100% (2/2)	50% (1/2)
	Mean % lysis (range)[b]	23.7 (13.0–37.2)	26.6 (10.6–56.8)	35.0 (12.2–53.0)	30.4 (13.7–47.1)	19.2	14.5 (10.0–19.0)	38.6

(B) A3 Supertype Peptides[b]

HLA		Exp-1_{10}	LSA-1_{105}	LSA-1_{94}	$SSP2_{523}$	CSP_{344}	LSA-1_{11}	LSA-1_{59}	$SSP2_{522}$
A3	Frequency of response	33.3% (1/3)	66.7% (2/3)	100% (3/3)	66.7% (2/3)	100% (3/3)	100% (3/3)	66.7% (2/3)	66.7% (2/3)
	Mean % lysis (range)	35.9 (17.7–49.5)	24.7 (12.5–41.8)	17.0 (11.7–21.5)	19.1 (10.6–29.3)	17.8 (14.1–20.4)	20.4 (13.6–29.0)	28.6 (13.6–56.9)	22.4 (18.9–26.5)
A33	Frequency of response	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)
	Mean % lysis (range)	26.6	23.6	55.4	29.3	16.2	17.7	31.8	35.4
A3 supertype overall	Frequency of response	50% (2/4)	75% (3/4)	100% (4/4)	75% (3/4)	100% (4/4)	100% (4/4)	75% (3/4)	75% (3/4)
	Mean % lysis (range)	33.6 (17.7–49.5)	24.4 (12.5–41.8)	28.1 (11.7–55.4)	21.1 (10.6–29.3)	17.3 (10.9–21.5)	19.9 (13.6–29.0)	29.2 (13.6–56.9)	25.0 (18.9–35.4)

(C) B7 Supertype Peptides

HLA		$Pfs16_{77}$	$SSP2_{539}$
B7	Frequency of response	100% (2/2)	100% (2/2)
	Mean % lysis (range)	21.4 (12.3–36.5)	26.7 (15.9–37.5)
B35	Frequency of response	100% (1/1)	100% (1/1)
	Mean % lysis (range)	16.9	14.5
B57	Frequency of response	100% (1/1)	100% (1/1)
	Mean % lysis (range)	21.4 (14.7–28.1)	16.5 (14.7–18.3)
B7 supertype overall	Frequency of response	100% (4/4)	100% (4/4)
	Mean % lysis (range)	20.7 (14.7–36.5)	20.2 (14.5–37.5)

Assays were conducted at an effector:target ratio of 30:1.

[a] Frequency of response represents the number of individuals positive in one or more assays. The number of positive individuals/total number of individuals tested is presented in parentheses.

[b] Mean % lysis represents the mean of positive values only.

and B53, 16.2%). Twenty-nine individuals were HLA-A69 positive but were not tested for recall CTL responses because of unavailability of an assay to discriminate between different subtypes of this allele. The overall fraction of individuals expressing at least one molecule from any of the three supertypes was 84.8%.

A representative subset of 53 naturally exposed individuals were tested for recall CTL responses as described above. Of these, 18 subjects expressed alleles included in the A2 superfamily; 24 were members of the A3 supertype; and 14 were in the B7 supertype. Overall, A2, A3, and B7 supertype–binding peptides were tested for reactivity in a total of 47, 63, and 24 assays, respectively. The frequency and magnitude of the CTL responses is summarized in Table 3. Recall CTL responses (percentage specific lysis $>10\%$) were detected for one of seven A2-binding peptides, five of eight A3-binding peptides, and two of two B7-binding peptides. Clear responses were detected in association with HLA-A*0201, A31, A33, A68, B7, and B35 alleles. Significant CTL responses were not detected in individuals expressing the HLA-A*0202, A*0205, A3, or B53 alleles.

The frequency of individuals responding varied between 0% (for six of the seven A2 and three of the eight A3 supertype peptides) and 66.7% (two of three $A31^+$ individuals tested for reactivity to the peptide $SSP2_{522}$) or 100% (one of one $A68^+$ individuals responding to peptides CSP_{344} or LSA-1_{11}) (Table 3). The magnitude of response (average specific lysis) was 14% for the A2 supertype peptides and ranged from 13% to 32% for the A3 supertype peptides and 14% to 20% for the B7 supertype peptides. In general, both the frequency and magnitude of responses were lower in the PBMCs of naturally exposed individuals (Table 3) than in volunteers immunized with irradiated sporozoites (Table 2). This finding is consistent with responses detected in other field studies (Doolan et al., 1991, 1993; Hill et al., 1992; Sedegah et al., 1992; Aidoo et al., 1995; Lalvani et al., 1996).

Degeneracy of Recall CTL Responses to A2, A3, and B7 Supertype Peptides

Next, the recall CTL data from both sporozoite-immunized and naturally exposed individuals (Tables 2 and 3) were examined in terms of degeneracy of CTL responses. Specifically, we examined whether each CTL epitope was recognized in the context of multiple HLA class I molecules derived from the same supertype. Degenerate antigenicity could not be examined at the CTL level for the A2 supertype peptides because the irradiated sporozoite–immunized volunteers expressed only HLA-A*0201 and the CTL reactivity of the naturally exposed individuals with these peptides was poor. By contrast, degeneracy of recall CTL responses to all A3-binding and B7-binding peptides was clearly demonstrated. The A3 supertype peptide CSP_{344} was recognized in the context of four of the four A3 supertype alleles tested (A3, A31, A33, and A68), peptides Exp-1_{10} and LSA-1_{11} in the context of three of three alleles (A3, A31, and A33, or A3, A33, A68, respectively), and peptide LSA-1_{59} in the context of two of two alleles (A3 and A33). Peptides LSA-1_{94} and $SSP2_{522}$ were recognized in the context of three of four alleles (A3, A33, and A68, or A3, A31 and A33, respectively), peptide LSA-1_{105} in two of three alleles (A3 and A33), and $SSP2_{523}$ in two of four alleles (A3 and A33). Similarly, the B7-binding peptides $Pfs16_{77}$ and $SSP2_{539}$ were recognized as CTL epitopes in the context of three of the five B7 supertype alleles (B7, B35, and B57).

Induction of Cytokine Responses by P. falciparum Degenerate CTL Epitopes

In addition to investigating whether the peptides under study could be recognized as CTL epitopes, we sought to measure the ability of such peptides to induce specific cytokine responses. In particular, we studied interferon-γ (IFNγ) and tumor necrosis factor α (TNFα), which have been implicated in protective immunity against malaria (Ferreira et al., 1986; Mellouk et al., 1987, 1991; Schofield et al., 1987a, 1987b; Nussler et al., 1991). Frozen PBMCs from all six irradiated sporozoite–immunized volunteers as well as fresh PBMCs from a subset of 85 naturally exposed individuals, including the 53 tested for recall CTL responses, were studied.

Significant peptide-induced responses for IFNγ and/or TNFα were observed for all HLA-A2, A3, and B7 degenerate, high-binding peptides tested (see Experimental Procedures for criteria and definition of significant responses). Figure 2 shows representative data illustrating peptide-specific induction of IFNγ from naturally exposed individuals expressing class I molecules of the HLA-A3 supertype and each of the eight peptides tested plus a control peptide (Exp-1_{83}). A summary of the data from naturally exposed individuals, incorporating all peptide-specific responses, is presented in Table 4. Specifically, the fraction of individuals responding varied between 6% (2 of 36 A2 supertype donors tested for IFNγ in response to peptide Exp-1_2) and 54% (23 of 43 A3 supertype donors tested for TNFα responding to peptide LSA-1_{11}). The magnitude of responses varied, with the most vigorous average responses observed in the case of the A2 supertype peptide Exp-1_{91} for IFNγ (860 pg/ml; range 420–1123 pg/ml) and the A2 supertype peptide $SSP2_{14}$ for TNFα (113 pg/ml; range 3–924 pg/ml). Overall, significant TNFα responses ($p < 0.05$) were detected for seven of seven A2, seven of eight A3, and two of two B7 supertype peptides (statistical data not shown). Significant IFNγ responses ($p < 0.05$) were detected for three of seven A2 supertype epitopes, five of eight A3 supertype peptides, and one of two B7 supertype epitopes. For PBMC cultures from individuals who did not express HLA-A2, the A2 supertype peptide Exp-1_{83} was used as the control peptide; in HLA-A2–positive cultures, the A3 supertype peptide CSP_{344} was used. Occasional responses were also noted in some of the "supertype-mismatched" control cultures. Nevertheless, the frequency and magnitude of observed responses to the control peptides were markedly lower than those observed to the supertype-matched peptides. Responses may be attributed to the fact that all peptides were preselected for degenerate binding to three or more class I alleles within a particular HLA supertype. Therefore, it is possible that these peptides may in fact bind to other alleles expressed in the Kenyan

Table 3. Frequency and Magnitude of CTL in Naturally Exposed Individuals

(A) A2 Supertype Peptides

HLA		CSP_{394}	Exp-1_{83}	Exp-1_{80}	Exp-1_{2}	CSP_{7}	Exp-1_{91}	$SSP2_{14}$
A201	Frequency of response	16.7% (1/6)	0% (0/3)	0% (0/2)	0% (0/6)	0% (0/3)	0% (0/3)	0% (0/9)
	Mean % lysis (range)	14.1						
A202	Frequency of response	0% (0/3)	—	0% (0/1)	0% (0/2)	0% (0/1)	0% (0/1)	0% (0/3)
A205	Frequency of response	0% (0/2)	—	—	—	—	—	0% (0/2)
A2 supertype overall	Frequency of response	9.1% (1/11)	0% (0/3)	0% (0/3)	0% (0/8)	0% (0/4)	0% (0/4)	0% (0/14)
	Mean % lysis (range)	14.1						

(B) A3 Supertype Peptides

HLA		Exp-1_{10}	LSA-1_{105}	LSA-1_{94}	$SSP2_{523}$	CSP_{344}	LSA-1_{11}	LSA-1_{59}	$SSP2_{522}$
A3	Frequency of response	0% (0/6)	0% (0/6)	0% (0/4)	0% (0/3)	0% (0/1)	—	0% (0/2)	0% (0/8)
A31	Frequency of response	50.0% (2/4)	—	0% (0/5)	0% (0/1)	50.0% (3/6)	—	—	66.7% (2/3)
	Mean % lysis (range)	20.1 (12.9–27.2)				18.6 (10.2–32.4)			15.5 (12.3–18.7)
A33	Frequency of response	0% (0/1)	0% (0/2)	50.0% (1/2)	0% (0/5)	33.3% (2/6)	20.0% (1/5)	0% (0/1)	—
	Mean % lysis (range)			13.7		25.0 (17.5–32.4)	12.7		
A68	Frequency of response	—	0% (0/1)	50.0% (1/2)	0% (0/2)	100.0% (1/1)	100.0% (1/1)	—	0% (0/1)
	Mean % lysis (range)			13.7		32.4	12.7		
A3 supertype overall	Frequency of response	20.0% (2/10)	0% (0/6)	9.1% (1/11)	0% (0/8)	41.7% (5/12)	20.0% (1/5)	0% (0/2)	22.2% (2/9)
	Mean % lysis (range)	20.1 (12.9–27.2)		13.7		17.7 (10.2–32.4)	12.7		15.5 (12.3–18.7)

(C) B7 Supertype Peptides

HLA		$Pfs16_{77}$	$SSP2_{539}$
B7	Frequency of response	40.0% (2/5)	42.9% (3/7)
	Mean % lysis (range)	20.3 (17.8–22.7)	16.0 (10.5–25.8)
B35	Frequency of response	50.0% (1/2)	50.0% (1/2)
	Mean % lysis (range)	14.2	14.2
B53	Frequency of response	0% (0/5)	0% (0/5)
B7 supertype overall	Frequency of response	30.0% (3/10)	28.6 (4/14)
	Mean % lysis (range)	18.2 (14.2–22.7)	15.7 (10.5–25.8)

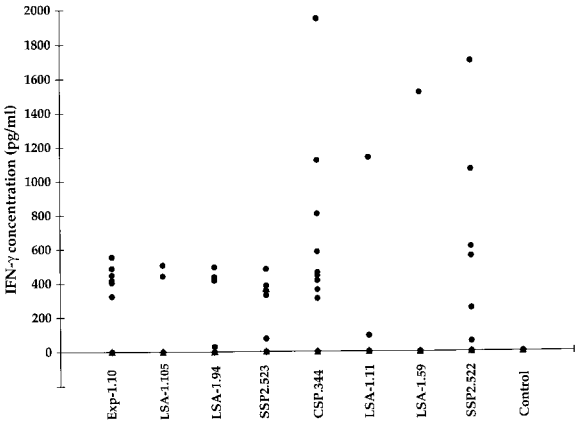

Figure 2. Induction of IFNγ by each A3 Supertype Peptide and Non-A3 Control Peptide

PBMCs from individuals naturally exposed to malaria who expressed any of the A3 supertype alleles were stimulated in vitro with each of the A3 binding peptides as described in Experimental Procedures. Supernatants were assessed for IFNγ production using a standard enzyme-linked immunosorbent assay. Concentrations were calculated by interpolation from standard curves based on recombinant cytokine dilutions. Any background level of cytokine production in cultures not stimulated with peptide was subtracted from peptide-induced responses.

study population that were not tested for in vitro binding capacity.

For the irradiated sporozoite–immunized volunteers, significant TNFα responses ($p < 0.05$) were detected for all A2 and B7 and seven of eight (not LSA-1_{105}) A3 supertype peptides (8 pg/ml; range 2–40 pg/ml). Significant IFNγ responses ($p < 0.05$) were detected for six of seven A2 (not CSP_{394}), four of eight A3 (Exp-1_{10}, LSA-1_{105}, LSA-1_{11}, and LSA-1_{59}), and one of two B7 ($Pfs16_{77}$) supertype peptides (369 pg/ml; range 40–711 pg/ml) (data not shown). There were no detectable TNFα or IFNγ responses to control peptides.

Degeneracy of Cytokine Responses to HLA A2, A3, and B7 Supertype Peptides

We next examined the data from both sporozoite-immunized and naturally exposed donors to determine whether each epitope could induce cytokines in the context of multiple HLA class I molecules derived from the same supertype (data not shown). Degeneracy of cytokine responses to all peptides, in particular the A3-binding and B7-binding peptides, was readily apparent. Three of the seven A2 supertype peptides (CSP_{394}, Exp-1_{83}, and Exp-1_2), were recognized in the context of three of the three A2 supertype alleles studied (A*0201, A*0202, and A*0205) and peptides Exp-1_{80}, CSP_7, Exp-1_{91}, and $SSP2_{14}$ were recognized in the context of two of the three alleles (A*0201 and A*0202). More striking, eight of the eight A3 supertype peptides were recognized in the context of all four A3 supertype alleles studied (A3, A31, A33, and A68), and one of the two B7 supertype epitopes ($SSP2_{539}$) was recognized in association with five of the five B7 supertype alleles (B7, B35, B51, B53, and B57). Peptide $Pfs16_{77}$, the other B7 supertype peptide, was recognized in association with four of the five alleles (B7, B35, B53 and B57).

Phenotype of Responding T Cells

The nonamer and decamer peptides in this study were selected on the basis of ability to bind to HLA class I alleles. Typically, class I binding peptides have a preferred size of 9 ± 1 amino acids, while peptides that bound to class II molecules range in size between 12 and 24 residues (Chicz and Urban, 1994), although peptides as short as seven (Rajadhyaksha and Thanavala, 1995) or eight (Suhrbier et al., 1991; Franco et al., 1994) amino acids may bind. Here, we assessed the ability of the 17 peptides to stimulate a lymphoproliferative response or stimulate release of interleukin-5 (IL-5), a lymphokine usually associated with class II responses. Little or no proliferation and no significant IL-5 release were observed in 691 and 702 independent cultures, respectively (data not shown). In addition, the activated T cell populations responsible for cytokine secretion were examined for expression of $CD3^+$, $CD4^+$, and $CD8^+$ cell surface markers. Activated T cells were almost exclusively $CD8^+$, with only a few $CD4^+$ T cells observed in only a small proportion of cultures (data not shown). In summary, the data were consistent with a $CD8^+$ T cell phenotype for the activated T cells.

Correlation between CTL and Cytokine Responses

Next, we examined the data for correlations between CTL activity and cytokine responses. In the case of irradiated sporozoite volunteers, 92.3% of the IFNγ responders and 83.9% of the TNFα responders for a given peptide were also positive for recall CTL responses. In contrast, 0% (IFNγ) and 3.4% (TNFα) of the naturally exposed individuals who responded to a specific peptide were able to mount similar recall CTL responses to that peptide. From a different perspective, of those individuals in whom a peptide-specific recall CTL response was detected to that peptide, TNFα and IFNγ were detected in 81.3% and 37.5% of vaccinee cultures, respectively, as compared to 5.3% and 0% of cultures from naturally exposed individuals.

Summary of Frequency of Responses against Degenerate HLA Supertype Epitopes

A summary of CTL, TNFα, and IFNγ responses for each individual peptide was compiled (Figure 3). Significant CTL, TNFα and IFNγ responses were detected for all peptides in one or more populations. Specifically, in irradiated sporozoite–immunized volunteers (Figure 3A), significant CTL responses were obtained to all 17 peptides; significant TNFα responses to all A2- and B7- and seven of eight A3-binding peptides; and significant IFNγ responses to six of seven A2, four of eight A3, and one of two B7 supertype epitopes. In naturally exposed individuals (Figure 3B), significant CTL responses were detected to one of seven A2, five of eight A3, and two of two B7 supertype peptides; significant TNFα responses to all A2-, A3-, and B7-binding peptides; and significant IFNγ responses to three of seven A2, five of eight A3, and two of two B7 supertype epitopes.

The frequency of peptide-specific CTL responses in

Table 4. Frequency and Magnitude of Cytokine Responses in Naturally Exposed Individuals

(A) A2 Supertype Peptides

Cytokine		CSP_{394}	Exp-1_{83}	Exp-1_{80}	Exp-1_{2}	CSP_{7}	Exp-1_{91}	$SSP2_{14}$	Control (CSP_{44})
TNFα	% Response	25.7% (9/35)	22.9 (8/35)	11.4% (4/35)	20.0% (7/35)	20.0% (7/35)	14.3% (5/35)	31.4% (11/35)	5.7% (2/35)
	Mean pg/ml	95.74	36.24	105.36	57.88	64.27	93.26	113.28	35.7
	Range	4.1–726.5	1.2–213.9	4.0–393.9	3.6–312.0	1.8–386.0	0.6–372.3	2.7–924.0	0–60.10
IFNγ	% Response	11.1% (4/36)	8.3% (3/36)	11.1% (4/36)	5.6% (2/36)	8.3% (3/36)	8.3% (3/36)	11.1% (4/36)	0% (0/36)
	Mean pg/ml	709.78	578.45	551.40	387.33	520.00	859.82	686.11	0
	Range	314.9–1481.0	375.9–877.2	286.3–1018.4	375.9–398.8	279.0–700.3	420.1–1123.4	399.5–1026.9	0
TNFα or IFNγ	% Response	33.3% (12/36)	30.6 (11/36)	16.7% (6/36)	25.0% (9/36)	22.2% (8/36)	19.4% (7/36)	38.9% (14/36)	5.6% (2/36)

(B) A3 Supertype Peptides

Cytokine		Exp-1_{10}	LSA-1_{105}	LSA-1_{94}	$SSP2_{523}$	CSP_{344}	LSA-1_{11}	LSA-1_{59}	$SSP2_{522}$	Control (Exp-1_{83})
TNFα	% Response	27.9% (12/43)	32.6% (14/43)	27.9% (12/43)	34.9% (15/43)	18.6% (8/43)	53.5% (23/43)	23.3% (10/43)	37.2% (16/43)	14.0% (6/43)
	Mean pg/ml	26.35	21.66	11.90	18.33	6.33	35.44	43.12	25.88	35.06
	Range	6.4–64.5	4.3–52.9	0.8–34.7	2.0–47.0	1.0–23.2	1.7–182.7	2.0–104.7	0.7–102.7	6.3–60.1
IFNγ	% Response	15.6% (7/45)	4.4% (2/45)	8.9% (4/45)	15.6% (7/45)	17.8% (8/45)	4.4% (2/45)	2.2% (1/45)	15.6% (7/45)	0% (0/45)
	Mean pg/ml	440.89	474.20	344.70	330.53	725.34	615.50	1516.10	759.51	0
	Range	324.3–554.5	442.8–505.6	29.9–495.2	76.3–484.1	311.7–1995.0	93.0–1138.0		59.6–1701.0	—
TNFα or IFNγ	% Response	37.8% (17/45)	33.3% (15/45)	28.9% (13/45)	42.2% (19/45)	28.9% (13/45)	51.1% (23/45)	24.4% (11/45)	51.1% (23/45)	13.3% (6/45)

(C) B7 Supertype Peptides

Cytokine		$Pfs16_{77}$	$SSP2_{539}$	Control (CSP_{344} or Exp-1_{83})
TNFα	% Response	12.2% (5/41)	9.8% (4/41)	7.3% (3/41)
	Mean pg/ml	33.30	35.87	22.57
	Range	5.3–136.7	14.1–102.4	6.1–39.9
IFNγ	% Response	6.7% (3/45)	11.1% (5/45)	0% (0/45)
	Mean pg/ml	504.67	599.97	0
	Range	393.1–612.7	72.7–911.3	—
TNFα or IFNγ	% Response	15.6% (7/45)	20.0% (9/45)	6.7% (3/45)

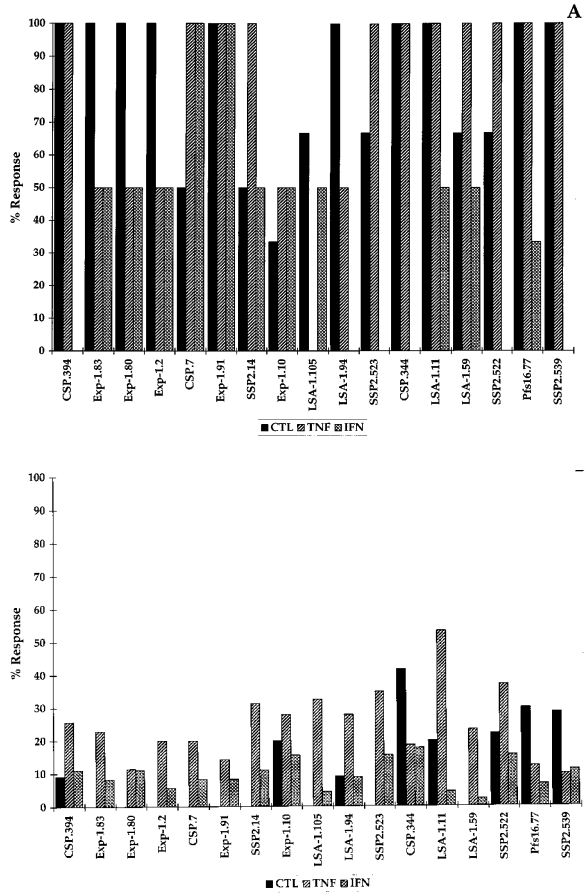

Figure 3. Summary of CTL, TNFα, and IFNγ Responses for each Peptide

(A) Summary of frequency of CTL, TNFα, and IFNγ responses in individuals immunized with irradiated sporozoites to each of the A2, A3, and B7 supertype peptides. PBMCs from volunteers immunized with irradiated sporozoites who expressed the appropriate alleles were stimulated in vitro with each of the peptides and tested for recall CTL, at an effector:target ratio of 30:1, and cytokine responses as described in Experimental Procedures. The number of individuals tested for each peptide varied, depending on the assay. For CTL assays: A2, n = 6; A3, n = 8; and B7, n = 7. For TNFα and IFNγ assays: A2, n = 2; A3, n = 2; and B7, n = 3.

(B) Summary of frequency of CTL, TNFα, and IFNγ responses in individuals naturally exposed to malaria to each of the A2, A3, and B7 supertype peptides. PBMCs from volunteers naturally exposed to malaria who expressed the appropriate alleles were stimulated in vitro with each of the peptides and tested for recall CTLs, at an effector:target ratio of 30:1, and cytokine responses as described in Experimental Procedures. The number of individuals tested for each peptide varied, depending on the assay. For CTL assays: A2, n = 3–11; A3, n = 2–12; and B7, n = 10–14. For TNFα assays, A2, n = 35; A3, n = 43; and B7, n = 41. For IFNγ assays, A2, n = 36; A3, n = 45; and B7, n = 45.

irradiated sporozoite–immunized volunteers was 50%–100%, 33.3%–100%, or 100%, for the A2, A3, or B7 supertype peptides, respectively. The range of A2-, A3- or B7-restricted TNFα responses was 50%–100%, 0%–100%, or 100%, and the range of IFNγ responses was 0%–100%, 0%–50%, or 0%–33.3% (Figure 3A). In naturally exposed individuals, the frequency of CTL responses was 0%–9.1%, 0%–41.7%, or 28.6%–30% for the A2, A3, or B7 supertype peptides, respectively. The range of A2-, A3- or B7-restricted TNFα responses was 11.4%–31.4%, 18.6%–53.5%, or 9.8%–12.2%, and the range of IFNγ responses was 5.6%–11.1%, 2.2%–17.8%, or 6.7%–11.1% (Figure 3B).

The overall frequencies of CTL, TNFα, and IFNγ responses for each supertype are presented in Table 5. In irradiated sporozoite volunteers, there was a markedly high prevalence of CTL responsiveness (6 of 6 of individuals responding in 73 of 120 [60.8%] of assays) and a high prevalence of cytokines (6 of 6 of individuals inducing either TNFα or IFNγ in a total of 34 of 38 [89.5%] of assays). In contrast, in naturally exposed individuals, the prevalence of CTL responsiveness was lower (10 of 53 of individuals responding in 19 of 134 [14.2%] of assays), as was the prevalence of cytokines (60 of 85 of individuals inducing either TNFα or IFNγ in a total of 217 of 702 [30.9%] of assays).

In both populations, an overall hierarchy of antigenicity was apparent, with the B7 supertype peptides being the most immunogenic, followed by the A3 supertype peptides, and finally the A2 supertype peptides.

In summary, the data indicate that in an immune population with a high prevalence of CTL responsiveness (73 of 120 assays [61%]), IFNγ and/or TNFα are induced in almost all (34 of 38 assays [89.5%]) cases of specific peptide stimulation, and CTL response is almost always associated with induction of IFNγ and TNFα. However, in a semi-immune or nonimmune population, the prevalence of CTL responsiveness is low (19 of 134 assays [14.2%]); the induction of cytokines is less frequent (30.9% of cases); and there is no significant association between CTL and cytokine responses (data not shown).

Summary of Breadth of Responses against Degenerate HLA Supertype Epitopes

A summary of the degeneracy of each of the 17 peptides in terms of binding capacity and antigenicity is presented in Table 6. Eight of eight A3 supertype peptides that exhibited highly degenerate binding capacity in vitro also demonstrated highly degenerate antigenicity at the level of both CTL and cytokine responses. In particular, CSP_{344} bound to four of five alleles of the A3 supertype and induced both CTL and cytokine responses in association with four of four alleles studied. Peptides Exp-1_{10} and LSA-1_{11} also induced a CTL response in three of three alleles tested and a cytokine response in four of four alleles, even though high-affinity binding was detected to only three of five and two of five alleles, respectively. These data suggest that, provided that a peptide can bind to a specific HLA molecule, subsequent antigenicity and immunogenicity may not directly correlate with the affinity of binding per se.

The B7 supertype peptides showed a similar pattern of highly degenerate antigenicity for both CTL and cytokines (Table 6). In contrast, a pattern of lower antigenicity was noted for the A2 supertype peptides, especially in terms of recall CTL responses (Table 6).

In summary, all peptides were recognized in the context of more than one HLA class I molecule, and three of seven A2, eight of eight A3, and one of two B7 supertype peptides were recognized in the context of all HLA alleles studied.

Table 5. Summary of Frequency of CTL, IFNγ, or TNFα Responses in Irradiated Sporozoite–Immunized or Naturally Exposed Individuals

	Irradiated Sporozoite–Immunized Volunteers				Naturally Exposed Individuals			
Supertype	CTL	IFNγ	TNFα	IFNγ or TNFα	CTL	IFNγ	TNFα	IFNγ or TNFα
A2	2/2[a] (18/42)[b]	2/2 (8/14)	2/2 (12/14)	2/2 (12/14)	1/19 (1/47)	10/36 (23/252)	17/35 (51/245)	23/36 (67/252)
A3	4/4 (43/64)	1/3 (4/18)	3/3 (14/18)	3/3 (16/18)	6/24 (11/63)	26/45 (38/360)	28/43 (110/344)	37/45 (134/360)
B7	4/4 (12/14)	1/3 (1/6)	3/3 (6/6)	3/3 (6/6)	4/14 (7/24)	6/45 (8/90)	7/41 (9/82)	12/45 (16/90)
Overall	6/6 (73/120)	2/5 (13/38)	5/5 (32/38)	5/5 (34/38)	10/53 (19/134)	40/85 (69/702)	44/85 (170/671)	60/85 (217/702)

[a] Number of individuals with a positive response to one or more peptides/total number of individuals tested.
[b] Number of positive assays/total number of assays conducted.

Table 6. Summary of Peptide Degeneracy at the Level of HLA Binding and CTL and Cytokine (IFNγ and/or TNFα) Responses

		Number of Alleles Recognized		
Supertype	Peptide	HLA Binding	CTL Responses	Cytokine Responses
A2	CSP_{394}	4/5	1/3	3/3
	Exp-1_{83}	3/5	1/1	3/3
	Exp-1_{80}	4/5	1/2	2/3
	Exp-1_{2}	3/5	1/2	3/3
	CSP_{7}	2/5	1/2	2/3
	Exp-1_{91}	2/5	1/2	2/3
	$SSP2_{14}$	5/5	1/3	2/3
A3	Exp-1_{10}	3/5	3/3	4/4
	LSA-1_{105}	3/5	2/3	4/4
	LSA-1_{94}	4/5	3/4	4/4
	$SSP2_{523}$	4/5	2/4	4/4
	CSP_{344}	4/5	4/4	4/4
	LSA-1_{11}	2/5	3/3	4/4
	LSA-1_{59}	3/5	2/2	4/4
	$SSP2_{522}$	3/5	3/4	4/4
B7	$Pfs16_{77}$	5/5	3/5	4/5
	$SSP2_{539}$	4/5	3/5	5/5

Discussion

In this study, we have used a combination of immunochemical and cellular analyses based on HLA-specific peptide-binding motifs to identify 17 degenerate $CD8^+$ T cell epitopes from a number of P. falciparum preerythrocytic-stage antigens that are restricted by A2 supertype, A3 supertype, and B7 supertype alleles. Peptide sequences from the P. falciparum antigens CSP, SSP2, LSA-1, and Exp-1 conforming to specific class I binding motifs were synthesized. Peptides that bound with good ($IC_{50} \leqslant 500$ nM) affinity to A*0201, A*0302, A*1101, or B*0702 molecules were tested for their capacity to bind multiple molecules of the corresponding supertype. Seventeen of 42 peptides tested exhibited degenerate binding and were subsequently tested for their capacity to elicit recall CTL and cytokine responses from PBMCs of volunteers either immunized with irradiated sporozoites or naturally exposed to malaria. Seventeen of seventeen peptides tested were antigenic; all of them were recognized in the context of more than one HLA allele; and at least 12 of them (71%) were recognized in the context of all HLA alleles studied.

The data presented here have several important implications. First, in terms of population coverage, the data support, in the case of a parasitic disease of medical importance such as malaria, the feasibility of a subunit vaccine comprising three degenerate peptide specificities. Of the 197 naturally exposed Kenyan individuals who were HLA typed, 39.0% expressed class I alleles classified as HLA-A2–like, 23.4% were HLA-A3–like, and 34.5% were HLA-B7–like. The overall fraction of individuals expressing at least one molecule from any of the three supertypes was 84.8%. These data are consistent with our previous predictions (Sidney et al., 1996a). Further studies will be required to examine whether these three supertypes will allow adequate population coverage in the majority of all racial and ethnic populations.

Second, these data illustrate that degenerate HLA binding capacity appears to be relatively frequent.

Thirty-six percent (5 of 14) of peptides tested that bound HLA-A*0201 with good affinity and were immunogenic in vitro, 28% (7 of 25) of peptides that bound to either A3 or A11, and 40% (2 of 5) of peptides that bound to HLA-B7 exhibited degenerate binding to three or more of the respective A2 supertype, A3 supertype, or B7 supertype alleles tested. In fact, one of the A2-binding peptides and one of the B7-binding peptides bound to all five of the alleles, and two A2-binding, three A3-binding, and one B7-binding peptides bound to four of the five alleles. These data suggest that peptides capable of degenerate binding capacity are relatively frequent and should therefore be identifiable in most antigenic systems.

The finding that each of the 17 peptides identified was antigenic for both recall CTL and cytokine responses is especially remarkable. Malaria peptide-specific CTLs were first identified in 1991 (Doolan et al., 1991; Malik et al., 1991), and CTLs restricted by defined HLA alleles were described in 1992 (Hill et al., 1992). Since then, a total of 19 HLA restricted CTL epitopes (reviewed by Doolan et al., 1996b) have been reported as a result of extensive research by three laboratories over a period of 6 years. Only 13 of these 19 epitopes are minimal. In the present study, we have identified 17 nonamer or decamer epitopes derived from five P. falciparum pre-erythrocytic-stage antigens, underscoring the power of our approach.

It should be noted that in the present study, peptides were preselected for high-affinity binding to selected class I HLA molecules, and A2-binding peptides were also screened in HLA-A*0201 transgenic mice. Moreover, peptides were also preselected for good binding to three or more alleles of the respective HLA superfamily. In fact, only a relatively small proportion of peptides fulfill such stringent selection criteria. Here, only 7 of 50 peptides tested for binding to HLA-A*0201, 8 of 203 peptides tested for binding to HLA-A3/A11, and 2 of 24 peptides tested for binding to HLA-B7 were selected for antigenicity studies. All of these peptides were recognized as epitopes for both CTL and cytokine responses.

In contrast, in studies utilizing nonquantitative assays and the antigens CSP, SSP2, LSA-1, Exp-1, and Pfs16, 1 of 60 HLA-B53, 3 of 10 HLA-B35, 1 of 60 HLA-B7, 3 of 23 peptides HLA-B8, 1 of 62 HLA-B17, and 3 of 61 HLA-A2 binding peptides were identified as CTL epitopes (overall 12 of 276; 4.4%) (Hill et al., 1992; Aidoo et al., 1995).

The high frequency of antigenicity detected in the case of HLA degenerate peptides might be explained, at least in part, by previous quantitative analysis of the peptide preferences of the transporters associated with antigen processing (TAP) (van Endert et al., 1995; Sidney et al., 1996a). Specifically, it has been noted that highly degenerate peptides conform to specific "supermotifs," and that "HLA supermotifs" are preferentially transported by TAP molecules. In other words, the high antigenicity of supertype peptides illustrates the biological consequences of similar (and perhaps coordinate) peptide specificities of TAP and HLA molecules (Sidney et al., 1996a). In this respect, it is worth noting that similarly, highly antigenic HLA degenerate, class I peptides have been observed recently in the case of epitopes derived from the hepatitis B virus (HBV) (Bertoni et al., unpublished data).

The data presented here also have implications for our understanding of the interplay between Plasmodium spp. infection and immunity to malaria. Even though antigen-specific CTL can eliminate infected hepatocytes from in vitro culture (Hoffman et al., 1989, 1990), the level of cytotoxicity observed in naturally exposed individuals is clearly not sufficient to eliminate all infected hepatocytes in vivo and thus protect against malaria. Accordingly, optimal vaccine design may be required to boost T cell or CTL precursor cells and enhance T cell responses. The data here demonstrate that it is possible to induce malaria-specific recall CTL responses of high magnitude (up to 57% specific lysis) as well as high frequency. Peptide-specific CTL responses were detected in a total of 100% (6 of 6) of individuals in 60.8% (73 of 120) of assays using PBMCs from sporozoite-immunized volunteers, but in only 18.9% (10 of 53) of individuals in 14.2% (19 of 134) of assays in which effector cells were derived from naturally exposed individuals. This pattern has been reported previously (Malik et al., 1991; Wizel et al., 1995a, 1995b) and may be a reflection of the high antigenic load achieved in the case of irradiated sporozoite immunization.

Although it is well established that antigen-specific $CD8^+$ T cells can completely protect against malaria in the absence of other parasite-specific immune responses (reviewed by Hoffman et al., 1996), a specific marker of protective immunity has not yet been identified. Several reports in both rodent and human systems suggest that the induction of CTLs against Plasmodium spp. proteins does not necessarily correlate with protection of the host. We have previously proposed (Doolan et al., 1996a; Hoffman et al., 1996) that the $CD8^+$ T cell activated by interacting with the MHC–peptide complex on the surface of the infected hepatocyte may secrete IFNγ, which in turn may induce the infected hepatocyte to produce nitric oxide, rendering the parasite noninfectious. Accordingly, in this investigation, we studied the ability of the 17 peptides to induce specific responses of selected cytokines implicated in protective immunity (specifically, IFNγ and TNFα). For all peptides tested, the capacity to recall specific responses of one or more of these cytokines was demonstrated. Moreover, with regard to recall CTL responses, the prevalence of cytokine responsiveness was much higher in an immune population than in a semi-immune or nonimmune population.

The epitopes identified here may be incorporated in a vaccine designed to protect humans against P. falciparum malaria via a $CD8^+$ T cell–mediated immune response. Such a vaccine may be composed of multiple constructs molecule made up of three distinct structural units: $CD8^+$ T cell epitopes, $CD4^+$ T helper epitopes, and lipid. Previous studies (Vitiello et al., 1995) have demonstrated that this type of lipopeptide construct is efficacious in inducing a CTL response in humans. Alternatively, $CD8^+$ and $CD4^+$ epitopes may be encoded in suitable DNA vaccines or recombinant infectious agents. In addition to circumventing the problem of genetic restriction of the immune response, such a multivalent vaccine incorporating a wide repertoire of specifici-

ties at the epitope level may prevent parasite escape from immune recognition because of strain variability and epitope mutation. Furthermore, a multivalent response is also likely to be more vigorous and efficacious in eliciting a protective immune response.

In conclusion, the data presented validate the concept of class I HLA supertypes at the biological level in the context of a disease of worldwide importance. Furthermore, they reveal a striking association between HLA degenerate binding capacity and antigenicity for class I–restricted responses. Finally, the results support the feasibility of a subunit malaria vaccine comprising as few as three peptide-binding specificities that would be potentially immunogenic in the majority of all racial and ethnic populations.

Experimental Procedures

Cell Culture

The following Epstein-Barr virus– transformed cell lines were used as sources of class I molecules: JY (A*0201), FUN (A*0203), CLA (A*0206), AMAI (A*6802, B*5301), GM3107 (A*0301, B*0701), BVR (A*1101), SPACH (A*3101), LWAGS (A*3301), KAS116 (B*5101), and KT3 (B*5401). The mouse B cell lymphoma P815 transfected with A*0202 was also utilized. C1R transfectants were used for the isolation of A*6801 and B*3501. These C1R transfectants were characterized by W. Storkus (Storkus et al., 1993) and Dr. M. Takaguchi (Ooba et al., 1989), respectively.

The HLA-A*0201–expressing cell line 771.221 was derived by transfecting the human class I null mutant B cell line, 721.221 (Shimizu and DeMars, 1989) with the HLA-A*0201 heavy chain gene as described previously (Celis et al., 1994). EHM, BVR, and JY cells are homozygous human Epstein-Barr virus–transformed B cell lines expressing A3, A11, and B7 respectively. K562, a natural killer cell–sensitive erythroblastoma line, was used for reduction of background killing in the ^{51}Cr release assay.

Cell culture medium consisted of RPMI 1640 containing HEPES and L-glutamine (Life Technologies, Grand Island, NY) supplemented with 2 mM L-glutamine (Irvine Scientific, Santa Ana, CA or Gibco, BRL Life Technologies, Grand Island, NY), 0.5 mM sodium pyruvate (Life Technologies), 100 U/100 μg/ml penicillin/streptomycin (Irvine Scientific, Santa Ana, CA), and 5 or 10% heat-inactivated human serum type AB (Gemini Bioproducts, Calabasas, CA, or ICN Biomedical, Costa Mesa, CA) or 10% heat-inactivated fetal calf serum (RPMI/10% FCS, Irvine Scientific, Santa Ana, CA).

Affinity Purification of Class I HLA-A Molecules and Peptide-Binding Assays

Cells were lysed at a concentration of 10^8 cells/ml in phosphate-buffered saline containing 1% NP-40 and 1 mM phenylmethylsulfonyl fluoride. The lysates were cleared of debris and nuclei by centrifugation at 10,000 × g for 20 min. MHC molecules were then purified by affinity chromatography as previously described (Sette et al., 1994; Sidney et al., 1995). Inactivated Sepharose CL4B and protein A–Sepharose were used as precolumns.

Purified class I peptide-binding assays were performed as previously described (Sette et al., 1994). In brief, purified human class I molecules (5 to 500 nM) were incubated with 1–10 nM ^{125}I-labeled probe peptide, iodinated by the chloramine T method (Buus et al., 1987), for 48 hr at room temperature in the presence of 1 μM human β_2-microglobulin (Scripps Laboratories, San Diego, CA) and a cocktail of protease inhibitors. Class I peptide complexes were separated from free peptide by gel filtration on TSK2000 columns, and the fraction of bound peptide was calculated as previously described (Rebai and Malissen, 1983). In inhibition assays, peptide inhibitors were typically tested at concentrations ranging from 120 μg/ml to 1.2 ng/ml. The data were then plotted and the dose yielding 50% inhibition was measured. Because under these conditions [label] $<$ [MHC] and $IC_{50} \geqslant$ [MHC], the measured IC_{50}s are reasonable approximations of the true K_D values.

Radiolabeled probe and standard control peptides used in the different assays are as follows. A $F_6 \rightarrow Y$ analog of the HBV core 18–27 peptide (sequence FLPSDYFPSV) (Ruppert et al., 1993) was used as the radiolabeled probe for the A*0201, A*0202, A*0203, and A*0206 assays. A $C_4 \rightarrow A$ analog of the HBV pol 646–654 peptide (sequence FTQAGYPAL) was used as the radiolabeled probe for the A*6802 assay. Radiolabeled probes used for A3 supertype alleles, A3CON1 peptide (sequence KVFPYALINK), and HBV core 141–151 peptide (sequence STLPETYVVRR), and B7 supertype alleles, B35CON2 peptide (sequence FPFKYAAAF), and A*0201 signal sequence $L_7 \rightarrow Y$ (APRTLVYLL), have been described elsewhere (Sidney et al., 1996b, 1996c).

Peptide Synthesis

Peptides were either synthesized at Cytel Corporation (San Diego, CA), as previously described (Ruppert et al., 1993) or, for large epitope libraries, purchased as crude material from Chiron Mimotopes (Chiron Corporation, Clayton, Victoria, Australia). Peptides synthesized at Cytel were purified to greater than 95% homogeneity by reverse-phase HPLC. The purity of these synthetic peptides was determined on an analytical reverse-phase column and their composition ascertained by amino acid analysis and/or mass spectrometry analysis.

Sequence Analysis

Complete sequences of the P. falciparum antigens CSP, SSP2, LSA-1, and Exp-1 were searched for the presence of conserved 9– or 10–amino acid sequences containing extended HLA-A*0201, A3, A11, or B7 binding motifs (Ruppert et al., 1993; Kubo et al., 1994; Sidney et al., 1995, 1996c). Multiple isolates were available for two of the aforementioned antigens. Motif-containing peptides that exhibited 100% conservancy in 80% or more of different isolates were considered for synthesis. These peptides were subjected to a customized computer algorithm analysis program that takes into account secondary effects of residues at each nonanchor position of the peptide (Ruppert et al., 1993; Sidney et al., 1996a; Sidney et al., unpublished data).

Selection and HLA Typing of Naturally Exposed Study Populations

Individuals were selected for study on the basis of presence of selected HLA class I alleles. Subjects were aged between 15 and 50 years, and informed consent was obtained in all cases.

Caucasian volunteers were exposed to the bites of between 1008 to 1576 (1.5×10^4 rad) γ-irradiated Anopheles stephensi mosquitoes infected by membrane feeding with either the NF54 strain (Ponnudurai et al., 1981) or the corresponding 3D7 clone (Walliker et al., 1987) of P. falciparum, as previously described (Egan et al., 1993). Subjects were exposed during 9–14 sessions over a period of 23 months.

Kenyan subjects were almost exclusively of the Luo ethnic group and were residents of 1 of 15 contiguous villages in the Asembo Bay area of Kenya, on the north and east shores of Lake Victoria. In this area, the year-round prevalence of P. falciparum infection among children 6 months to 6 years of age has been documented as 94.4%–97.8% (Beier et al., 1994; McElroy et al., 1994; Beadle et al., 1995). A total of 197 Kenyans were HLA typed using commercially available serological typing plates (72-Ti-Black, One Lambda, Canoga Park, CA). HLA-$A2^+$ individuals were further subtyped using an allele-specific polymerase chain reaction technique. Eighty-five individuals were selected for subsequent CTL and cytokine studies (43 females and 42 males, mean age 27.8 years, range 16–48 years). Subjects reported an average of 2.1 episodes of clinical malaria within the previous year. Parasitemia at the time of blood collection was assessed by examination of Giemsa-stained thick blood films, and smears were considered negative after examination of 200 oil-immersion fields. Sixteen of the 85 subjects were parasitemic (mean parasitemia 4.5%). These donors were not excluded from analysis because positive CTL responses were detected for 2 of the individuals, and positive cytokine responses for the majority. The human immunodeficiency virus (HIV) infection status of individuals was assessed using a commercially available kit designed to detect both HIV-1 and HIV-2 (Abbott Laboratories, Abbott Park, IL), according

to the manufacturer's specifications. Of the 85 subjects selected for CTL and cytokine studies, 26% tested positive for HIV-1 or HIV-2. They were not excluded from the study because there was no associated immunosuppression apparent with HIV infection. The average mitogen response of HIV-infected individuals was equivalent to the response of uninfected individuals; peptide-specific cytokine responses were detected in all but one of the HIV-infected individuals; and peptide-specific CTL responses were detected in two individuals.

Induction and Assay of CTLs from Irradiated Sporozoite–Immunized and Naturally Exposed Individuals

In vitro induction of peptide-specific CTLs was achieved as described previously (Doolan et al., 1991; Sedegah et al., 1992), using frozen PBMCs as effector cells. In brief, PBMCs were cultured at a concentration of 3–4 $\times$ 10^6 cells/2 ml in 24-well plates in the presence of each MHC-matched peptide (10 μg/ml) or an MHC-mismatched peptide (10 μg/ml) for 7 days. Human rIL-2 (50 U/ml, Cetus) was added 2 days after the initiation of the culture. Cytolytic activity was assessed by conventional chromium release using autologous or MHC-mismatched phytohemagglutinin P-activated lymphoblasts (PHA blasts) as target cells. PHA blasts were generated by stimulating PBMCs with 0.2% (v/v, according to the manufacturer's specifications) PHA (Sigma, St. Louis, MO). Target cells were incubated with or without peptide (10 μg/ml) and labeled with 100 μCi ^{51}Cr (sodium chromate solution, Dupont–NEN, Boston, MA) for 90 min, washed three times, and plated (in triplicate) at a concentration of 5000 cells/well in a round-bottom 96-well plate, in the presence of 5–10 μg/ml peptide and effector cells. CTL activity was measured in a standard 6 hr chromium release assay. The percentage lysis was determined as (experimental release $-$ medium control release)/(maximum release $-$ medium control release) $\times$ 100. The percentage specific lysis was determined by subtracting the percentage lysis (control) from percentage lysis (specific peptide). Results were expressed as the mean of triplicate determinations. A positive response was defined as percentage specific lysis greater than 10%.

Induction and Assay of Cytokine Responses from Irradiated Sporozoite–Immunized and Naturally Exposed Individuals

Fresh PBMCs were cultured at a concentration of 1 $\times$ 10^6 cells/ml per well in 24-well plates (Costar, Cambridge, MA) in the presence of each MHC-matched peptide (10 μg/ml), an MHC-mismatched peptide (10 μg/ml; A2-binding control peptide for the A3 and B7 cultures; A3-binding peptide for the A2 cultures; HBV-derived A2-binding control peptide for A2, A3, and B7 cultures), no peptide, or mitogen (PHA at 10 μg/ml). Cell-free supernatants were collected at 48 hr, 72 hr, 5 days, and 7 days and stored at -70°C until analysis. IFNγ was measured by enzyme-linked immunosorbent assay as described previously (Steel et al., 1984). TNFα was assayed using commercially available kits (Endogen, Woburn, MA), according to the manufacturer's specifications. Samples were considered positive if the optical density reading exceeded 1.5 times that of the blank, and concentrations were calculated by interpolation from standard curves based on recombinant cytokine dilutions run in parallel on the same plate. Any background level of cytokine production in cultures not stimulated with peptide was subtracted from peptide-induced responses.

Analysis of Lymphoproliferative Responses of Peptide-Activated T Cells

Fresh PBMCs (2 $\times$ 10^5) were cultured in quadruplicate in 0.2 ml of medium in a round-bottom 96-well tissue culture plate in the presence of 10 μg/ml of each peptide, without peptide (negative control), or with mitogen (PHA at 10 μg/ml) for 6 days. Wells were then pulsed with 1.0 μCi [^{3}H]methyl thymidine (Dupont–NEN) overnight and uptake assessed by liquid scintillation spectroscopy (Beckman LS6800). Results were expressed as a stimulation index (counts per minute in sample/counts per minute in control without peptide).

Phenotypic Staining of Peptide-Activated T Cells

Fresh PBMCs cultured at a concentration of 1 $\times$ 10^6 cells/ml for 7 days in the presence of 10 μg/ml of each peptide, without peptide, or with mitogen (PHA) were harvested and 50,000 cells aliquoted in quadruplicate wells of 12-well slides. Slides were air-dried and stored at -70°C prior to analysis. For each sampe analyzed, one well was stained with fluorescein isothiocynate–-conjugated anti-CD3$^+$ (Becton Dickinson, San Jose, CA), anti-CD4$^+$ (Becton Dickinson) or anti-CD8$^+$ (Becton Dickinson) for 30 min at room temperature, or left unstained, washed three times with phosphate-buffered saline, and mounted. Stained cells were quantitated visually using an Olympus microscope (Japan).

Statistical Analysis

The prevalence of dichotomous outcome variables (frequency of peptide-specific CTL responses, proliferative T cell responses, or cytokine responses) was assessed using the Pearson chi-square test (two-tailed) and Fisher's exact test (two-tailed). Nonparametric continuous outcome variables (mean peptide-specific cytokine 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). Bivariate correlations (associations between CTLs, cytokines, and binding affinity), Pearson correlation coefficients and p values were calculated using the Pearson (two-tailed) and Spearman (two-tailed) tests. Analysis was conducted using the SPSS statistical program (SPSS, Chicago, IL).

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References

Aidoo, M., Lalvani, A., Allsopp, C.E.M., Plebanski, M., Meisner, S.J., Krausa, P., Browning, M., Morris-Jones, S., McAdam, S., Gotch, F., et al. (1995). Identification of conserved antigenic components for a cytotoxic T lymphocyte-inducing vaccine against malaria. Lancet *345*, 1003–1007.

Beadle, C., McElroy, P.D., Oster, C.N., Beier, J.C., Oloo, A.J., Onyango, F.K., Chumo, D.K., Bales, J.D., Sherwood, J.A., and Hoffman, S.L. (1995). Impact of transmission intensity and age on *Plasmodium falciparum* density and associated fever: implications for malaria vaccine design. J. Infect. Dis. *172*, 1047–1054.

Beier, J.C., Oster, C.N., Onyango, F.K., Bales, J.D., Sherwood, J.A., Perkins, P.V., Chumo, D.K., Koech, D.V., Whitmire, R.E., Roberts, C.R., et al. (1994). *Plasmodium falciparum* incidence relative to entomologic inoculation rates at a site proposed for testing malaria vaccines in western Kenya. Am J. Trop. Med. Hyg. *50*, 529–536.

Buus, S., Sette, A., Colon, S., Miles, S., and Grey, H.M. (1987). The relation between major histocompatibility complex (MHC) restriction

and the capacity of Ia to bind immunogenic peptides. Science *235*, 1353–1358.

Celis, E., Tsai, V., Crimi, C., DeMars, R., Wentworth, P.A., Chesnut, R.W., Grey, H.M., Sette, A., and Serra, H.M. (1994). Induction of anti-tumor cytotoxic T lymphocytes in normal humans using primary cultures and synthetic peptide epitopes. Proc. Natl. Acad. Sci. USA *91*, 2105–2109.

Chicz, R.M., and Urban, R.G. (1994). Analysis of MHC-presented peptides: applications in autoimmunity and vaccine development. Immunol. Today *15*, 155–160.

del Guercio, M.-F., Sidney, J., Hermanson, G., Perez, C., Grey, H.M., Kubo, R.T., and Sette, A. (1995). Binding of a peptide antigen to multiple HLA alleles allows definition of an A2-like supertype. J. Immunol. *154*, 685–693.

Doolan, D.L., Houghten, R.A., and Good, M.F. (1991). Location of human cytotoxic T cell epitopes within a polymorphic domain of the *Plasmodium falciparum* circumsporozoite protein. Int. Immunol. *3*, 511–516.

Doolan, D.L., Saul, A.J., and Good, M.F. (1992). Geographically restricted heterogeneity of the *Plasmodium falciparum* circumsporozoite protein: relevance for vaccine development. Infect. Immun. *60*, 675–682.

Doolan, D.L., Khamboonruang, C., Beck, H.P., Houghten, R.A., and Good, M.F. (1993). Cytotoxic T lymphocyte (CTL) low-responsiveness to the *Plasmodium falciparum* circumsporozoite protein in naturally-exposed endemic populations: analysis of human CTL response to most known variants. Int. Immunol. *5*, 37–46.

Doolan, D.L., Sedegah, M., Hedstrom, R.C., Hobart, P., Charoenvit, Y., and Hoffman, S.L. (1996a). Circumventing genetic restriction of protection against malaria with multi-gene DNA immunization: $CD8^+$ T cell, interferon-γ, and nitric oxide dependent immunity. J. Exp. Med. *183*, 1739–1746.

Doolan, D.L., Wizel, B., and Hoffman, S.L. (1996b). Class I restricted cytotoxic T lymphocyte responses against malaria- elucidation on the basis of HLA peptide binding motifs. Immunol. Res. *15*, 280–305.

Doolan, D.L., and Hoffman, S.L. (1997). Multi-gene vaccination against malaria: a multi-stage, multi-immune response approach. Parasitol. Today *13*, 171–178.

Egan, J.E., Hoffman, S.L., Haynes, J.D., Sadoff, J.C., Schneider, I., Grau, G.E., Hollingdale, M.R., Ballou, W.R., and Gordon, D.M. (1993). Humoral immune response in volunteers immunized with irradiated *Plasmodium falciparum* sporozoites. Am. J. Trop. Med. Hyg. *49*, 166–173.

Ferreira, A., Schofield, L., Enea, V., Schellekens, H., van der Meide, P., Collins, W.E., Nussenzweig, R.S., and Nussenzweig, V. (1986). Inhibition of development of exoerythrocytic forms of malaria parasites by gamma-interferon. Science *232*, 881–884.

Franco, A., Appella, E., Kagnoff, M.F., Chowers, Y., Sakaguchi, K., Grey, H.M., and Sette, A. (1994). Peripheral T cell response to A-gliadin in celiac disease: differential processing and presentation capacities of Epstein-Barr-transformed B cells and fibroblasts. Clin. Immunol. Immunopathol. *71*, 75–81.

Hill, A.V.S., Allsopp, C.E.M., Kwiatkowki, D., Anstey, N.M., Twumasi, P., Rowe, P.A., Bennett, S., Brewster, D., McMichael, A., and Greenwood, B.M. (1991). Common West African HLA antigens are associated with protection from severe malaria. Nature *352*, 595–600.

Hill, A.V.S., Elvin, J., Willis, A.C., Aidoo, M., Allsopp, C.E., Gotch, F.M., Gao, X.M., Takiguchi, M., Greenwood, B.M., Townsend, A.R., et al. (1992). Molecular analysis of the association of HLA-B53 and resistance to severe malaria. Nature *360*, 434–439.

Hoffman, S.L., Isenbarger, D., Long, G.W., Sedegah, M., Szarfman, A., Waters, L., Hollingdale, M.R., van der Miede, P.H., Finbloom, D.S., and Ballou, W.R. (1989). Sporozoite vaccine induces genetically restricted T cell elimination of malaria from hepatocytes. Science *244*, 1078–1081.

Hoffman, S.L., Weiss, W., Mellouk, S., and Sedegah, M. (1990). Irradiated sporozoite vaccine induces cytotoxic T lymphocytes that recognize malaria antigens on the surface of infected hepatocytes. Immunol. Lett. *25*, 33–38.

Hoffman, S.L., Franke, E.D., Hollingdale, M.R., and Druilhe, P. (1996). Attacking the infected hepatocyte. In Malaria Vaccine Development, S.L. Hoffman, ed. (Washington, DC: ASM Press), pp. 35–76.

Khusmith, S., Sedegah, M., and Hoffman, S.L. (1994). Complete protection against *Plasmodium yoelii* by adoptive transfer of a $CD8^+$ cytotoxic T cell clone recognizing sporozoite surface protein 2. Infect. Immun. *62*, 2979–2983.

Kubo, R.T., Sette, A., Grey, H.M., Appella, E., Sakaguchi, K., Zhu, N. Z., Arnott, D., Sherman, N., Shabanowitz, J., Michel, H., et al. (1994). Definition of specific peptide motifs for four major HLA-A alleles. J. Immunol. *152*, 3913–3924.

Lalvani, A., Hurt, N., Aidoo, M., Kibatala, P., Tanner, M., and Hill, A.V.S. (1996). Cytotoxic T lymphocytes to *Plasmodium falciparum* epitopes in an area of intense and perennial transmission in Tanzania. Eur. J. Immunol. *26*, 773–779.

Malik, A., Egan, J.E., Houghton, R.A., Sadoff, J.C., and Hoffman, S. L. (1991). Human cytotoxic T lymphocytes against *Plasmodium falciparum* circumsporozoite protein. Proc. Natl. Acad. Sci. USA. *88*, 3300–3304.

McCutchan, T.F., and Waters, A.P. (1990). Mutations with multiple independent origins in surface antigens mark the targets of biological selective pressure. Immunol. Lett. *25*, 23–26.

McElroy, P.D., Beier, J.C., Oster, C.N., Beadle, C., Sherwood, J.A., Oloo, A.J., and Hoffman, S.L. (1994). Predicting outcome in malaria: correlation between rate of exposure to infected mosquitoes and level of *Plasmodium falciparum* parasitemia. Am. J. Trop. Med. Hyg. *51*, 523–532.

Mellouk, S., Maheshwari, R.K., Rhodes-Feuillette, A., Beaudoin, R.L., Berbiguier, N., Matile, H., Miltgen, F., Landau, I., Pied, S., Chigot, J. P., et al. (1987). Inhibitory activity of interferons and interleukin 1 on the development of *Plasmodium falciparum* in human hepatocyte cultures. J. Immunol. *139*, 4192–4195.

Mellouk, S., Green, S.J., Nacy, C.A., and Hoffman, S.L. (1991). IFN-gamma inhibits development of *Plasmodium berghei* exoerythrocytic stages in hepatocytes by an L-arginine-dependent effector mechanism. J. Immunol. *146*, 3971–3976.

Nussler, A., Pied, S., Goma, J., Renia, L., Miltgen, F., Grau, G.E., and Mazier, D. (1991). TNF inhibits malaria hepatic stages in vitro via synthesis of IL-6. Int. Immunol. *3*, 317–321.

Ooba, T., Hayashi, H., Karaki, S., Tanabe, M., Kano, K., and Takiguchi, M. (1989). The structure of HLA-B35 suggests that it is derived from HLA-Bw58 by two genetic mechanisms. Immunogenetics *30*, 76–80.

Ponnudurai, T., Leeuenberg, A.D., and Meuwissen, J.H. (1981). Chloroquine sensitivity of isolates of *Plasmodium falciparum* adapted to in vitro culture. Trop. Geogr. Med. *33*, 50–54.

Rajadhyaksha, M., and Thanavala, Y. (1995). Delineation of the minimal hepatitis B surface antigen-specific B- and T-cell epitope contained within an anti-idiotype-derived pentadecapeptide. Proc. Natl. Acad. Sci. USA *92*, 1575–1579.

Rebai, N., and Malissen, B. (1983). Structural and genetic analyses of HLA class I molecules using monoclonal xenoantibodies. Tissue Antigens *22*, 107–117.

Rodrigues, M.M., Cordey, A.-S., Arreaza, G., Corradin, G., Romero, P., Maryanski, J.L., Nussenzweig, R.S., and Zavala, F. (1991). $CD8^+$ cytolytic T cell clones derived against the *Plasmodium yoelii* circumsporozoite protein protect against malaria. Int. Immunol. *3*, 579–585.

Romero, P., Maryanski, J.L., Corradin, G., Nussenzweig, R.S., Nussenzweig, V., and Zavala, F. (1989). Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria. Nature *341*, 323–325.

Ruppert, J., Sidney, J., Celis, E., Kubo, R.T., Grey, H.M., and Sette, A. (1993). Prominent role of secondary anchor residues in peptide binding to HLA-A2.1 molecules. Cell *74*, 929–937.

Schofield, L., Ferreira, A., Nussenzweig, V., and Nussenzweig, R.S. (1987a). Antimalarial activity of alpha tumor necrosis factor and gamma-interferon. Fed. Proc. *46*, 760.

Schofield, L., Villaquiran, J., Ferreira, A., Schellekens, H., Nussenzweig, R.S., and Nussenzweig, V. (1987b). Gamma-interferon, $CD8^+$ T cells and antibodies required for immunity to malaria sporozoites. Nature *330*, 664–666.

Sedegah, M., Sim, B.K. L., Mason, C., Nutman, T., Malik, A., Roberts, C., Johnson, A., Ochola, J., Koech, D., Were, B., and Hoffman, S.L. (1992). Naturally acquired $CD8^+$ cytotoxic T lymphocytes against the *Plasmodium falciparum* circumsporozoite protein. J. Immunol. *149*, 966–971.

Sette, A., Vitiello, A., Reherman, B., Fowler, P., Nayersina, R., Kast, W.M., Melief, C.J.M., Oseroff, C., Yuan, L., Ruppert, J., et al. (1994). The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. J. Immunol. *153*, 5586–5592.

Shimizu, Y., and DeMars, R. (1989). Production of human cells expressing individual transferred HLA-A, -B, -C genes using an HLA-A, -B, -C null human cell line. J. Immunol. *142*, 3320–3328.

Sidney, J., del Guercio, M.-F., Southwood, S., Engelhard, V.H., Appella, E., Rammwnsee, H., Falk, K., Rotzschke, O., Takiguchi, M., Kubo, R.T., et al. (1995). Several HLA alleles share overlapping peptide specificities. J. Immunol. *154*, 247–259.

Sidney, J., Grey, H.M., Kubo, R.T., and Sette, A. (1996a). Practical, biochemical and evolutionary implications of the discovery of HLA class I supertypes. Immunol. Today *17*, 261–266.

Sidney, J., Grey, H.M., Southwood, S., Celis, E., Wentworth, P.A., del Guercio, M.-F., Kubo, R.T., Chesnut, R.W., and Sette, A. (1996b). Definition of an HLA-A3-like supermotif demonstrates the overlapping peptide binding repertoires of common HLA molecules. Hum. Immunol. *45*, 79–93.

Sidney, J., Southwood, S., del Guercio, M.-F., Grey, H.M., Chesnut, R. W., Kubo, R.T., and Sette, A. (1996c). Specificity and degeneracy in peptide binding to HLA-B7-like class I molecules. J. Immunol. *157*, 3480–3490.

Steel, C., Lujan-Trangay, A., Gonzalez-Peralta, C., Zea-Flores, G., and Nutman, T.B. (1984). Transient changes in cytokine profiles following Ivermectin treatment of Onchocerciasis. J. infect. Dis. *170*, 962–970.

Storkus, W.J., Zeh, H.J.I., Maeurer, M.J., Salter, R.D., and Lotze, M.T. (1993). Identification of human melanoma peptides recognized by class I restricted tumor infiltrating T lymphocytes. J. Immunol. *151*, 3719–3727.

Suhrbier, A., Rodda, S.J., Ho, P.C., Csurhes, P., Dunckley, H., Saul, A., Geysen, H.M., and Rzepczyk, C.M. (1991). Role of single amino acids in the recognition of a T cell epitope. J. Immunol. *147*, 2507–2513.

van der Most, R.G., Sette, A., Oseroff, C., Alexander, J., Murali-Krishna, K., Lau, L.L., Southwood, S., Sidney, J., Chesnut, R. W., Matloubian, M., et al. (1996). Analysis of cytotoxic T cell responses to dominant and subdominant epitopes during acute and chronic Lymphocytic Choriomeningitis virus infection. J. Immunol. *157*, 5543–5554.

van Endert, P.M., Riganelli, D., Greco, G., Fleischhauer, K., Sidney, J., Sette, A., and Bach, J.F. (1995). The peptide-binding motif for the human transporter associated with antigen processing. J. Exp. Med. *182*, 1883–1895.

Vitiello, A., Ishioka, G., Grey, H., Rose, R., Farness, P., LaFond, R., Yuan, L., Chisari, F., Furze, J., Bartholomeuz, R., and Chesnut, R. (1995). Development of a lipopeptide-based therapeutic vaccine to treat chronic HBV infection: induction of a primary cytotoxic T lymphocyte response in man. J. Clin. Invest. *95*, 341–349.

Walliker, D., Quakyi, I.A., Wellems, T.E., McCutchan, T.F., Szarfman, A., London, W.T., Corcoran, L.M., Burkot, T.R., and Carter, R. (1987). Genetic analysis of the human malaria parasite *Plasmodium falciparum*. Science *236*, 1661–1666.

Weiss, W.R., Mellouk, S., Houghten, R.A., Sedegah, M., Kumar, S., Good, M.F., Berzofsky, J.A., Miller, L.H., and Hoffman, S.L. (1990). Cytotoxic T cells recognize a peptide from the circumsporozoite protein on malaria-infected hepatocytes. J. Exp. Med. *171*, 763–773.

Weiss, W.R., Berzovsky, J.A., Houghten, R., Sedegah, M., Hollingdale, M., and Hoffman, S.L. (1992). A T cell clone directed at the circumsporozoite protein which protects mice against both *P. yoelii* and *P. berghei*. J. Immunol. *149*, 2103–2109.

Wentworth, P.A., Vitiello, A., Sidney, J., Keogh, E., Chesnut, R.W., Grey, H.M., and Sette, A. (1996). Differences and similarities in the A2.1-restricted cytotoxic T cell repertoire in humans and human leukocyte antigen-transgenic mice. Eur. J. Immunol. *26*, 97–101.

Wizel, B., Houghten, R., Church, P., Tine, J.A., Lanar, D.E., Gordon, D. M., Ballou, W.R., Sette, A., and Hoffman, S.L. (1995a). HLA-A2 restricted cytotoxic T lymphocyte responses to multiple *Plasmodium falciparum* sporozoite protein 2 epitopes in sporozoite-immunized volunteers. J. Immunol. *155*, 766–775.

Wizel, B., Houghten, R.A., Parker, K.C., Coligan, J.E., Church, P., Gordon, D.M., Ballou, W.R., and Hoffman, S.L. (1995b). Irradiated sporozoite vaccine induces HLA-B8-restricted cytotoxic T lymphocyte responses against two overlapping epitopes of the *Plasmodium falciparum* sporozoite surface protein 2. J. Exp. Med. *182*, 1435–1445.

World Health Organization (1994). World malaria situation in 1992. Wkly. Epidemiol. Rec. *69*, 309–314.