Cytotoxic T Lymphocytes in Humans Exposed to *Plasmodium falciparum* by Immunization or Natural Exposure*

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1 The Problem of Malaria and Efforts to Control the Disease

Malaria is one of the most important infectious diseases in the world. It is estimated that 2.1 billion people live in areas of the world where malaria is transmitted and that there are 100–300 million new cases of malaria and one

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* The opinions and assertions 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 the Naval Service at large.

Current Topics in Microbiology and Immunology, Vol. 189

to two million deaths caused by malaria every year (WHO 1991). In the past 10–20 years, the severity of the malaria problem has worsened in many areas because of the emergence of drug-resistant strains of the parasite, resistance to insecticides of the *Anopheles* sp. mosquitoes that transmit the disease, socioeconomic problems that have led to a decreased capacity to optimally utilize existing tools to combat the disease, and movement of nonimmune populations into areas where malaria is transmitted. Accordingly, there are now renewed efforts to control this disease. A major focus of research efforts is to develop vaccines against malaria, including vaccines designed to produce protective cytotoxic T lymphocytes (CTL; HOFFMAN et al. 1991). To understand how such vaccines might work, it is important to understand the life cycle of the parasite.

2 Life Cycle of *Plasmodium falciparum*

Plasmodium falciparum is transmitted to humans by the bite of female *Anopheles* sp. mosquitoes. The mosquito injects sporozoites that pass to the liver, where they enter hepatocytes within 30 min (FAIRLEY 1947). It is unknown whether the sporozoites pass through another cell, such as a reticuloendothelial cell, on the way from the circulation to hepatocytes. Most of the uninucleate sporozoites develop in hepatocytes during 6–10 days to mature liver stage schizonts, which have 10000–40000 uninucleate merozoites. After rupturing out of hepatocytes, each merozoite can invade an erythrocyte, where during the next 48 h each uninucleate merozoite can develop to a mature erythrocytic stage schizont with an average of 16 merozoites. These erythrocytic stage schizonts then rupture, thus releasing merozoites, each of which can then reinvade an erythrocyte, initiating the cycle of amplification, rupture, and reinvasion that leads to increasing levels of parasitemia and the clinical manifestations of malaria. Some of the parasites within infected erythrocytes do not develop to erythrocytic stage schizonts, but instead become sexual forms of the parasite called gametocytes. If a mosquito ingests blood infected with gametocytes, the gametocytes develop during an average of 14 days to sporozoites that can be inoculated into other humans. Parasites that cause malaria in rodents such as *P. yoelii* and *P. berghei* have a similar life cycle, but develop much more rapidly in the liver (40–48 h). Since sporozoites and merozoites in circulation are extracellular and mature human erythrocytes are not known to have major histocompatibility antigens on their surface, infected hepatocytes are considered the primary, and perhaps the only, target of CTL against *P. falciparum*.

3 Rationale for Work on CD8$^+$ CTL Against Preerythrocytic *Plasmodium* Species Antigens

Immunization of mice (NUSSENZWEIG et al. 1969) and humans (CLYDE et al. 1973; RIECKMANN et al. 1979) with radiation attenuated *Plasmodium* sp. sporozoites protects against challenge with live sporozoites, but does not protect against challenge with infected erythrocytes. The protective immunity induced by the irradiated sporozoite vaccine must therefore be directed against extracellular sporozoites in the circulation (antibodies) or against parasites within hepatocytes (antibodies or cellular immune responses). This is consistent with the observation that irradiated sporozoites develop only partially within hepatocytes and never mature to the late liver schizont stage. Because the sera from mice (VANDERBERG et al. 1969) and humans (CLYDE et al. 1973) immunized with irradiated sporozoites precipitates the surface coat of sporozoites, for many years antibodies against sporozoites were thought to be the major immune effectors responsible for this protection. However, in 1977 it was reported that mu-suppressed mice (mice that could not produce antibodies) immunized with irradiated sporozoites were protected against challenge (CHEN et al. 1977), and several years ago it was shown that adoptive transfer of T cells from mice immunized with irradiated sporozoites protected naive mice against challenge with live sporozoites at a time when the mice had no detectable circulating antisporozoite antibodies (EGAN et al. 1987). Furthermore, in some strains of mice the immunity induced by immunization with irradiated sporozoites was completely reversed by in vivo depletion of CD8$^+$ T lymphocytes (SCHOFIELD et al. 1987; WEISS et al. 1988). The antibodies induced by this form of immunization were not adequate to protect on their own, and it appeared that CD8$^+$ CTL were required for this protective immunity. CTL must recognize their target peptides in combination with major histocompatibility complex (MHC) proteins. These data indicated that the protective CTL could not be directed against extracellular sporozoites in the circulation and must be directed against *Plasmodium* sp. peptides in association with class I MHC molecules on the surface of infected hepatocytes. However, when these discoveries were made, no *Plasmodium* sp. antigen had ever been detected on the surface of infected hepatocytes, inflammatory infiltrates had been shown to be infrequent in the livers of mice infected with malaria (MEIS et al. 1987), and there was considerable debate regarding whether hepatocytes express class I MHC molecules and whether immune CTL could pass from the sinusoids into the space of Disse and attack infected hepatocytes. Within a year it was shown that mice immunized with irradiated *P. berghei* sporozoites and challenged with live sporozoites developed antigen-specific, CD8$^+$ T cell-dependent infiltrates in their livers and that spleen cells from these mice eliminated *P. berghei*-infected hepatocytes from in vitro culture in an MHC-restricted and antigen specific manner (HOFFMAN et al. 1989a). These studies demon-

strated that *Plasmodium* sp. peptides were recognized on infected hepatocytes by T cells and indicated that immune cells could reach, recognize, and destroy infected hepatocytes. They did not indicate which specific *Plasmodium* sp. antigens on hepatocytes were recognized by CTL.

Another intriguing observation supporting the role of CTL in protection against malaria was made several years later. An association was noted between the presence of HLA Bw53 (class I) and protection against severe malaria in Gambians (HILL et al. 1991). HLA Bw53 was found in 16.9% of cases of severe malaria, in 25.4% of controls with mild malaria, and in 25% of adults without malaria. The relative risk of severe malaria among individuals with HLA Bw53 compared with those without this allele was 0.59.

HLA Bw53 is found in 15%–40% of the population of sub-Saharan Africa, but is found in less than 1% of Caucasians and Orientals; like the sickle cell trait, it may have been selected because it protects against severe malaria. The presence of HLA Bw53 is not as protective as the HbS carrier state; only 1.2% of patients with severe malaria in the Gambia study had HbS, while 12.9% of controls with mild malaria carried HbS (relative risk 0.08). Nonetheless, the decreased association of HLA Bw53 with severe malaria suggests that naturally acquired CTL against *Plasmodium* sp. proteins protect against severe malaria. The logical targets for CTL are infected hepatocytes. Most deaths occur in children less than 5 years of age, and these children are not protected against developing malaria infections. Thus, such data raise the possibility that under natural conditions of exposure, CTL against infected hepatocytes reduce mortality by reducing the burden of infection, not by preventing infection. This is consistent with the recent report that use of insecticide-impregnated bednets does not reduce the incidence of parasitemia in Gambian villages, but has a dramatic effect on mortality (ALONSO et al. 1991).

4 $CD8^+$ CTL Against the Circumsporozoite Protein

4.1 Rationale for Work

When work began in earnest on CTL in malaria, the only characterized sporozoite or liver (pre-erythrocytic) stage antigen available for study was the circumsporozoite (CS) protein (YOSHIDA et al. 1980). Kumar reported that B10.BR mice immunized with irradiated *P. falciparum* sporozoites and recombinant vaccinia expressing the *P. falciparum* CS protein (PfCSP) produced CTL against a single 23-amino acid region from the carboxy terminus of the CS protein, Pf 7G8 CS368–390 (KPKDELDYENDIEKKICK-MEKCS; KUMAR et al. 1988). It was next reported that BALB/c mice immunized with *P. berghei* (ROMERO et al. 1989) or *P. yoelii* (WEISS et al. 1990)

sporozoites produced CTL against 12-amino acid (residues 249 260, NDDSYIPSAEKI) and 16-amino acid (residues 281 296, SYVPSAE-QILEFVKQI) regions of the respective CS proteins. WEISS demonstrated that the CTL eliminated infected hepatocytes from in vitro culture; the CTL recognized this 16-amino acid peptide on the surface of infected hepatocytes with class I MHC. ROMERO reported that adoptive transfer of CTL clones against the analogous epitope on the *P. berghei* CS protein completely protected against challenge in vivo. RODRIGUES (RODRIGUES et al. 1991) subsequently reported that adoptive transfer of CTL clones against the *P. yoelii* CS protein protected even if transferred 3 h after sporozoites were inoculated, at a time when the sporozoites had left the circulation and were within hepatocytes. Infected hepatocytes were clearly the target of these CTL. RODRIGUES also showed that protective clones could be found in the liver in apposition to infected hepatocytes (RODRIGUES et al. 1992), but that only CTL clones expressing the adhesion molecule CD44 were protective. This suggests that in addition to epitope specificity and cytolytic capacity, homing is critical for protection. This view has been supported by studies in the *P. yoelii* and *P. berghei* rodent model system that show that the induction of CTL by active immunization does not indicate that an animal will be protected (FLYNN et al. 1990; SEDEGAH et al. 1992a; SATCHIDANANDAM et al. 1991).

The mechanism by which the CTL eliminate infected hepatocytes has as yet not been established. Schofield showed that treatment of fully immune A/J mice with antibodies to γ-interferon abrogated the irradiated sporozoite-induced protective immunity (SCHOFIELD et al. 1987). This was not the case in BALB/c mice immunized with irradiated *P. berghei* sporozoites (HOFFMAN et al. 1989a). However, Weiss has recently shown that protective immunity conferred by adoptive transfer of a $CD8^+$ CTL clone against a 10-amino acid peptide (SYVPSAEQIL) on the *P. yoelii* CS protein is eliminated by in vivo treatment with anti-γ-interferon (WEISS et al. 1992). Weiss has also shown that this $CD8^+$ CTL clone protects against both *P. yoelii* and *P. berghei*, despite the fact that the two CS proteins vary by two amino acids at the site of the CTL epitope (WEISS et al. 1992).

In addition to the adoptive transfer experiments, it has been shown that immunization of BALB/c mice with recombinant *Salmonella typhimurium* expressing the *P. berghei* CS protein induces protective immunity in 50%–75% of mice and that this protection is eliminated by in vivo depletion of $CD8^+$ T cells (AGGARWAL et al. 1991). Likewise, immunization of BALB/c with recombinant P815 mastocytoma cells expressing the *P. yoelii* CS protein also induces $CD8^+$ T cell-dependent protective immunity in 50%–75% of recipient mice (KHUSMITH et al. 1991).

4.2 CD8$^+$ T Cell-Dependent Cytolytic Activity Against the *P. falciparum* Circumsporozoite Protein

4.2.1 Volunteers Immunized with Radiation-Attenuated Sporozoites

The studies in the rodent model systems provided a compelling rationale for developing vaccines for humans that would induce similar CTL against the PfCSP. However, at the time such work was contemplated, no one had shown that humans produced CTL to any *Plasmodium* sp. protein, and in fact, there was only one report in the world's literature demonstrating that humans produced CTL against any parasite protein (YANO et al. 1989). MALIK and collaborators (1991) developed an assay for demonstrating CD8$^+$ CTL against the PfCSP. To provide effector cells, humans were immunized by the bites of previously irradiated *Anopheles stephensi* mosquitoes carrying NF54/3D7 *P. falciparum* sporozoites in their salivary glands (EGAN et al. 1993). Peripheral blood mononuclear cells were harvested and stimulated in vitro with recombinant vaccinia virus expressing the PfCSP (7G8 clone of *P. falciparum*) for 6 days. These effectors were then incubated with autologous Epstein-Barr virus (EBV)-transformed B lymphocytes that had been transiently transfected with the gene encoding the 7G8 PfCSP 48 h previously. Using this method, MALIK and colleagues demonstrated that three of four immunized volunteers produced CTL against the PfCSP and that this cytolytic activity was antigen specific, genetically restricted, and dependent on CD8$^+$ T lymphocytes. They reported that cytolytic activity could not be demonstrated each time an assay was run; six of nine, seven of 11, and two of 12 assays were positive in the three individuals shown to have cytolytic activity. They also reported the presence of cytolytic activity 13 weeks after last immunization in one volunteer.

Stimulating with vaccinia-infected cells in vitro was consumptive of large numbers of human cells. Since a peptide including amino acids 368–390 of the Pf 7G8 CS (Table 1) had previously been shown to include a CTL epitope in B10.BR mice (KUMAR et al. 1988), this peptide was studied in the same system. Three of the four volunteers were shown to have CTL directed at one or more epitopes included within this peptide. In one volunteer, overlapping 20-amino acid peptides representing the entire carboxy terminus of the 7G8 CS protein were studied, and only peptide 368–390 was able to label target cells for killing by CTL. CTL from this volunteer recognized both the 7G8 *P. falciparum* sequence and the sequence based on the immunizing sporozoites (3D7) that differed by one amino acid (Table 1). It has not been established whether the variant amino acid residue is actually included within the CTL epitope. However, this finding is of potential importance because of concerns regarding polymorphism of this epitope and the reasons why polymorphism has developed at this site. If the polymorphism is the result of immune selection and CTL against one sequence do not recognize CTL against variant

Table 1. Variation in the region of the only know human CD8$^+$ CTL epitope on the *P. falciparum* circumsporozoite (CS) protein

Strain or isolate	Amino acid residue										
	367	368	369	370	371	372	373	374	375	376	377
7G8 T9/101;406_{3-5}; T4R	**N**	K	**P**	**K**	D	**E**	L	**D**	Y	**E**	**N**
NF54/3D7; $XP_{8,9}$; $X_{10,11}$; T9-98; $406_{1,7,8}$; 427_{1-10}; 419_{10}; GAM1										A	
AE7, PNG2						Q					
ItG2: W/L; PNG1, PNG X 22; T9-94; WEL	D					Q					
MCK$^+$; AE28; GAM2; MS2; Palo Alto; $366_{1,5,8-10}$ 399_{1-10}; LE5						Q				A	
HB3; X5; $XP_{12,13}$; PNG4; BRA1	G		S								
PNG3	D					Q		C			S
GAM3; $366_{2-4,6,7}$; 406_{10}; 419_{1-9};						Q		N			
GAM4; 406_2				R						A	
GAM5; $406_{3,9}$										A	D

In mice and humans, a peptide including amino acid residues 368–390 of the 7G8 *P. falciparum* CS protein (Pf 7G8 CS 368–390, KPKDELDYENDIEKKICKMEKCS; DAME et al. 1984) has been shown to include a CD8$^+$ CTL epitope. No variation has been identified from amino acids 378–390. Amino acids 367–377 of the CS protein of the 7G8 clone of *P. falciparum* are shown, and identified variations in this region (LOCKYER et al. 1989; DOOLAN et al. 1992; SHI et al. 1992) are listed. Amino acid 367 is also included, because there are data suggesting that there is a CTL epitope in the region from amino acid 351–370 (DOOLAN et al. 1991), and no variation has been shown from amino acid residues 351–366, but variation has been identified at amino acid residue 367.

sequences, the usefulness of a vaccine designed to produce CTL against only one variant will be limited. The importance of polymorphism at important CD8$^+$ CTL sites remains to be elucidated. However, variation has been identified in isolates from around the world (Table 1; LOCKYER et al. 1989; DOOLAN et al. 1992; SHI et al. 1992), suggesting that the variation is the result of immune selection (GOOD et al. 1988).

Having established that volunteers immunized with irradiated *P. falciparum* sporozoites produced CTL against the PfCSP, the investigators asked whether the demonstration of CTL by their assay indicated that a volunteer would be protected. As predicted by previous murine studies (FLYNN et al. 1990; SEDEGAH et al. 1992a; SATCHIDANANDAM et al. 1991), the presence of CTL against this epitope did not indicate that a volunteer would be protected aginst challenge, and the absence of cytolytic activity did not indicate that a volunteer would not be protected (MALIK et al. 1991).

Table 2. HLA types of the four Kenyans naturally exposed to malaria and three individuals immunized with irradiated *P. falciparum* sporozoites who have been shown to have CD8$^+$ T cell dependent cytolytic activity against peptide Pf CS 368–390 (MALIK et al. 1991, SEDEGAH et al. 1992)

Volunteer	Class I				Class II			CTL
	A	B	Bw4/w6	C	DR	DRw52/53	Dq	
6	2, 32	w48, 45	w6, w6	w6	w8, w11	w52	w7,w1	+
9	30, w34	w42, w48	w6, w6	w4	ND	ND	ND	+
10	2, 28	51, 15	w4, w4	w4	w11, 7	w52, w53	w7, w2	+
8	30, 32	w58, -	w4	w6	w11, -	w52	w1, w7	+
1H	11, 24	35, -	ND	w4, -	[a]	[a]	w1	+
3W	1, 3	8, 35	ND	w4,	1, 7	w53, -	w1, w2	+
4R	1, 28	44, w57	ND	w6, -	7, w11	w52, w53	w3	+

[a] Not typeable by conventional methods.

4.2.2 Kenyans Naturally Exposed to Malaria

Knowing that humans immunized with irradiated sporozoites produced CD8$^+$ CTL against the region of PfCSP including amino acids Pf 7G8 CS 368–390, Sedegah and coworkers set out to determine whether humans naturally exposed to malaria produced CTL against this region of the CS protein. This region was of particular interest because it had been previously shown that there was a correlation between lymphocyte proliferation to peptides Pf 7G8 CS 361–380 and 371–390 and resistence to reinfection with malaria among Kenyan adults (HOFFMAN et al. 1989; MASON et al., unpublished). Eleven Kenyans were selected because their lymphocytes had been shown to proliferate after stimulation with one of these peptides and because they were also resistant to reinfection with malaria (nine of the 11 volunteers). They were studied at a single time point at the end of a period of low malaria transmission, and four of the 11 volunteers were shown to have cytolytic activity against autologous EBV-transformed B cells pulsed with the Pf 7G8 CS 368–390 peptide. In three of the four cases, the cytolytic activity was reversed by depletion of CD8$^+$ T cells, but in no case did depletion of CD4$^+$ T cells reduce cytolytic activity (SEDEGAH et al. 1992b). Individuals of various HLA phenotypes have been shown to have CD8$^+$ T cell-dependent cytolytic activity against target cells pulsed with peptide 368–390 (Table 2). Thus far, minimal CTL epitopes have not been identified and T cell clones against this region of the CS protein have not been produced. Thus, restriction elements have not been clearly delineated.

4.2.3 HLA B35-Restricted Activity Among Gambians Naturally Exposed to Malaria

Using the techniques described in Sect. 5.1 below, HILL and colleagues (1992) eluted peptides from HLA B35. In addition to a proline at position 2, they found these peptides generally included a tyrosine at position 9. They

found ten peptide sequences from the four pre-erythrocytic stage proteins described below that had prolines at positions 2 and tyrosines at position 8 (six sequences), position 9 (one sequence), or position 10 (three sequences). Six of these sequences were from PfCSP, and four of the six were from variants of the first eight to ten amino acids of Pf CS 7G8 368–390, the previously identified human CTL epitope (see Sects. 4.2.1 and 4.2.2 above). One of eight Gambians tested had cytolytic activity against peptide Pf CS 7G8 368 375 (KPKDELDY). Cells from this volunteer that were stimulated with peptide KPKDELDY did not lyse targets pulsed with peptides KPKD**Q**L**N**Y or K**S**KDELDY, sequences from other known variants (Table 1). Cells from a second volunteer recognized peptide K**S**KDELDY, but did not recognize the variant peptides KPKDELDY, KPKD**Q**LDY, or KPKD**Q**L**N**Y (Table 1). This cytolytic activity was inhibited by an anti-CD8 monoclonal antibody. These data demonstrate for the first time that single amino acid changes at the site of PfCSP CTL epitopes can eliminate recognition by CTL. Identification of a minimal CTL epitope in the region of Pf CS 7G8 368 390 with the greatest polymorphism supports the hypothesis that this polymorphism has arisen secondary to immune pressure (GOOD et al. 1988).

5 CD8$^+$ CTL Against Liver Stage Antigen-1

5.1 HLA Bw53-Restricted, CD8$^+$ T Cell-Dependent Cytolytic Activity Against Liver Stage Antigen-1 in Gambians Naturally Exposed to Malaria

Elegant studies that followed up the demonstration of an association between HLA Bw53 and lack of severe malaria (see Sect. 3 above) have recently been reported (HILL et al. 1992). The investigators sequenced peptides eluted from HLA B53 and found that most peptides had a proline at position 2. They then synthesized 60 8- to 10-amino acid peptides from four *P. falciparum* pre-erythrocytic stage antigens, Pf CSP (DAME et al. 1984), TRAP/Pf SSP2 (ROBSON et al. 1988; ROGERS et al. 1992a), liver stage antigen-1 (LSA-1; GUERIN-MARCHAND et al. 1987; ZHU and HOLLINGDALE 1991), and Pfs16 (MOELANS et al. 1991), that had prolines in position 2. These peptides were studied to determine whether they bound to HLA B53 in an HLA assembly assay. Eight peptides were found to bind, three from PfCSP, two from TRAP/PfSSP2, one from LSA-1, and two from Pfs16. Six individuals in one village and nine individuals in a village with much lower transmission of malaria were tested during a period of low malaria transmission. Using a classical restimulation CTL assay, they showed that none of the individuals had CTL against the PfCSP, PfSSP2/TRAP, and Pfs16 peptides.However, three of six volunteers in the first village and one of nine volunteers in the second village had HLA B53, peptide-specific, CD8$^+$ T

cell-dependent cytolytic activity against the LSA-1 peptide referred to as ls6 (KPIVQYDNF). This epitope was shown to be invariant in nine *P. falciparum* isolates that they sequenced. These findings suggest that CTL against this LSA-1 peptide may be involved in the partial protection against severe malaria associated with HLA Bw53.

5.2 HLA B35-Restricted CTL Against Liver Stage Antigen-1

One of eight Gambians was shown to have cytolytic activity against the LSA-1 peptide (ls8, KPNDKSLY) eluted from HLA B35 as described in Sects. 4.2.3 and 5.1 above (HILL et al. 1992). There was no indication as to whether the cytolytic activity was dependent on $CD8^+$ T cells.

6 $CD4^+$ CTL Against the Circumsporozoite Protein

6.1 Rationale for Work

DEL GIUDICE and colleagues immunized mice subcutaneously at the base of the tail with a peptide including amino acids 59–79 of the *P. yoelii* CS protein (YNRNIVNRLLGDALNGKPEEK) and using regional lymph node cells produced a $CD4^+$ T cell clone that recognized this peptide and a shorter peptide (PyCS 61–70 in BALB/c mice and PyCS 59–70 in C57BL/6 mice; DEL GIUDICE et al. 1990). This clone eliminated infected hepatocytes from culture (RENIA et al. 1991) and adoptively transferred protection against *P. yoelii*. The clone was never shown to have cytolytic activity against target cells pulsed with the peptide, but the in vitro activity against infected hepatocytes was not reversed by anticytokine treatment. It is not certain whether these clones are directly cytolytic or whether they mediate protection through another mechanism. Nonetheless, working in the less infective, but uniformly lethal, *P. berghei* system, CORRADIN and colleagues have found that immunization of mice at the base of the tail with the analogous *P. berghei* peptide presented as a branched chain polymer protects mice (unpublished observation). The mechanism of this protection has not been established, but such data identify protective $CD4^+$ T cells against an epitope amino terminal of the repeat region of the *P. yoelii* CS protein.

6.2 $CD4^+$ CTL Against the *P. falciparum* Circumsporozoite Protein in Volunteers Immunized with Irradiated *P. falciparum* Sporozoites

Moreno and colleagues have recently reported the production of a $CD4^+$ T cell clone with cytolytic activity from an individual immunized with radiation

Table 3. Variation in the region of the only known human CD4$^+$ CTL epitope on the *P. falciparum* circumsporozoite (cs) protein

Strain or isolate	*Amino acid residue (7G8 sequence)*													
	329	330	331	332	333	334	335	336	337	338	339	340	341	342
7G8	**K**	H	I	**E**	**Q**	Y	L	**K**	**K**	I	**K**	N	S	**I**
NF54/3D7; $XP_{8,9,}$ $X_{10,11,}$; $427_{1\ 4,6\ 10}$				K	F			N			Q			L
T4; T4R; T9-101; WEL; W/L; PNG X 22; T9-94; MCK$^+$; AE28; AE7; HB3; X_5; $XP_{12,13}$; PNG2; BRAI											Q			L
ItG2G1; ItG2					K						Q			L
366_1; $399_{1\ 10}$; $406_{1\ 9}$ 419_{10};	Q				K				T					L
T9-98									T					L
427_5								N	T					L
LE5, 366_9	Q				K				T		Q			L
MS2, Palo Alto Brazil X4; BRA2	Q				K				R		Q			L
$366_{2\ 7}$	Q				K				Q		R			L
$366_{8.10}$	Q				K				Q		Q			L
406_{10}; $419_{1\ 9}$	Q				K				Q					L
GAM1	Q				K				T		Q			L
GAM2	Q				K				T					L
GAM3	Q				K			Q			Q			L
GAM4	Q				K			Q						L
GAM5								N	T		Q			L
GAM6	Q				K			Q			R			L

In humans, a peptide including amino acid residues 326–345 of the NF54/3D7 *P. falciparum* CS protein (Pf 3D7 CS 326–345, EYLN**KIQ-NSLSTEW**SPCSVT; Caspers et al. 1989; Campbell 1989) that is analogous to amino acid residues 333–352 of the 7G8 CS protein seqence (Dame et al. 1984) has been shown to include a CD4$^+$ CTL epitope (Moreno et al. 1991). The minimal epitope recognized in the context of HLA DR7 has been shown to be amino acids Pf 3D7 CS 330–339 (KIQNSLSTEW) (= Pf 7G8 337–346). No variation has been identified in amino acids Pf 3D7 CS 336–345 (Pf 7G8 CS 343–352). Amino acids 329–342 of the 7G8 clone of *P. falciparum* are shown, and identified variations among isolates in the region (Lockyer et al. 1989; Doolan et al. 1992; Shi et al. 1992) are listed. Amino acid residues 329–332 are included, since Pf 7G8 326–345 (PSDKHIEQYLKKIKNSISTE) has been shown to include a T-helper epitope, and no variation has been shown in amino acids 326–328 of the 7G8 sequence.

attenuated *P. falciparum* sporozoites (MORENO et al. 1991). They have mapped the epitope to residues 330–339 (KIQNSLSTEW) of the NF54/3D7 sequence of the PfCSP (CASPERS et al. 1989; CAMPBELL 1989) and demonstrated that this peptide is recognized in the context of HLA DR7. This epitope corresponds to amino acids 337–346 of the 7G8 sequence of the PfCSP (DAME et al. 1984) and is found in a polymorphic region of the CS protein (Table 3; LOCKYER et al. 1989; DOOLAN et al. 1992; SHI et al. 1992). It has therefore been suggested that, as for the $CD8^+$ CTL epitope described above, the variation in this region is a result of immune selection.

7 $CD4^+$ CTL Against Other Pre-erythrocytic Antigens in Murine Models

TSUJI and colleagues (1990) immunized mice with irradiated *P. berghei* sporozoites, stimulated spleen cells with an extract of blood stage *P. berghei* parasites, and derived a $CD4^+$ T cell clone that produces interleukin 2 (IL-2) and γ-interferon when stimulated with blood stage parasite extract. The clone also lyses target cells pulsed with blood stage *P. berghei* extract. More importantly, adoptive transfer of this clone protects against sporozoite challenge, but not against blood stage challenge. This strongly suggests that the clone is recognizing a parasite antigen expressed in infected hepatocytes. Neither the *P. berghei* antigen recognized by this clone, nor its *P. falciparum* analog has been identified.

8 CTL Against the *P. falciparum* Circumsprozoite Protein Among Australians Naturally Exposed to Malaria

Doolan and colleagues stimulated peripheral blood mononuclear cells from Australians who had been previously exposed to malaria (DOOLAN et al. 1991) with a mixture of CS protein peptides. Their studies showed that after two cycles of stimulation, cells from three of 42 donors had cytolytic activity against a mixture of overlapping 20-amino acid peptides spanning amino acid residues 341–412 (Pf CSP 7G8). Like the results with volunteers immunized with irradiated sporozoites (MALIK et al. 1991), cytolytic activity could not be demonstrated each time an assay was run, suggesting to the investigators that there is a low frequency of these cells in the peripheral blood. They did not report on whether the activity was dependent on a particular subset of T lymphocytes or genetically restricted, but two of the three positive donors were HLA Bw57. They produced a T cell line from one

donor and demonstrated that the line lysed target cells pulsed with peptides Pf 7G8 CS 351 370, 371 390, or 376 395. These data indicate that there is at least one CTL epitope within residues Pf7G8 CS 351 370 and at least one more within residues Pf 7G8 CS 371 390. However, the T cell subset specificity and genetic restriction of this cytolytic activity remains to be established.

9 Rationale for Work on CD8$^+$ CTL Against Sporozoite Surface Protein-2

Immunization with irradiated sporozoites completely protects against malaria, but none of the subunit *P. berghei* or *P. yoelii* CS protein vaccines have given protection comparable to the irradiated sporozoite vaccine. Furthermore, in the human studies the presence of CTL against the PfCSP did not guarantee that the individual would be protected and, likewise, one individual who was not shown to have CTL was protected against challenge (MALIK et al. 1991). Considering the complexity of sporozoites, it was not logical to assume that all protection induced by the whole organism vaccine was mediated by CTL against a single short stretch of amino acids on a single protein. Thus, there has been considerable effort to identify additional targets of irradiated sporozoite-induced protective immunity. Mice were immunized with irradiated *P. yoelii* sporozoites, and a monoclonal antibody directed at a 140-kDa sporozoite protein was produced (CHAROENVIT et al. 1987). The gene encoding this protein was cloned and sequenced (HEDSTROM et al. 1990; ROGERS et al. 1992a), and the protein was named sporozoite surface protein-2 (PySSP2). To determine whether immunization with irradiated sporozoites not only produced antibodies, but also CTL against PySSP2, a 1.5-kb fragment of the gene encoding PySSP2 was transfected into P815 mouse mastocytoma cells. When these transfected cells were used as targets in CTL assays, mice immunized with irradiated sporozoites were shown to produce CTL against PySSP2 (KHUSMITH et al. 1991). KHUSMITH et al. subsequently produced CD8$^+$ CTL clones against PySSP2 and showed that adoptive transfer of one of these clones completely protected against challenge, establishing that CTL against PySSP2 could completely protect against this highly infectious parasite in the absence of any other parasite specific immune responses (S. KHUSMITH, unpublished). Like the anti-CS protein CTL clones, these clones protected when adoptively transferred into mice 3 h after sporozoite inoculation, indicating that they were attacking infected hepatocytes. Mice were also immunized with the P815 cells expressing PySSP2, and approximately 50% were protected against challenge (KHUSMITH et al. 1991). KHUSMITH then went on to show that although immunization with the *P. yoelii* CS protein or PySSP2 vaccines gave only

partial protection against malaria (50%–75%), immunization with transfected P815 cells expressing, *P. yoelii* CSP and PySSP2 produced 100% protection (KHUSMITH et al. 1991). Furthermore, as after immunization with irradiated sporozoites, this protective immunity was completely reversed by in vivo depletion of $CD8^+$ T cells.

The gene encoding the *P. falciparum* SSP2 (PfSSP2) has now been identified and characterized (ROGERS et al. 1992b) and shown to be the previously described thrombospondin-related anonymous protein (TRAP; ROBSON et al. 1988). Work is in progress to identify CTL epitopes on PfSSP2 and to produce vaccines that will induce protective CTL in humans against PfSSP2 and PfCSP.

10 Conclusion

The study of human CTL against *Plasmodium* sp. peptides is in its infancy. There is a compelling rationale for work on the identification, characterization, and induction of class I-restricted, $CD8^+$ CTL against pre-erythrocytic stages of *P. falciparum*. The data supporting work on $CD4^+$ CTL is not as complete, but is also solid. Work is now, or will soon be, underway in a number of laboratories to use synthetic peptide, recombinant protein, live vector-delivered antigen and plasmid DNA vaccines to induce CTL in humans against PfCSP, PfSSP2, PfLSA-1, and other *P. falciparum* proteins expressed in infected hepatocytes. In parallel studies, a number of scientists are attempting to characterize further the qualities of CTL associated with protection, to identify and characterize additional CTL epitopes on these proteins, and to optimize vaccine delivery systems in the rodent model systems. Duplicating the non-strain-specific, sterile, consistently protective immunity induced in humans by the irradiated sporozoite vaccine will almost certainly require constructing vaccines that induce a essentially all vaccinees protective CTL against at least one epitope that is present in all strains of parasites. It will probably also require induction of protective antibody responses.

Acknowledgements. This work was supported in part by the Naval Medical Research and ONR grant N00014-89-J-1856 to the Pan American Health Organization.

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