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Immunology

Life-spans of human T-cell responses to determinants from the circumsporozoite proteins of *Plasmodium falciparum* and *Plasmodium vivax*

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ABSTRACT **The longevity of specific human memory T-cell responses is largely unknown. However, a knowledge of the duration of memory is important for understanding immunity to an organism and for planning vaccine intervention. To address this, we have examined T-cell memory to malaria by determining T-cell responses by subjects recently exposed to peptides spanning the circumsporozoite (CS) proteins of two species of malaria-causing organisms, *Plasmodium falciparum* and *Plasmodium vivax*. Responses to vivax CS peptides by exposed Thai subjects were more frequent than responses by nonexposed individuals, permitting identification of determinants seen by vivax-induced responses. At the population level, there appears to be life-long memory, as the time since individuals were exposed did not diminish responsiveness to these determinants. In contrast, falciparum-exposed subjects were largely indistinguishable from nonexposed controls in responsiveness to falciparum CS determinants. However, a single peptide (F16: DNEKLRKPKHKKLKQPGDGN) was recognized significantly more frequently by *P. falciparum*-exposed than nonexposed Thai subjects. T cells responsive to this peptide were CD450$^+$ and produced γ-interferon. In contrast to the response to the vivax determinants and the other falciparum determinants, responsiveness to F16 was undetectable or minimal 2 years after exposure. Our data provide the average life-spans of certain malaria-specific T cells and are consistent with, but do not prove, the hypothesis that antigenic persistence (in the form of *P. vivax* hypnozoites) correlates with persistence of human T-cell memory.**

T-cell memory is poorly understood, and controversial issues include the longevity of T-cell memory and the mechanism by which longevity can be attained (1–3). For the latter, two postulates have been proposed. First, memory is maintained by long-lived nonproliferating memory cells. Second, continual stimulation—by the original antigen, cross-reactive antigens, cytokines released in the vicinity by other cells, or perhaps anti-idiotypic T cells—maintains memory in the form of continually dividing specific T cells (4–8). Although the concept of particularly long-lived memory responses may be favored, the existence of such cells has not been demonstrated (1). However, the two mechanisms are not mutually exclusive.

Most studies investigating memory T-cell longevity have been conducted in animals (4–7), and those investigating memory in humans have studied cell pools defined by putative memory markers (8). We are interested in T-cell-mediated immunity to malaria and have developed a system whereby we can define functional memory responses without reliance on surface activation markers (refs. 9 and 10 and see below). *Plasmodium falciparum* and *Plasmodium vivax* are responsible for most malaria in humans, and *P. vivax* is distinct from *P. falciparum* in having dormant liver forms responsible for relapses (hypnozoites). Hypnozoites are known to express the circumsporozoite (CS) protein (13), a target of protective antibodies (14, 15) and T cells (16–20) and a candidate vaccine antigen (21).

Human T-cell-recognized epitopes have been identified in the *P. falciparum* and *P. vivax* CS proteins (9, 22–27), and these studies have revealed critical differences between the two species. A study with Caucasians exposed previously to *P. falciparum* found that nonexposed controls could respond at equal frequencies to the lymphoproliferative determinants recognized by the exposed group (23). γ-Interferon (γ-IFN) production induced by CS peptides, and frequencies of the peptide-specific and recombinant CS protein-specific T-cell responses were identical between the two groups (28). The responsiveness of nonexposed people to falciparum CS peptides has been attributed to cross-reactive T cells induced by exposure to common environmental organisms (10, 29), as generally proposed by Beverley (30).

A different pattern of responsiveness was found when a similar study with vivax malaria-exposed and -nonexposed Caucasians, and *P. vivax* CS peptides was performed (9). The vivax malaria-exposed subjects responded to many more vivax CS peptides than did nonexposed subjects. Epitopes recognized by vivax-induced responses could be defined, and specific T-cell memory was very long-lived (up to 50 years) in the absence of reinfection; we hypothesized that memory was maintained by hypnozoites acting as antigen depots.

It was not clear whether the failure to define determinants recognized by malaria-induced responses to the falciparum CS protein was due to absence of such determinants or to the short life-span of T-cell memory. Consequently, we decided to study groups from Thailand with more recent malaria exposure. We confirmed that vivax-exposed subjects respond better to vivax-CS peptides than do nonexposed subjects, and that responses to falciparum CS peptides are very similar between exposed and nonexposed subjects. However, there was an exception: we found that recent infection with *P. falciparum* correlated with a response by T cells to a single determinant.

SUBJECTS, MATERIALS, AND METHODS

Subjects and Pattern of Malaria Exposure. Five Khon Muang and Karen Thai groups of adults were recruited: vivax malaria-exposed Karen (n = 40; 33% female; mean age 31 years; mean time since last malaria attack 1.31 years);

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Abbreviations: CS, circumsporozoite; γ-IFN, γ-interferon; TT, tetanus toxoid; PPD, purified protein derivative; PBL, peripheral blood lymphocytes.

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vivax-exposed Khon Muang (n = 40; 25% female; mean age 34.1 years; mean time since last malaria attack 0.65 year); falciparum-exposed Khon Muang (n = 48; 21% female; mean age 39 years; mean time since last malaria attack 0.98 year); nonexposed Karen (n = 25; 26% female; mean age 32 years); and nonexposed Khon Muang (n = 29 for vivax study and n = 31 for falciparum study; 41% and 37% female, respectively; mean age 35 and 37 years, respectively). A falciparum-exposed Karen group of children was subsequently recruited (n = 38; 34% female, mean time since last attack 0.97 year). All subjects were recruited through malaria clinic or hospital records, which documented the species with which they were infected. The last attack of malaria was noted to the nearest year. None of the subjects recalled malaria-like illnesses which they did not report or get medically treated within the last 5 years and none take antimalarial drugs. Approximately 90% of malaria infections were reported to have been contracted outside the village of residence, suggesting that indigenous malaria at the three locations from which the subjects were recruited is quite low.

Antigens. Peptides (see legends to Figs. 1 and 3) were synthesized as previously described (31) and were assessed to be pure by HPLC. None of the peptides were toxic to lymphocytes. Two recombinant proteins were also used, rPfCSP (32) and rPvCSP (33).

Lymphoproliferation Assays. Peripheral blood lymphocytes (PBL) were isolated as previously described (22). Between 2 $\times 10^5$ and 1.5 $\times 10^5$ cells were placed in each well of a 96-well microtiter plate. Quadruplicate wells received each antigen, and 12–24 wells received no antigen. Peptides were tested at 30 and 3 μg/ml, while control antigens tetanus toxoid (TT) and purified protein derivative (PPD) were used at 0.3 limit flocculation unit (Lf)/ml and 60 μg/ml, respectively. After 6 days, each well was pulsed with 0.5 μCi (1 μCi = 37 kBq) of [^{3}H]thymidine and subsequently harvested, and radiolabel uptake was determined by liquid scintillation spectroscopy. Means of wells with antigen were divided by means of wells without antigen to yield the stimulation index (SI). Proliferation was assessed to be significant at an SI of 3 or above.

γ-IFN Production. Fifty-microliter aliquots were removed from each well of randomly selected lymphoproliferation assays on day 4 of incubation, and the presence of γ-IFN was assessed [kit from CSL (Melbourne, Australia)].

Monoclonal Antibodies. Monoclonal antibodies used included OKT4 (anti-CD4), OKT8 (anti-CD8), FMC44 (anti-CD45RA), and UCHL1 (anti-CD45RO). UCHL1 and FMC44 were kind gifts of Heddy Zola (Flinders Medical Centre, Adelaide, Australia) and P. Beverley (London).

Specific T-Cell Subset Depletion. T cells were purified as described (28) and CD4$^+$, CD8$^+$, CD45RO$^+$, or CD45RA$^+$ T-cell subsets were then removed (10). Depleted cells were plated out according to the regime applied to bead-treated undepleted cells. Mitomycin C-treated non-T cells were plated out at 10,000 cells per well to act as antigen-presenting cells.

ELISAs Detecting Serum Antibodies to Crude Asexual Stage Parasite Extract. Antigen was prepared as described (34) and specific antibody in sera diluted 1:100 then determined as described (35). Sera were considered to have significant levels of antibodies if their ODs exceeded the mean + 3SD of the nonexposed sera of the relevant ethnic group.

Statistical Analysis. Comparisons of exposed and nonexposed responses to particular peptides were performed by an approximate testing procedure for differences between two proportions equivalent to χ^2 testing of a 2 × 2 contingency table (36).

RESULTS

To confirm parasite exposure, antibodies to crude *P. falciparum* extract were determined; such antibodies cannot discriminate falciparum from vivax exposure, as previously shown (37). Of the exposed subjects, 43–60% had significant levels of antibodies. Presence of antibodies did not correlate with either the number of attacks documented or the duration since last attack ($P > 0.05$), as previously reported (37). Four nonexposed subjects (one Caucasian, one Karen, and two Khon Muang subjects) exhibited significant levels of antibodies. Such cross-reactivity has been previously reported to occur for malaria (37) and other antigens (38).

Responses to Vivax-Induced CS Epitopes Do Not Fade with Time. Four groups were tested with the vivax CS peptides: (*i*) 40 vivax-exposed Khon Muang, (*ii*) 29 nonexposed Khon Muang, (*iii*) 40 vivax-exposed Karen, and (*iv*) 25 nonexposed Karen. Peptides were tested at 30 and 3 μg/ml, and Fig. 1 indicates the responsiveness of each group. Exposed Khon Muang generally responded better at 3 μg/ml, while the exposed Karen responded better to 30 μg/ml. Nonexposed groups responded equally to both concentrations.

As a group, vivax malaria-exposed subjects responded better than nonexposed subjects for both Karen and Khon Muang ($P < 0.05$). Comparison of responses of exposed and nonexposed subjects to each peptide (t test) permitted iden-

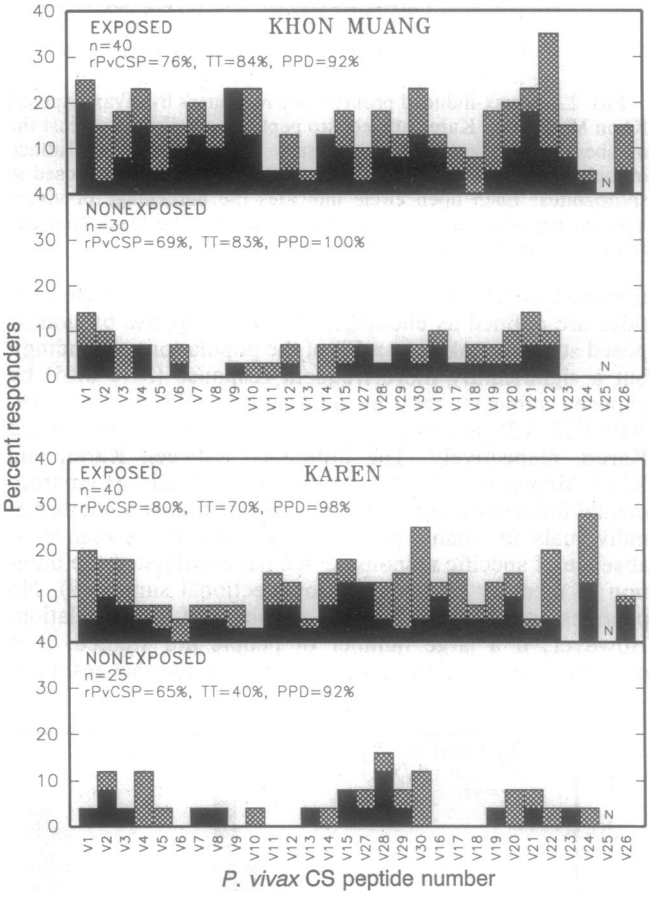

FIG. 1. Percent lymphoproliferative responses (SI ≥ 3) to each vivax CS peptide by vivax-exposed and -nonexposed Khon Muang and Karen. The cross-hatched bars represent the percentage of subjects who responded maximally at a concentration of 30 μg/ml, whereas the filled bars represent the percentage who responded maximally at 3 μg/ml. N, not tested. Peptides used include the following: (*i*) V1–V10 and V18–V26, previously described (9), which span the nonrepeat sequence of the Belem CS protein (39). (*ii*) Peptides V11–V15, previously described (9), and V27–V30, which represent the Belem (39) and VK247 (40) types of repeat sequences, respectively. Sequences of V27–V30 are V27, ANGAGNQPGANGAGNQPG; V28, ANGAGNQPGEDGAGNQPG; V29, EDGAGNQPGANGAGNQPG; and V30, ANGAGNQPGANGAGGQAA. (*iii*) Peptides V16 and V17, previously described (9) which represent an insert sequence found in the NK strain (41).

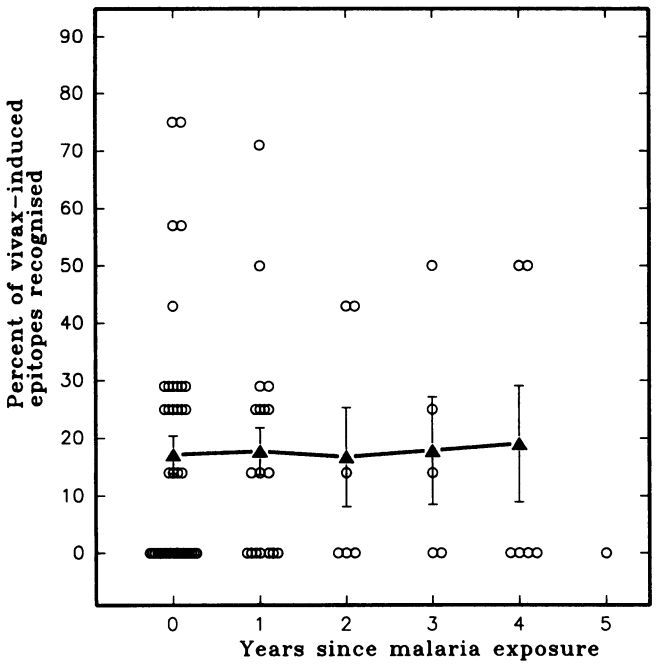

FIG. 2. Vivax-induced proliferative responses by vivax-exposed Khon Muang and Karen subjects to peptides are plotted against the number of years since malaria exposure. The last documented attack of malaria is taken as the last occasion the subject was exposed to sporozoites. Each open circle indicates the percentage of vivax-induced peptides each subject can recognize. The black triangles represent the mean and SEM of each year group.

tification of possible vivax-induced responses. These peptides are defined as eliciting a minimal response by nonexposed subjects (taken as ≤5% of the population responding), but a significantly more frequent response ($P < 0.05$) by exposed subjects; they include peptides V5, V7, V8, V9, V10, V23, V26, and V12, V16, V17, V24 for Khon Muang and Karen, respectively. The difference between Karen and Khon Muang may be due to disparate genetic or environmental influences. It was not possible to follow a number of individuals for many years to determine the presence or absence of specific responses. We have estimated the duration of memory by using a cross-sectional survey (9). No peptides are recognized by all members of the population. However, if a large number of people are studied, it is possible to plot the fraction of the population that responds to a given peptide as a function of time since last exposure. This approach can be expanded to look at all peptides as a group and ask how many different peptides are recognized by the population as a function of time since last exposure (9). Derived data will not give the life-span of a T-cell response for any given individual but will give the mean duration of such life-spans for the population. Longitudinal studies would be required to measure the life-span of a T-cell response for any given individual. Fig. 2 indicates the distribution of all vivax-induced responses by both Karen and Khon Muang subjects over time relative to their last documented attack of malaria. On average, these responses do not appear to diminish over time, supporting our previous data for a Caucasian population (9). Neither Khon Muang nor Karen show a different pattern when analyzed separately.

***P. falciparum*-Specific Responses.** Two groups of adults were tested with the falciparum CS peptides at 30 and 3 μg/ml: (*i*) 48 falciparum-exposed Khon Muang, and (*ii*) 31 nonexposed Khon Muang. Fig. 3 indicates the average responsiveness to each peptide at the optimal concentration (30 μg/ml) by both adult groups tested. Both groups were able to respond to peptides F23 and F24, which are immunodominant T-cell sites for Gambians and Caucasians, respectively (22, 23). Neither could recognize F28, a site known to be immunodominant for Gambians (22). Interestingly, the peptide representing the major NANP repeats (F34) proved to be immunodominant for the Khon Muang. Neither the Gambians nor the Caucasians were able to recognize this peptide significantly (22, 23). The "minor" repeats (peptide F35) were not recognized by less than 5% of either exposed or nonexposed subjects, although this peptide was recognized by a sporozoite-immunized volunteer (32).

Identification of a Unique Falciparum-Induced Response. Relative to the nonexposed subjects, falciparum-exposed Khon Muang respond significantly more frequently to only one peptide, F16 ($P < 0.02$, significant after correction for continuity). We define this response as falciparum-induced, as nonexposed subjects respond minimally. Nonexposed subjects respond more frequently to most peptides than exposed, although this is not statistically significant ($P > 0.05$). However, one peptide (F6) was recognized significantly better by the nonexposed subjects after correction for continuity ($P < 0.02$). The poorer responses by the exposed may reflect immunosuppression resulting from malaria infection (43).

Falciparum-Induced Epitope Induces γ-IFN Production. Supernatants removed on day 4 from the proliferation assays with 33 *P. falciparum*-exposed and 22 nonexposed Khon

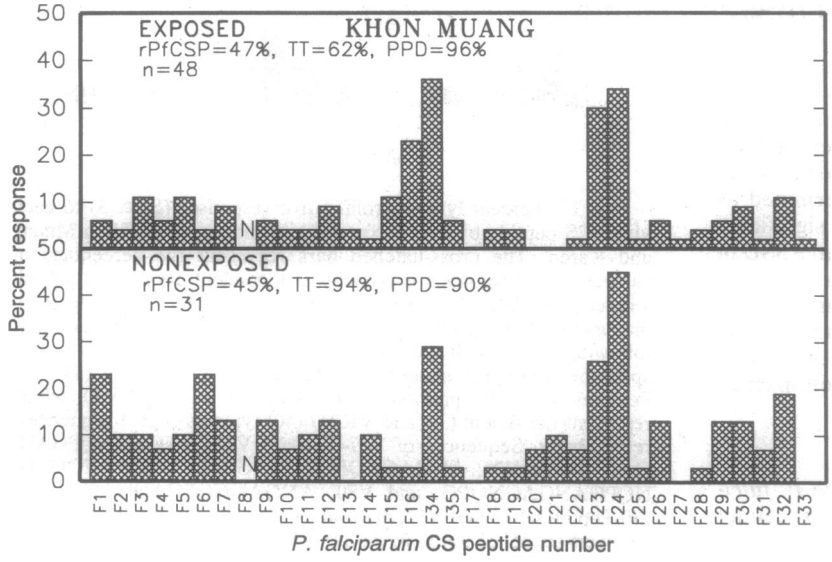

FIG. 3. Percent lymphoproliferative responses (SI ≥ 3) to each falciparum CS peptide by falciparum-exposed and -nonexposed Khon Muang. Only responses at 30 μg/ml are shown. N, not tested. Peptides used include the following: (*i*) Nonrepeat sequences spanning the 7G8 CS protein (42). Residue numbers are F1, 1–20; F2, 11–30; F3, 21–40; F4, 26–45; F5, 31–50; F6, 36–55; F7, 41–60; F8, 46–65; F9, 51–70; F10, 61–80; F11, 66–85; F12, 71–90; F13, 76–95; F14, 81–100; F15, 91–110; F16, 101–120; F17, 286–305; F18, 291–310; F19, 296–315; F20, 301–320; F21, 311–330; F22, 321–340; F23, 326–345; F24, 331–350; F25, 341–360; F26, 351–370; F27, 356–370; F28, 361–380; F29, 371–390; F30, 376–395; F31, 381–400; F32, 386–405; and F33, 391–412. (*ii*) The major and minor repeats of falciparum are represented by peptides F34, NPNANPNANPNANPNANPNA, and F35, NPNANPNVDPNANPNVDPNA, respectively (42).

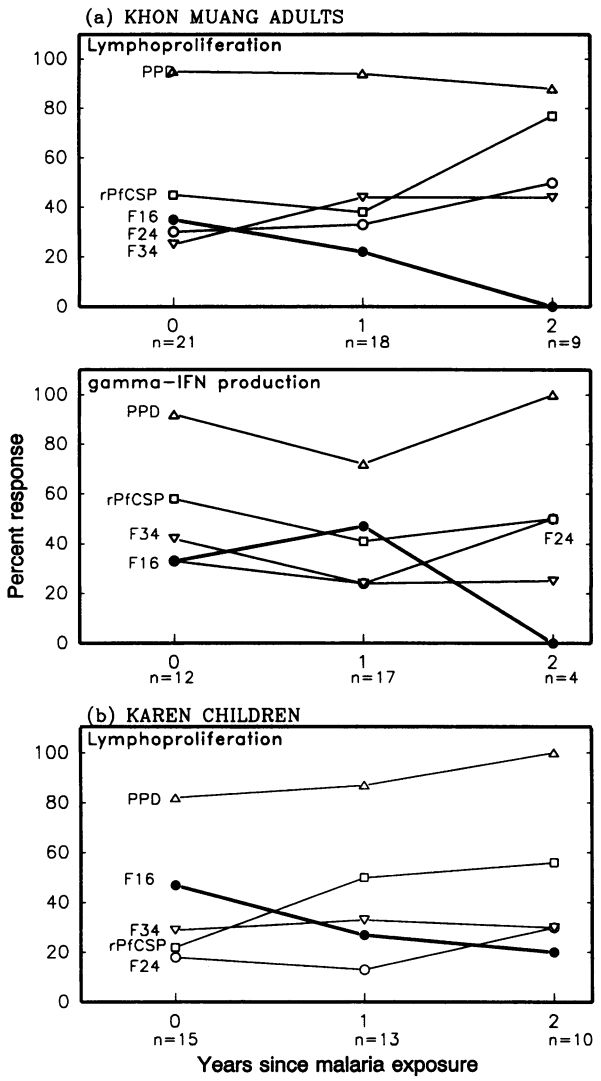

FIG. 4. Falciparum-exposed Khon Muang adults and Karen children were ranked according to the last malaria attack experienced, which is taken as their last exposure to sporozoites. The percent response to several antigens is plotted against the time since exposure. Antigens include F16, the two immunodominant peptides F24 and F34, rPfCSP, and the control antigen PPD. (*a*) Khon Muang adults. (*b*) Karen children. Subjects exposed to malaria 2 or more years before the study are grouped, but 78% of Khon Muang adults and 10% of Karen children were exposed more than 2 years ago.

Muang were randomly selected and tested for the presence of γ-IFN produced in response to peptides F16, F24, F32, and F34, rPfCSP, and PPD. Supernatants from negative control wells (no antigen) were also tested. Except for F24 in the nonexposed group, there is good correlation between proliferative responses and γ-IFN production at the population level; there was, however, no correlation at the level of the individual. Interestingly, the amounts of γ-IFN produced in response to F16 were greater than those secreted in response to any of the other peptides or rPfCSP. Three of the four subjects with T cells producing more than 0.30 international unit of γ-IFN in response to F16 were exposed to malaria less than a year ago. One of the two nonexposed subjects demonstrating proliferation in response to F16 was negative for γ-IFN production to this peptide; the other was not tested. No other nonexposed subjects produced γ-IFN in response to F16.

Estimation of Duration of F16-Induced T-Cell Memory. Responsiveness to F16 and other immunodominant peptides by exposed Khon Muang was plotted against time since the last documented attack of malaria (Fig. 4*a*). Proliferative responses by Karen children to selected antigens were analyzed similarly (Fig. 4*b*). Although we did not have a nonexposed Karen children group, we found that <5% of nonexposed Karen adults responded to F16 (not shown). For both Khon Muang adults and the Karen children, responses to the control antigen PPD, the immunodominant peptides F24 and F34, and the recombinant protein remain equivalent or increase over time. A similar pattern was found for Khon Muang adult responses to all peptides (data not shown). However, responses to F16 decreased over time. While the response of children to F16 did not completely extinguish over two years, the response of adults did.

Phenotypic Analysis of Memory T Cells. To investigate the activation phenotype of T cells capable of responding to various falciparum peptides, lymphocytes of nine subjects of both falciparum-exposed and -nonexposed groups were depleted of $CD45RA^+$ or $CD45RO^+$ T cells, and the responses by depleted and bead-treated cells to TT, PPD, rPfCSP, F1, F6, F16, F23, F24, F28, F32, and F34 were assessed. On average, 96.6% of $CD45RA^+$ T cells (range 93–99%) and 94% of $CD45RO^+$ cells (range 91–98%) were removed. All responses to PPD and TT by both groups, and all responses by exposed subjects to the peptides, were by cells expressing the activation marker ($CD45RO^+$). Responses by nonexposed individuals were mainly by $CD45RO^+$ T cells, except for two peptides, F16 and F32, which stimulated $CD45RA^+$ T-cell responses by several different subjects. The two nonexposed subjects responding to F16 were the only two subjects demonstrating a response to F16 in the population proliferative study.

The contributions of $CD4^+$ or $CD8^+$ T cells were also assessed by specific depletion. Most responses (91%) were by $CD4^+$ T cells.

DISCUSSION

From comparing responses of falciparum malaria-exposed and -nonexposed individuals, we have defined a CS protein determinant recognized significantly more frequently by exposed subjects. We have denoted this response as falciparum- or F16-induced. T-cell responses to this epitope (F16) are undetectable or minimal, by our methods, beyond 2 years since the last malaria infection. In contrast, responses to falciparum CS peptides recognized by both exposed and nonexposed subjects do not diminish with time since the last exposure to malaria. Contrary to the case for F16-induced responses, responses by vivax-exposed subjects to vivax-induced determinants remain undiminished by time since malaria infection, as reported previously for a Caucasian group (9).

That F16-induced T cells are memory cells is supported by two additional sources of evidence: (*i*) we have demonstrated that T cells from recently exposed subjects produce high levels of γ-IFN in response to F16 [secretion of high levels of γ-IFN is known to be a feature of memory T cells (44, 45)]; and (*ii*) T cells from malaria-exposed subjects responsive to F16 are of the $CD45RO^+$ phenotype. In contrast, however, T cells from the two nonexposed people who respond to F16 are of the $CD45RA^+$ phenotype, which suggests that responsiveness to this determinant by these people may be due to *in vitro* priming. CD45RA is believed to be a marker for naive T cells, while CD45RO is a marker for memory/activated T cells (11), although the difference between memory and activated T cells cannot be resolved as yet (12).

We have previously shown in a Caucasian group that: (*i*) responses that were unique to vivax-exposed donors were life-long (9); and (*ii*) falciparum-specific responses were identical between exposed and nonexposed groups (23). In the falciparum-exposed group, the average time since most recent infection was 14 years, compared with a mean time of

1 year for the Khon Muang in this study, which probably explains why we were not able to define a response unique to the falciparum-exposed Caucasian individuals. We hypothesized that the persistence of the vivax-specific responses might result from antigenic persistence in the liver (9), reasoning that persistence of antigen is associated with maintenance of memory in animal models (6) and the CS protein of *P. vivax* is thought to be expressed in the hypnozoite stage (13). Our present vivax data confirm our original finding in a different population; however, we now define a response that is unique to donors recently exposed to *P. falciparum*. Our data overall define the longevity of different malaria-specific memory T-cell responses and are consistent with, but do not prove, the hypothesis that T-cell memory is dependent on the continuing presence of antigen or cross-reactive stimulation. We do not know why the response to F16 fades over 2 years, but a likely possibility is that the F16 epitope is not commonly found in environmental organisms, only in the falciparum CS protein, and the lack of persistent forms in falciparum is responsible for the disappearance of memory. Other explanations, however, cannot be excluded at this stage. There may be an environmental antigen which closely mimics falciparum CS protein (explaining the widespread response of nonexposed donors to CS peptides) and immune responses to these other epitopes may regulate the response to F16 in a unique manner. Thus, longevity of memory needs to be defined in other systems.

In this study, we have used functional criteria supported by the presence of activation markers on reactive T cells to define a population of memory cells. This has permitted us to subsequently track its presence after exposure to antigen and provide an estimate of T-cell memory life-span. This is in contrast with most other human studies estimating T-cell longevity, which have relied solely on surface expression of activation markers (mainly CD45RA/RO). We are able to determine that the longevity of the F16-induced population of T cells is in the range of 1–2 years. This supports an estimate of the longevity of $CD45RO^+$ T cells in cancer patients after radiotherapy (8). In contrast, naive T cells (defined as $CD45RA^+$) can persist in humans for more than 10 years (8). Thus, it is possible from these data and ours that T-cell memory (at least that defined by lymphoproliferation and γ-IFN production) resides within a relatively short-lived population of cells which maintain a memory response by continual turnover. However, we cannot exclude the possibility that memory T cells which are long-lived in the absence of antigen are present but undetectable by our methods. This matter remains to be resolved.

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