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Natural amino acid polymorphisms of the circumsporozoite protein of *Plasmodium falciparum* abrogate specific human CD4+ T cell responsiveness*

Sequence polymorphism has been reported for virtually all malaria antigens and, in the case of the circumsporozoite (CS) protein, this variation is in the form of point mutations concentrated primarily in several regions recognized by T cells. The factors responsible for the variation are unknown. We studied the T cell responses to all known variants in malaria-exposed Thais. Memory CD4+ T cells responded to variants of a polymorphic immunodominant region (denoted Th2R), and CD4+ T cell clones specific for one Thai Th2R variant were generated. There was minimal cross-reactivity to any of the naturally occurring variants, including the other Thai variant, and competition studies performed with the clones using analog peptides demonstrated that all the substitutions of the polymorphic residues modulate either the binding of the peptide to major histocompatibility complex (MHC) molecules or the recognition by the T cell receptor of the peptide-MHC complex. Our data suggest that CD4+ T cells may be able to select parasites expressing variant sequences and have implications for development of a CS-based vaccine.

1 Introduction

The circumsporozoite (CS) protein is a coat protein of malaria sporozoites and is the best characterized of the anti-sporozoite vaccine candidates. All species of malaria have CS proteins which conform to a specific structure [1], the most notable feature of which is a large central segment composed of multiple tandemly repeated units. In *Plasmodium falciparum*, the repeats form a well-conserved [2, 3] and highly immunodominant [4] B cell epitope. However, while antibody, which interacts with the circulating sporozoite, may contribute in part to sporozoite immunity, T cell immunity focusing on the intra-hepatocytic parasite may be more important [5].

CS-specific CD8+ murine T cells can transfer immunity into naive recipients [6–8] and immunization of mice with CS-recombinant *Salmonella* can induce protective CD8+ T cells [9, 10]. However, not all sporozoite-immunized mice rendered CD8-deficient lose immunity [11] and recently it was shown that synthetic peptide immunization could induce protective CD4+ T cells [12]. T cells probably act by killing the parasite after hepatocyte invasion either

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Abbreviations: CS: Circumsporozoite **SI:** Stimulation index

Key words: Malaria / Circumsporozoite protein / T cells / Polymorphism

by direct cytotoxicity [7] or through secretion of parasiticidal lymphokines (including interferon-γ and IL-6) [8, 12–18].

A number of human T cell epitopes have been identified on the *P. falciparum* CS protein [19–24]. Most occur in the non-repetitive regions of the protein, and the most immunodominant of those map to two foci of known amino acid diversity, denoted as Th2R and Th3R (Fig. 1). The precise mapping of T cell epitopes to variable domains of the protein suggested that CD8+ T cells could have selected the variation by killing sporozoite-infected hepatocytes [19]. This was proposed when it was found that the rate of non-synonymous (coding change) nucleotide substitutions in regions of polymorphism is significantly higher than synonymous (silent) mutations, indicating a strong biological pressure at the protein level [25]. A recent study analyzing ten variant *P. falciparum* CS nucleotide sequences reaches similar conclusions and also shows that the non-synonymous mutations are restricted to the T cell domains [34]. Moreover, the non-synonymous substitutions cause a change in amino acid charge nine times more frequently than expected by chance [34]. Charge differences may alter the ability of peptides to bind to the MHC and/or the TCR, thus affecting T cell responsiveness.

It has been suggested that CD8+ T cells could select parasites expressing variation in the CS protein [25]. However, only a single CD8+ T cell site has been identified from one mouse [35] and three human [20–23] studies, and this is located in the Th3R region. It is possible that CTL have selected the variation in this region, as CTL from two individuals raised to the CTL determinant were able to discriminate between four of the naturally occurring variants [23]. However, the human CTL studies suggest that few people naturally exposed to malaria sporozoites contain CS-specific CD8+ cells [20–23]. In contrast, the frequency of CS-specific CD4+ T cells in endemic populations and the number of CD4+ T cell sites are much greater. It is possible that CD4+ cells could have selected variants.

```
326      331              345    350           361                   380
PSDKHIEQYLKKIKNSISTEWSPCSVTCGNGIQVRIKPGSANKPKDELDYENDIE
```

```
A   ---Q--EK--KR-Q--L---        A   ------N-PK-Q-D-AN---
B   ---K--EQ--KK-K--I---        B   ------N-PK-E-D-EN---
C   ---Q--EK--QK-K--L---        C   ------N-PK-E-N-EN---
D   ---K--KE--NK-Q--L---        D   ------N-PK-E-D-AD---
E   ---Q--EK--QK-R--L---        E   ------N-PK-Q-D-EN---
F   ---Q--EK--KT-K--L---        F   ------N-PR-E-D-EN---
G   ---K--EQ--KT-K--L---        G   ------N-PK-Q-N-EN---
H   ---Q--EK--KT-Q--L---        H   ------G-SK-E-D-EN---
I   ---K--EQ--KK-Q--L---        I   ------N-PK-E-D-AN---
J   ---K--EK--NT-Q--L---        J   ------D-PK-Q-D-EN---
K   ---Q--EK--QK-Q--L---
L   ---K--EK--KK-Q--L---        Th3R361-380
```

Th2R326-345

```
A   -EK--KR-Q--L--------
B   -EQ--KK-K--I--------
C   -EK--QK-K--L--------
D   -KE--NK-Q--L--------
E   -EK--QK-R--L--------
F   -EK--KT-K--L--------
G   -EQ--KT-K--L--------
H   -EK--KT-Q--L--------
I   -EQ--KK-Q--L--------
J   -EK--NT-Q--L--------
K   -EK--QK-Q--L--------
L   -EK--KK-Q--L--------
```

Th2R331-350

```
A1  -EK--KR-Q--L
A2      -KR-Q--L----
A3          Q--L--------
```
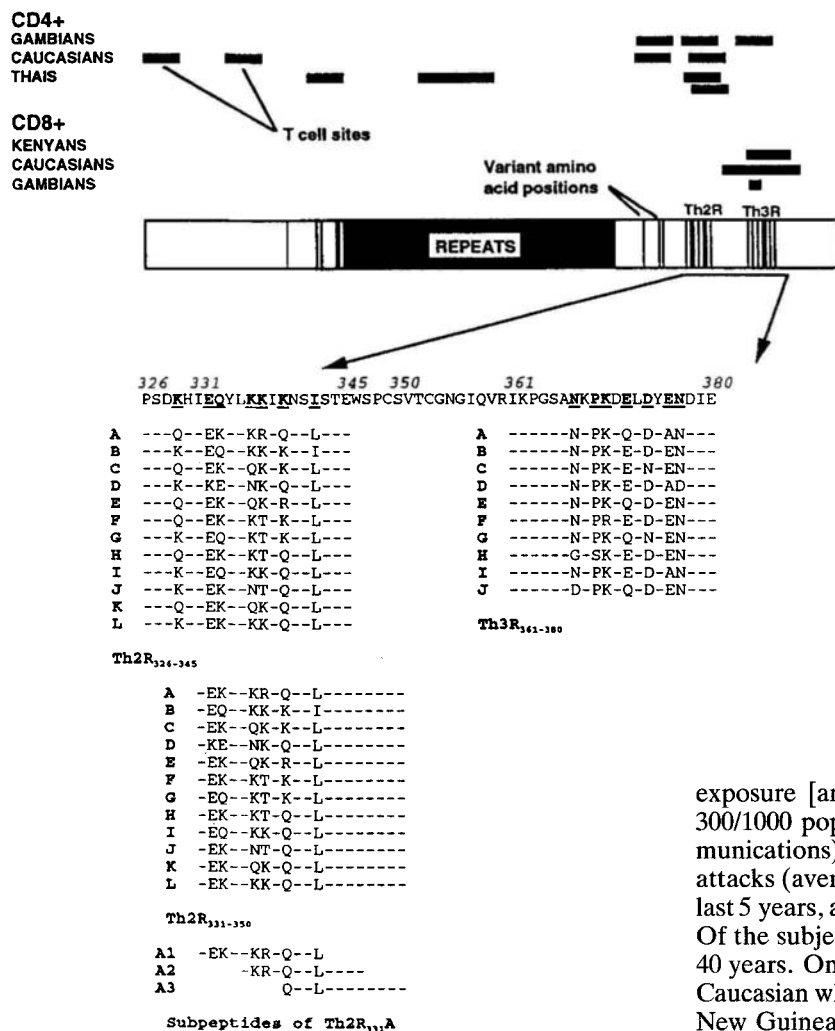
Subpeptides of Th2R331A

Figure 1. Schematic representation of the CS protein. The correlation of T cell determinants [19–24] with point mutations [25–33] is indicated. Further, the region of Th2R and Th3R is expanded to provide the sequences of the peptides used in the study, which include 12 variant peptides each of $Th2R_{326-345}$ and $Th2R_{331-350}$, 10 variants of $Th3R_{361-380}$, and three 12-mer subpeptides of the A variant of $Th2R_{331-350}$.

exposure [annual parasite incidence ranging from 50 to 300/1000 population (C. Khamboonruang, personal communications)]. All subjects had experienced one or more attacks (average three) of *P. falciparum* malaria within the last 5 years, as shown by hospital and malaria clinic records. Of the subjects, 77 % were male, and the average age was 40 years. One additional blood donor, number 002, was a Caucasian who was highly exposed to sporozoites in Papua New Guinea for 7 years, 8 years previously.

Although this would be difficult to prove, it would suggest that T cells should be able to discriminate variant natural sequences, some of which differ by only one or a few amino acids. Such discrimination by CD4$^+$ T cells would have obvious implications for vaccine development. Consequently, we examined the specificity of CD4$^+$ T cells from malaria-exposed Thais for a panel of variant peptides representing the 119 isolates/clones sequenced in the Th2R region and the 98 isolates/clones sequenced in the Th3R region. We found that the T cell responses of Thais were able to discriminate between Th2R variants and that substitutions of the six variant Th2R amino acid residues affected either or both MHC binding and TCR recognition by T cell clones. Further, given the immunodominance of the polymorphic sites and consequently their desirability for inclusion in a subunit vaccine, these data indicate strategies may have to be developed to overcome immune evasion facilitated by the natural polymorphism of the CS protein.

2 Materials and methods

2.1 Volunteers

Malaria-exposed native Thai adults ($n = 48$) were recruited from regions of northern Thailand with high malaria

2.2 Synthetic peptides and recombinant CS protein

Thirty-four 20-mer peptides representing the natural and published variants [25–33] of three regions of the *P. falciparum* CS protein, Pf326–345, Pf331–350, and Pf361–380, were synthesized (Fig. 1). All peptides were synthesized by the teabag method [37] and were assessed to be pure by HPLC. None of the peptides were toxic, as determined by comparing proliferative responses to tetanus toxoid to responses against a combination of peptide at 30 μg/ml with tetanus toxoid.

The Pf326–345 and Pf330–350 peptide sequences overlap within the Th2R domain, and Pf361–380 represents Th3R. Twelve variant sequences are known for each of the Th2R regions and ten for Th3R. Sequences from Asian [29–31], African [25, 26, 28, 31], South American [27, 31, 32] and Papua New Guinean [32, 33] isolates are included, and eight Thai isolates [29–31, 33] are represented in peptides Th2R-A, -I, and Th3R-A, -B, -I, and -J. The amino acid numbering derives from the 7G8 sequence [27].

2.3 Lymphoproliferation assays

Proliferation assays were carried out as previously described [19]. Briefly, PBL were isolated from samples of

venous blood by density gradient centrifugation over Ficoll. Peptides were tested at 30 µg/ml in quadruplicate microtiter wells with 200 000 PBL/well. Several peptides were also tested at 3 µg/ml. PPD at 60 µg/ml was used as a positive control antigen. After pulsing with [³H]thymidine on day 6, the uptake of the radiolabel was measured by β-counting. The means of quadruplicate wells with peptide were divided by means of the negative control wells (between 20 to 40 wells) to yield the stimulation index (SI).

2.4 T cell clones and proliferation assays

Clones from one subject were generated by an adaptation of the classical cloning method previously described [38]. Briefly, a line was generated as follows: proliferation assays with 50 wells each containing 200 000 PBL and 30 µg/ml Th2R₃₃₁A were split after 6 days, one split pulsed with [³H]thymidine and wells with antigen were compared to means of 20 control wells without antigen. Nine wells with counts five times greater than the mean of the controls were selected and pooled. This line was restimulated once with antigen, and cells were then cloned by limiting dilution (0.3 and 1 cell/well). All clones propagated had a greater than 90 % chance of being clonal [39].

Other clones were established by split-pulsing limit dilution assays a described [40], with cell numbers ranging from 10 000 to 200 000 PBL/well and 50 wells of antigen and 12 control wells per cell concentration. Positive wells with a greater than 90 % chance of being clonal [39] were selected.

All clones were expanded and maintained by cycles of antigen stimulation with mitomycin C-treated autologous PBL, followed by a 8–14-day rest with 50 U/ml IL-2. Clones were tested with antigen in triplicate with between 5000 and 15 000 T cells and 40 000 mitomycin C-treated autologous PBL/well. Pulsing with [³H]thymidine occurred on day 4. Each peptide was tested at concentrations of 0.3, 3, 10 and 30 µg/ml, and recombinant *P. falciparum* CS protein was tested at 5 µg/ml.

2.5 Phenotyping by depletions or FACS analysis and MHC allele-restriction analysis

Phenotyping of T cells for the presence of CD4, CD8, CD45RA or CD45RO antigens on the T cell surface was conducted by specifically depleting cells using mAb and magnetic beads as previously described [41]. T cell receptor usage was determined by FACS analysis after staining with FITC-labeled anti-α/β or γ/δ antibodies, as previously described [41]. Restricting HLA antigens for T cell clones were mapped by testing the clones with the parent peptide in the presence of mitomycin C-treated autologous or allogeneic PBL.

2.6 Competition assays

All competition experiments were carried out as previously described [42–44]. Briefly, between 5000 and 15 000 clone cells/well were added to 40 000 mitomycin C-treated autologous PBL/well. Pulsing with [³H]thymidine occurred on

day 4. The peptide for which the clones are specific was tested in triplicate at four concentrations (0.3, 3, 10 and 30 µg/ml). Sometimes an additional concentration (20 µg/ml) was also tested. To observe competition, each non-cross-reactive heterologous peptide was added at 30 µg/ml to all concentrations of the parent. Where competition occurs, there is a shift of the curve to the right. Competition is defined as occurring when the addition of analog significantly (by *t*-test, $p < 0.05$) reduces the response to the parent by 40 % or more, at all concentrations where the stimulation index (SI) of parent alone is above 3. A peptide is defined as not competing when the addition of a peptide does not significantly ($p > 0.05$) decrease the response of the clone to the parent peptide. There are a number of analogs which show statistically significant competition at some or all concentrations but at levels below 40 %, and these peptides are not included in later analyses.

Alternative competition experiments, previously described [44], were also used; here the competitor peptides were titrated from 0.3 to 30 µg/ml in the presence of the parent peptide at a suboptimal concentration (3 µg/ml) and the responses compared to the response to the parent alone at 3 µg/ml. Where competition occurs, the response to parent decreases with increased competitor concentration.

3 Results

3.1 Lymphoproliferative assays with malaria-exposed donors

Forty-eight malaria-exposed Thais were tested for proliferative T cell responses to all 34 variant peptides. The optimal concentration of the peptides was 30 µg/ml (96 % of all responses to three peptides, Th2R₃₂₆B, Th2R₃₃₁B and Th3RB, tested at both 30 and 3 µg/ml, were to the higher concentration). The phenotype of precursor T cells specific for selected peptides was shown by T cell subset depletion to be CD45RO⁺ (a marker for memory T cells) and CD4⁺ (data not shown). Fig. 2 indicates the responsiveness of the individuals to each peptide at 30 µg/ml. All Th3R peptides were poorly immunogenic (recognition by no more than 6 % of the population tested). This lack of immune recognition of Th3R has been previously documented for malaria-exposed Caucasians [24]. However, the two Th2R series of variant peptides were recognized frequently, with the Th2R₃₃₁₋₃₅₀ series being generally more immunogenic. Fine differences affected the T cell responses of the subjects to the Th2R peptides. First, no individual was able to respond to all variants. Also, variations are able to alter the sites of minimal epitopes within the Th2R region. For example, for the F, G and H variants, many more individuals responded to the peptides containing residues 331–350 compared to peptides with residues 326–345. These three peptides are distinct from the other variants by bearing both residues K336 and T337. Similarly for the J peptides, many more individuals responded to the 326–345 peptide relative to the 331–350 peptide. There was no preferential response to the peptide sequences known to occur in Thailand (Th2R-A and -I), which may be due to lack of geographical restriction of the variants as previously suggested [30]; Th2R-A is also known to occur in Brazil [31], and Th2R-I in Nigeria [27].

Figure 2. Lymphoproliferative responses to variants of Th2R$_{326-345}$, Th2R$_{331-350}$, and Th3R$_{361-380}$ by 48 malaria-exposed Thais. Responses above 3 and 5 SI are shown. The percent of responders to each peptide is shown at the bottom of the figure.

3.2 Cross-reactivity assays with T cell clones

Seventeen clones were generated from four malaria-exposed people. Three were Thais (subjects 620, 633 and 639), and one was a Caucasian (002). The parent peptide for all clones was Th2R$_{331}$A (Fig. 1), which is a sequence known to occur in Thailand [33]. The clones from subject 620 were raised by cloning from a line, while the clones from the remaining subjects were generated by cloning from limiting dilution assays (see Sect. 2.4). Phenotyping of selected clones by specific depletion of cells using mAb and magnetic beads or FACS analysis showed the clones were all CD4$^+$ and used TCR α/β receptors. DR2 was shown to be the restricting allele for all three clones from the Caucasian donor (Table 1).

Figure 3. Cross-reactive responses by T cell clones from three Thais (620, 633, 639) and one Caucasian (002) raised to Th2R$_{331}$A and tested with 11 heterologous Th2R$_{331-350}$ peptides and occasionally three 12-mer nested peptides spanning the sequence of Th2R$_{331}$A (N = not tested). All responses shown are those at 30 μg/ml, which is the optimal concentration for all clones, as exemplified by the top Fig. indicating clone 620A12 tested with concentrations ranging from 0.3 to 30 μg/ml.

Fig. 3 indicates the responses of the clones tested against the panel of Th2R$_{331-350}$ variant peptides at 30 μg/ml, which are representative of the cross-reactive abilities of the heterologous peptides, as indicated by clone 620A12. The clones showed little or no cross-reactivity to the variants. Clones from two of the Thais did not cross-react to any of the variants, while clones from the Caucasian and the remaining Thai could recognize a few variant peptides.

Twelve-mer subpeptides spanning Th2R$_{331}$A (Fig. 1) were also tested with most clones. In all but one case, the clones

Table 1. MHC allele-restriction of proliferative response to Th2R$_{331}$A by 002 clones

APC donor								Stimulation index 002A9	002F11	002H6
				HLA antigens of donor						
Autologous		DR2		DRw6[a)]	DQw1		DRw52	20.2	8.8	13.6
Allogeneic 1	DR1			DRw14	DQw1		DRw52	0.5	0.6	0.5
Allogeneic 2		DR2			DQw1			20.3	49.0	11.4
Allogeneic 3			DR3	DRw13	DQw1	DQw2	DRw52	2.0	0.7	1.0

a) DRw6 is subtyped into DRw13 and DRw14.

were not able to recognize any of the subpeptides. Only one clone, 620D9, was able to respond appreciably to one subpeptide, Pf339–350 (A3), although the response was lower than to the parent peptide (Fig. 3). The overlap of four amino acids may be insufficient for minimal epitope mapping.

3.3 Competition studies with T cell clones

Competition studies were all performed by titrating parent peptide in the presence of competitor peptide (see Sect. 2.6). Where competition occurs, the curve of parent peptide alone shifts to the right, indicating that more parent peptide is needed to restore the response (Fig. 4). The competition experiments were verified by conducting, with a few peptides and selected clones, alternative competition
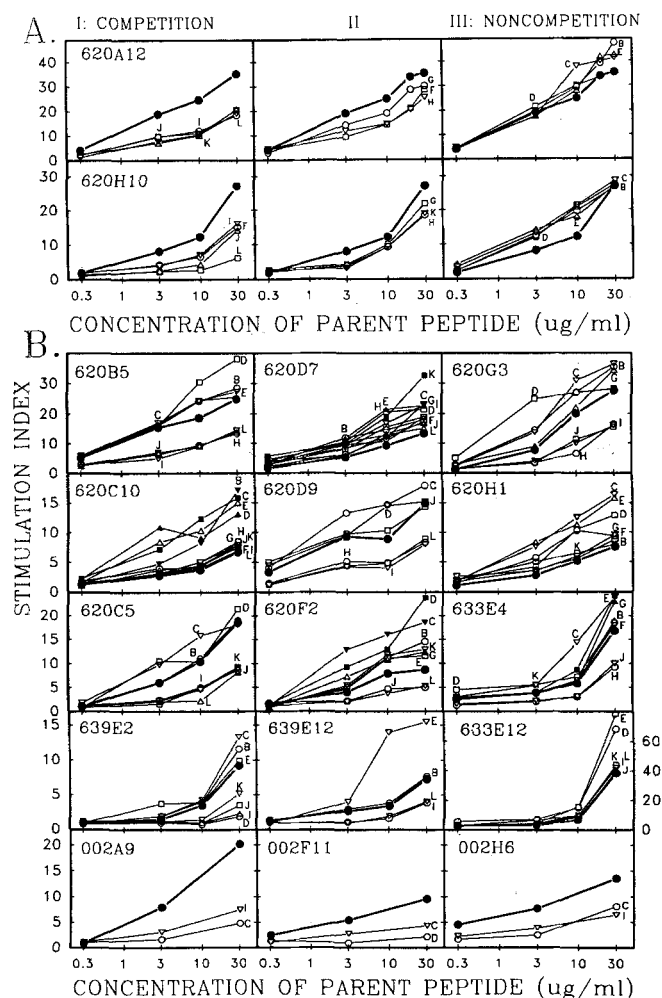

Figure 4. Competition studies with T cell clones and non-cross-reactive peptides. These competition assays were conducted by titrating parent peptide over a range of 0.3 to 30 µg/ml in the presence of 30 µg/ml heterologous peptide. Thick lines indicate parent peptide titrated alone. Heterologous peptides (B to L) are indicated by their letters. (A) Clones 620A12 and 620H10: column I indicates peptides that are competitive; column III shows peptides that are non-competitive; and column II shows peptides that fall in between these categories. (B) Responses to competitive and non-competitive peptides shown for all remaining clones.
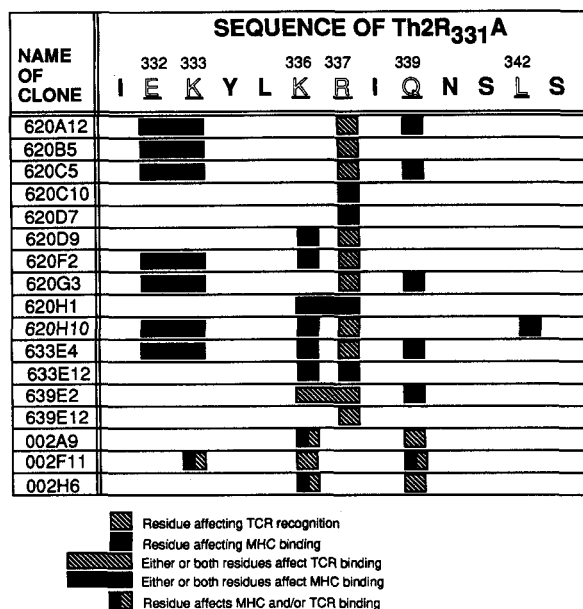
experiments (described in Sect. 2.6) and in all cases the inhibition outcome was identical to that of the previous competition experiments (data not shown).

Examples of competition and non-competition are shown in columns I and III of Fig. 4A, respectively, and responses of remaining clones are given in Fig. 4B. Some analogs show statistically significant competition at some or all concentrations, but at levels below 40% (see Sect. 2.6). These peptides are not included in later analyses, nor in Fig. 4B, but examples are given in column II of Fig. 4A. Where competition satisfying both criteria occurs, the degree varies with the competing peptide, indicating that various substitutions have a greater or lesser effect in impeding the response to the parent. Some non-competitive peptides enhance the response to the parent peptide (*e.g.* 620D7 with most analogs and 639E12 with Th2R$_{331}$E). Such enhancements of responses have been previously reported [43]. Since both parent and heterologous peptides are incubated with the clones together, it is possible that the additional peptide reduces protease degradation of the parent peptide and thus more parent can bind the MHC molecule.

The contribution of a particular polymorphic residue to MHC/TCR binding can be established from the pattern of heterologous peptides that cross-react or compete. Residues of cross-reactive peptides different from the parent do not affect MHC binding nor TCR recognition of the MHC-peptide complex [45]. Peptides which do not cross-react and also do not compete cannot bind to the MHC, indicating that one or more of the polymorphic residues different from the parent are part of the agretope [44, 46–54] or have side-chains inhospitable to the binding of the peptide to the MHC [44, 49–52]. However, if a non-cross-reactive peptide can compete with the parent, it binds to the MHC but one or more of the residues different from the parent affect recognition of the peptide-MHC complex by the TCR. This non-recognition may be due to several possibilities: first, critical TCR contact residues may be altered (*i.e.* the epitope is changed) [43, 46–49, 54–56]. Second, the polymorphic residue of the competing peptide may bind the TCR but is ineffective in maintaining a stable connection, preventing the activation of the T cell (causing TCR antagonism [57]). Third, the substitution abrogating TCR recognition alters the conformation of the peptide within the MHC so that the TCR cannot recognize the complex [50, 56].

The primary data permitting the analyses of critical residues affecting MHC and TCR interaction for all clones given in Fig. 3 and 4, and these analyses are summarized in Fig. 5, with an example described in Fig. 6. For the various T cell clones, all six polymorphic residues of Th2R$_{331-350}$ were found to affect the binding of the peptide to the MHC, and three polymorphic residues, K336, R337, and Q339 were shown to affect TCR recognition of the peptide-MHC complex.

Using the naturally occurring sequences we have focused on only the polymorphic residues. However, non-polymorphic residues are also involved in MHC/TCR binding, as none of the clones responded significantly to a 12-mer subpeptide of Th2R$_{331-350}$A, Pf331–342 (denoted A1), which contains all of the variant residue positions (Fig. 3).

SEQUENCE OF Th2R$_{331}$A

NAME OF CLONE	332	333			336	337		339			342	
	I	E	K	Y	L	K	R	I	Q	N	S	L	S
620A12													
620B5													
620C5													
620C10													
620D7													
620D9													
620F2													
620G3													
620H1													
620H10													
633E4													
633E12													
639E2													
639E12													
002A9													
002F11													
002H6													

Residue affecting TCR recognition
Residue affecting MHC binding
Either or both residues affect TCR binding
Either or both residues affect MHC binding
Residue affects MHC and/or TCR binding

Figure 5. Summary of the analyses of cross-reaction and competition patterns of T cell clones with analogs, indicating which of the naturally occurring polymorphic residues affect MHC binding and TCR recognition.

Competitive peptides   Non-competitive peptides

```
332 336 339                 332 336 339
-EK--KR-Q--L--------  A     -EK--KR-Q--L--------  A
    333 337  342                333 337  342

-EK--KT-K--L--------  F     -EQ--KK-K--I--------  B
-EQ--KK-Q--L--------  I     -EK--QK-K--L--------  C
-EK--NT-K--L--------  J     -KE--NK-Q--L--------  D
-EK--KK-Q--L--------  L     -EK--QK-R--L--------  E
```

Figure 6. Competition data of clone 620H10 is analyzed as an example, indicating that one polymorphic residue affects TCR recognition (R337) and three or four others affect binding to the MHC (E332 and/or K333, and K336 and L342). None of the analog peptides are cross-reactive, and competitive and non-competitive peptide sequences are shown. Residues of the competitive peptides shown in bold type indicate all substitutions of the parent sequence, several of which must be responsible for abrogating TCR recognition of each analog. Residue R337 must be a contact point for the TCR since peptide L differs from the parent only at this position. Only this residue is noted in Fig. 5 as an epitopic residue, as the roles of K333, K336 and Q339 are not clearly defined. Residues of the competitive peptides shown in bold type do not modulate their MHC binding, and should not be responsible for the lack of MHC binding of the non-competitive peptides, assuming that the substitutions act independently. Only substitutions not found in the competitive peptides are shown in bold type in the non-competitive peptides, several of which are likely to be responsible for abrogating MHC binding. Thus, peptide B cannot bind the MHC because of substitution at L342. Peptide C does not bind because of substitution at K336, and peptide D does not bind because of substitutions at one or both of E332 or K333. The potential role of the Q339 substitution of peptide E in modulating MHC binding cannot be further defined because of the presence of the substitution at L342. This latter possibility is not noted in Fig. 5.

4 Discussion

The T cell clones unambiguously demonstrate that, for at least the individuals tested and clones generated, there is widespread lack of cross-reactivity between these naturally occurring sequences, some of which differ in only one amino acid. For T cells to exert biological pressure for diversity of the CS protein, it is essential that most clones from a given individual can discriminate two different sequences. If a number of clones did not discriminate, then those clones could destroy variant parasites and selection would not occur. We have shown that 11 from 11 clones from one individual could discriminate the variant peptide A from all other variants, and that all clones tested from any individual could discriminate peptide A from most other peptides. Unpublished preliminary data with polyclonal lines specific for other variants generated from six exposed Thais also indicates that cross-reactivity to analogs is similarly limited. Further, lymphoproliferative data with many more subjects indicates those individuals capable of recognizing many variants are few, and the predominant trend is that there is immunological discrimination of the variants. These data confirm and extend early studies using proliferative mouse cultures [26] and a single human proliferative clone [58], which showed limited cross-reactivity to the two variants of the Th2R parent peptide (7G8 sequence) known at the time. A recent study has also found that three CD4+ T cell clones, one from each of three sporozoite-immunized humans and specific for a peptide closely overlapping variant Th2R$_{331}$D, show variable cross-reactivity to a bank of natural analog peptides closely overlapping those used here, with one clone capable of proliferating to all 11 variant peptides, and two others proliferating to 3 and 5 variants [59].

By conducting competition studies with the clones, we have shown that epitope mutations of *P. falciparum* CS protein affect both MHC and TCR binding. Individual residues present in an immunogenic peptide may contribute either to MHC binding, TCR interaction, spacer function or be redundant; it is unlikely that it is chance that all six variant residues in the Th2R$_{331-350}$ sequence play critical roles in the MHC/TCR function of the randomly selected clones in our study. This data contrasts with recent work examining the binding ability of the variants of Th2R$_{331-350}$ to purified soluble DR1 and DR4 which showed the analogs were all able to bind [59]. Although we have not been able to determine the restricting allele(s) for the Thai clones, we found that responsiveness of clones from the Caucasian donor was restricted by DR2. This may partly explain our contradictory results, and suggests that immune responses to variant Th2R determinants may be affected by both the specific epitope sequence and tissue type.

These data are consistent with the concept that CD4+ T cells may be able to select escape mutants expressing variant CS sequences. Such selection would occur in the liver and would be reliant on hepatocytes expressing CS peptide in association with MHC class II molecules. Hepatocytes are known to be able to express these MHC molecules during infection [60]. If T cells have selected variants, then hepatocytes must be able to process CS protein and present the epitope to T cells. We have not tested whether human hepatocytes can process CS protein; however, we know that human T cells specific for Th2R peptide can respond to recombinant CS protein processed by peripheral blood APC [41]. Further, CS-specific CD4+ T cells can passively protect mice and were shown to recognize sporozoite-infected hepatocytes *in vitro* [12].

These cells killed parasites by non-cytolytic methods, suggesting that selective killing could be by localized lymphokine production. We have shown that CD4$^+$ T cells specific for Th2R$_{331-350}$ produce IFN-γ ([41, 61], and others have also shown that T cells specific for the same peptide have cytolytic ability [36]; consequently it is possible that the selective action of T cells may be implemented by either mechanism.

Although the data are consistent with selection of variants by CD4$^+$ T cells, we cannot prove this concept because of technical difficulties. Nevertheless, our data indicate the potential for variation to affect vaccine-induced T cell responses. Variation which affects both MHC binding and TCR interaction with MHC-peptide may also contribute to the widespread lack of natural sporozoite immunity [62]. However, observations reported here will help in the development of new strategies to overcome the consequences of natural variation.

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