

## CD4 T Cell Responses to a Variant Antigen of the Malaria Parasite *Plasmodium falciparum*, Erythrocyte Membrane Protein-1, in Individuals Living in Malaria-Endemic Areas

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*Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) is a variant antigen on the surface of malaria-infected red blood cells. Antibody responses to PfEMP-1 correlate with immunity, and, therefore, PfEMP-1 may be a good candidate for a malaria vaccine. However, the specificity of CD4 T cells required for a protective variant-specific antibody response is not known. We have measured the CD4 T cell response to 3 different regions that are relatively homologous among different PfEMP-1 variants. The response to the cysteine-rich interdomain region was unusual in that the majority of donors, whether malaria exposed or not, had positive CD4 T cell, interleukin-10, and interferon- $\gamma$  responses. The CD4 T cell response to the exon 2 and duffy binding-like domain proteins was significantly greater in malaria-exposed donors than in unexposed Europeans, which suggests that these regions contain peptides recognized by T cells, which thus may be useful as components of a vaccine.

*Plasmodium falciparum* infections are characterized by a slow development of resistance to disease and a lack of sterilizing immunity to infection [1]. Two explanations for this pattern of immunity have been proposed: (1) that buildup of immunity occurs through cumulative exposure to the spectrum of diverse antigenic parasite types [2] and (2) that slow acquisition of immunity to cross-reactive antigenic determinants occurs.

One target of the protective immune response is the variant surface antigen, *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1). PfEMP-1, a family of proteins (20–350 kDa) expressed on the surface of infected erythrocytes, undergoes antigenic variation and mediates cytoadherence of the infected erythrocyte to the host endothelium. These molecules are coded for by a family of 50 *var* genes that are located on most chromosomes of the parasite [3–9]. The *var* genes are composed of 2

exons, one of which codes for multiple extracellular domains and the other for an intracellular domain (figure 1). In addition, there is recombination between these genes [10], which gives rise to a much larger number of PfEMP-1 molecules in the natural parasite population. The extent of the *var* repertoire in the field is unknown.

As far as it has been studied, the antibody response to PfEMP-1 is variant specific, and there is a gradual acquisition of antibody responses to different variants. Antibodies of children in endemic areas of *P. falciparum* transmission recognize only a small proportion of the circulating variants, but adults have a much wider range of antibody specificities [2, 11–13], which suggests that there is a significant correlation between anti-PfEMP-1 antibodies and clinical immunity. PfEMP-1 could, therefore, be considered a good candidate for a malaria vaccine. However, the variant nature of the antibody response is a limitation, and it would be important to determine whether there were cross-reactive or shared epitopes on PfEMP-1, both for antibody and for CD4 T cells, which will be needed to provide specific B cell help. Recently, cross-reactive epitopes recognized by antibodies have been described [14], but no data currently exist on the nature and specificity of T cell responses to PfEMP-1.

In this article, we have investigated the CD4 T cell response to 3 regions of PfEMP-1 in adults and children in an area of Kenya endemic for *P. falciparum*, to determine whether PfEMP-1 T cell responses are induced by exposure to the parasite. The regions selected for this first study were duffy binding-like (DBL $\alpha$ ) and cysteine-rich interdomain region (CIDR $\alpha$ ) domains from the extracellular part of the molecule coded for by exon 1 and a region of the relatively conserved exon 2 domain (figure 1).

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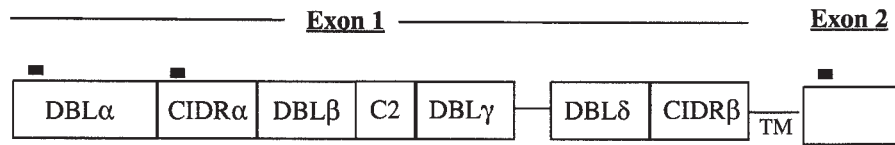
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**Figure 1.** Diagrammatic representation of *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) showing the 2 exons and the extracellular domains. The regions from which recombinant protein fragments were expressed for T cell assays are shown (■). CIDR, cysteine-rich interdomain region; DBL, duffy binding-like domain; TM, transmembrane region.

The choice of exon 2, DBL $\alpha$ , and CIDR $\alpha$  was based primarily on the fact that, although polymorphic, these 3 regions show the most homology among different *var* genes: exon 2 > DBL $\alpha$  > CIDR $\alpha$  [3, 15]. In addition to CD4 T cell proliferation, the levels of 2 cytokines, interleukin (IL)-10 and interferon (IFN)- $\gamma$ , were also measured. IFN- $\gamma$  is thought to play a role both in the development of protective immunity [16–18] and in the inflammatory response, which may be involved in the pathology of malaria [19, 20]. IL-10 is an antiproliferative cytokine [21] and is produced *in vitro* in response to some malaria peptides [22, 23]. We have determined whether both cytokines are present in the same cultures together and whether cytokine production correlates in any way with the proliferation, or lack of it, observed in response to each of the PfEMP-1 fragments tested.

## Materials and Methods

**Antigen preparation and purification of 3 individual *var* gene sequences.** The 3 antigens used were recombinant protein fragments of PfEMP-1; the extracellular DBL $\alpha$  domain was obtained from a PfEMP1 gene isolated from an R29 laboratory isolate (accession no. Y13402), the CIDR $\alpha$  from a PfEMP1 gene from the Malayan Camp laboratory isolate (accession no. U27338) [5], and a fragment of an intracellular exon 2 region from part of a pfEMP1 gene isolated from the A4 laboratory isolate (accession no. AJ413950).

A region of DBL $\alpha$  that codes for 332 aa (aa 128–459) of the R29 *var* gene from the IT lineage was amplified by the 3' primer 5'-TGC-ACCACATATATGAGACAAAATCTGTGTG-3' and the 5' primer 5'-GGGGATCCTTATCAATATCCTGAAGGACCAAAAGT-3', cloned into the expression vector pET (Novagen; CN BioSciences), and expressed in protease-deficient *Escherichia coli* BL21. Inclusion bodies were enriched by centrifugation after lysis by sonication and were solubilized in 8 M urea, 100 mM 2-mercaptoethanol (2-ME), and 20 mM sodium acetate (NaAc; pH 5.0) by sonication on ice. The solution was cleared by centrifugation and was applied to a gel filtration column (Superdex 2HR 10/30 fast protein liquid chromatography; Pharmacia). Fractions that contained proteins of appropriate molecular weight were pooled and were dialyzed stepwise against one of the following: (1) 20 mM NaAc (pH 5.0), 50 mM 2-ME, and 4 M urea; (2) 20 mM NaAc (pH 5.0), 25 mM 2-ME, and 2 M urea; (3) 20 mM NaAc (pH 5.0), 12.5 mM 2-ME, and 1 M urea; or (4) 20 mM NaAc. The dialysate was clarified by centrifugation for 15 min at 22,000 g at room temperature.

Polymerase chain reaction was used to generate a 717-bp fragment of the exon 2 domain (3' primer, 5'-CCGGAATTCATATAT-

GTACCAGGTAGTCC-3'; and 5' primer, 5'-CCGGAATTCCTAG-TCACCACATATGTGTCTCA-3'), coding for 239 aa, and a 702-bp fragment from the CIDR $\alpha$  region, coding for 234 aa (aa 576–809; 3' primer, 5'-CGGGAATTCGAAGACAAAATTATGTCTATA-ATGC-3'; and 5' primer, 5'-CGGGAATTCCTAATCCGCTTTTT-CCTCCTGG-3'). Sequences were verified (MWG Biotech) and were cloned into pGEX-6P1 (Pharmacia Biotech), resulting in glutathione S transferase (GST) fusion proteins. Proteins were expressed in protease-deficient *E. coli* BL21. The GST fusion proteins were purified on glutathione sepharose 4b columns [24]. While still bound to the glutathione sepharose 4b beads, the recombinant proteins were cleaved off GST and were eluted by use of PreScission protease enzyme, according to the manufacturer's protocol (Pharmacia Biotech). The recombinant proteins (i.e., CIDR $\alpha$  and exon 2) were further purified by gel filtration over a Superdex 200 column with PBS (pH 7.4) as the mobile phase. Purity was assessed to be >95% by SDS-PAGE and Coomassie blue staining. Mass spectrometry (Applied Biosystems) confirmed the presence of signals in the region of the expected molecular ion and determined the average molecular weight of CIDR $\alpha$  and exon 2 to be 27,531 and 27,009 Da, respectively. Elution corresponding to the PreScission protease digest of lysed BL21 *E. coli* transfected with empty pGEX-6P1 that was purified on glutathione sepharose 4b beads was used as the negative control in preliminary assays (see *Cell preparation and in vitro proliferation assays* [below]).

Lipopolysaccharide contamination, as assessed by E-TOXATE (Sigma), was negligible. Proteins were dissolved in tissue-culture grade water, filter-sterilized through a 0.2- $\mu$ m filter, and stored at  $-80^{\circ}\text{C}$  before use.

**Donors.** Malaria-exposed donors were women aged 20–54 years and children aged 3–5 years (40% boys and 60% girls) living in a rural area to the north of Kilifi town, Kenya. None of the women was pregnant at the time blood was taken. The area has prolonged seasonal *P. falciparum* transmission (10 and 30 infective bites per person per year) after the short and long rains in the months of October–November and March–July, respectively. At the end of the long dry season, blood samples of 2.5–3.5 and 5 mL were collected into heparinized tubes (Leo Laboratories) from children and mothers, respectively. A blood film was prepared from all donors for parasite determination. Unexposed control donors (aged 20–45 years; 32% men and 68% women) were European volunteers, none of whom had ever traveled in a malaria-endemic area.

**Cell preparation and *in vitro* proliferation assays.** Peripheral blood mononuclear cells (PBMC) were separated over Ficoll-Paque (Pharmacia Biotech) by use of standard procedures. Plasma was taken from each sample before PBMC separation to determine the presence of isolate-specific antibodies (see below). PBMC were labeled with PKH26 (Sigma), as described elsewhere [25]. In brief,

cells were washed twice in sterile PBS, pelleted (415 g for 5 min at room temperature), and resuspended at  $5 \times 10^6$  cells in 250  $\mu\text{L}$  of diluent for PKH26 labeling. The cells were incubated with 1.5 mM (final concentration) PKH26 dye at room temperature for 1 min. Dye incorporation into the lipid bilayer of PBMC was stopped by adding twice the volume of 100% human AB serum. Cells were washed 3 times, and dye incorporation was assessed on the flow cytometer.

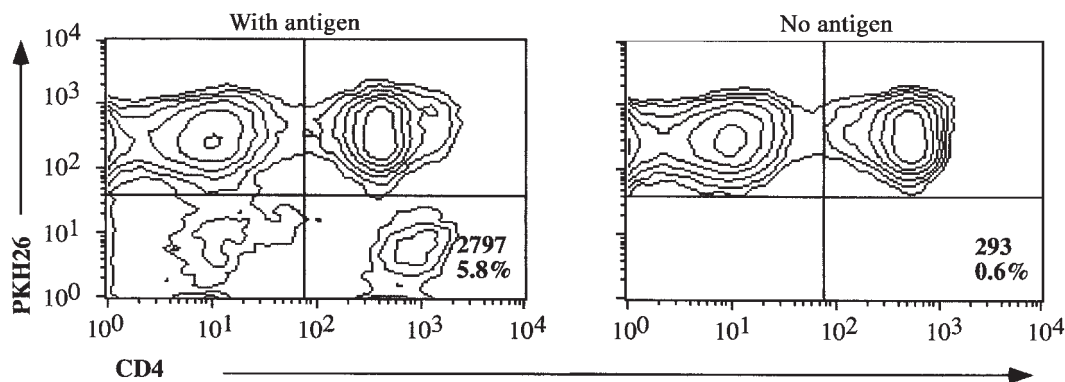
PBMC were plated out at  $2\text{--}3 \times 10^5$  cells/well in a 96-well U-bottomed plate (Nunc; Gibco) in RPMI 1640 that contained 10% heat-inactivated human AB serum (North London Blood Transfusion Service), 2 mM glutamine, 100 U/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin, and 10 mM HEPES (Gibco) and were cultured in a final volume of 200  $\mu\text{L}$ . The PfEMP-1 fragments were used at a final concentration of 0.1  $\mu\text{g}/\text{mL}$ . Initially, eluates from lysed bacterial lysate transfected with "empty" vector and medium only were both used as controls. However, because extensive pretesting showed that neither division of CD4 T cells nor cytokine production occurred in the presence of the eluates from the control bacterial lysate, medium only was used in the experiments shown here. Each condition was set out in triplicate. Phytohemagglutinin (PHA; Sigma) at 2.0  $\mu\text{g}/\text{mL}$  was used as a positive control for cell viability. Plates were incubated for 7 days at 37°C in a humidified atmosphere of 5%  $\text{CO}_2/95\%$  air. The numbers of total lymphocytes and CD3 and CD4 T cells dividing in response to the antigens were determined by flow cytometry [25]. Supernatants were removed for the measurement of cytokines before cytometric analysis.

**Antibodies for flow cytometry.** The antibodies used to determine the phenotype of the dividing cells were as follows: anti-CD3, UCHT1 (mouse IgG1; Coulter and Sigma) and SK7 (mouse IgG1; Becton Dickinson); anti-CD4, 13B8.2 (mouse IgG1; Coulter), SFC121T4D11 (mouse IgG1; Coulter), RPA-T4 (mouse IgG1; Becton Dickinson), and Q4120 (mouse IgG1; Sigma); and anti-CD8, B9.11 (mouse IgG1; Coulter), SFC121ThyD3 (mouse IgG1; Coulter), RPA-T8 (mouse IgG1; Becton Dickinson), and OKT8 (mouse IgG2a; ATCC). Anti-mouse IgG1, 679.1Mc7 (mouse IgG1), was used as an isotype control. Cells were washed in sterile PBS

that contained 0.5% wt/vol bovine serum albumin (BSA), 5.0 mM EDTA (pH 8.0), and 0.01% wt/vol sodium azide (Sigma) and were incubated with the appropriate combinations of fluorescein isothiocyanate (FITC), allophycocyanin (APC), R-phycoerythrin/cyanin 5.1 (PE-CY5), or R-phycoerythrin/texas red (ECD) directly conjugated antibodies for 30 min. Excess antibody was removed with PBS that contained 0.5% BSA and 1%  $\text{NaN}_3$ , and the cells were fixed in 1% paraformaldehyde in PBS. All antibody labeling was carried out in situ in the culture plates.

**Measurement of proliferation by flow cytometry.** Flow cytometry was performed on a FACScalibur and analyzed by Cell Quest software (Becton Dickinson) or on a Coulter EPICS R XL with XL System II and analyzed with FCSPress software. The number of cells dividing in response to antigen or medium control was determined, as described elsewhere [25]. Viable lymphocyte populations were defined by use of forward and 90° light scatter. Fluorescence intensity of the membrane dye halves with each cell division [26]. The relative number of viable cells recovered in the microwells at the end of the culture period was determined by setting the flow cytometer to count the number of events acquired in 30 s. An estimate of the magnitude of this response was made by comparing the number of divided T cells recovered in the cultures with and without antigen. Analysis that used the FITC-, APC-, PE-, peridinin chlorophyll protein-, PE-CY5-, or ECD-conjugated antibodies defined the proportions of the different cell subsets within the divided (low PKH26 fluorescence) and undivided (high PKH26 fluorescence) populations; thus, the number of cells in each population that had divided could be calculated (an example is shown in figure 2).

The number of CD4 T cells that divided in the absence of antigen (background) varied widely between individuals (range, 13–3000 cells). Therefore, the CD4 T cell response for each individual was calculated as a stimulation index (i.e., the ratio of mean no. of CD4 T cells that divided in response to an antigen and mean no. of CD4 T cells that divided in the control wells without antigen for each individual). SEs of the geometric means of triplicate counts were generally in the range of 10%–20% of the mean. A stimu-



**Figure 2.** Representative CD4 T cell response to recombinant malaria antigen (*left*) or without antigen (*right*). Contour plots show fluorescent intensity (measured in standard fluorescence units) of the membrane dye PKH26 (phycoerythrin) and CD4 T cells (fluorescein isothiocyanate). CD4 T cells with low PKH26 fluorescence intensity have divided (*lower right quadrants*). Nos. show no. of events (cells) and percentage of CD4 T cells collected in 30 s from microwells of peripheral blood mononuclear cells cultured in the presence or absence of antigen, as described in Materials and Methods.

lation index  $\geq 2$  was defined as a positive response. The definition of the positive response was set at this low level so that any small positive responses were included, as described elsewhere [27].

**Detection of cytokines by ELISA.** Supernatants from the PBMC cultures were tested for the presence of IFN- $\gamma$  and IL-10 by use of paired antibody kits (Pharmingen/Becton Dickinson), according to the manufacturer's instructions. The sensitivities of the ELISA assays were 4 pg/mL for both IFN- $\gamma$  and IL-10.

Cytokine levels in the cultures of PBMC without antigen (medium control) did not differ from those in PBMC cultured with the eluates from the control bacterial lysates (data not shown). Therefore, the cytokine response in the presence of medium was used for the background response. The background levels differed widely between individual donors (range, 0–200 pg/mL for both IL-10 and IFN- $\gamma$ ). For this reason, the concentration of cytokine produced by each donor in the presence of antigen was taken as the net level after subtraction of the background level of that donor without antigen. SEMs were usually in the range of 15%–20%.

The response of a donor was defined as positive when the mean concentration of cytokine produced in triplicate wells containing antigen was significantly greater (i.e.,  $P < .05$ , Student's *t* test) than the mean of triplicate wells without antigen of that donor.

**Variant-specific antibodies.** Antibody responses to different parasites were tested on a panel of 7 fields and 2 laboratory isolates (A4 and FCR3) of *P. falciparum*, using methods described elsewhere [28]. In brief, cultured trophozoites were combined at 5% hematocrit with each plasma sample in PBS with 0.1% BSA. After incubation, cells were washed in PBS with 0.1% BSA. Bound human immunoglobulin was assessed by addition of FITC-conjugated goat anti-human IgG, and infected red blood cells were stained with ethidium bromide. Samples were assessed by flow cytometry, and the percentage of infected cells recognized was determined with the background (normal noninfected serum) subtracted. For each parasite isolate, the antibody response was considered to be positive when  $> 5\%$  of the infected erythrocytes were recognized.

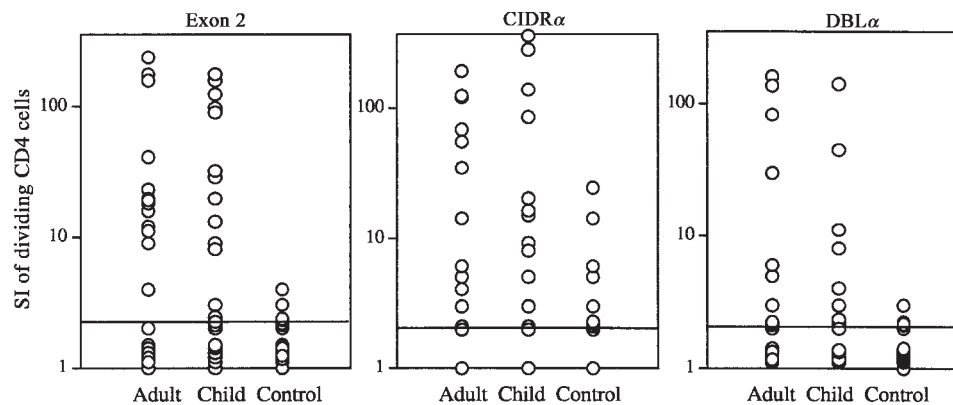
**Statistics.** The magnitudes of the CD4 T cell response (no. of dividing CD4 T cells) and of the cytokine response (picograms per milliliter in the supernatant) of exposed adults and children versus

unexposed control donors were compared with an unpaired, non-parametric 2-tailed Mann-Whitney *U* test.

## Results

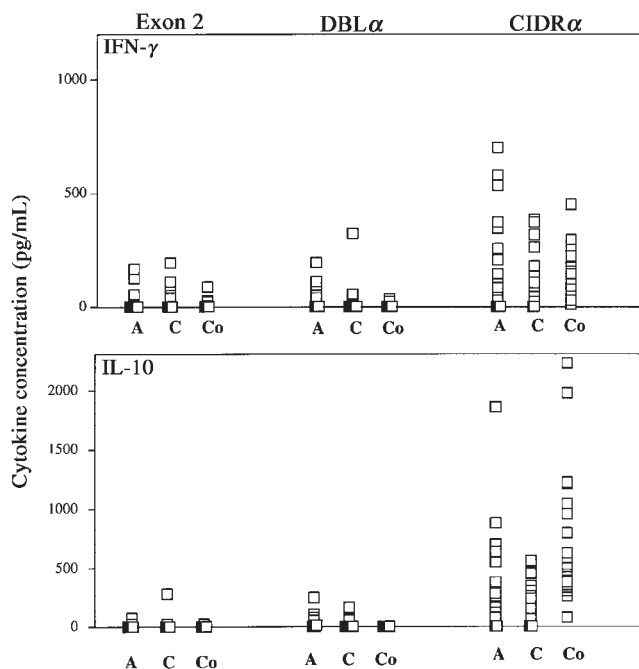
**CD4 T cell response of exposed donor to exon 2 and DBL $\alpha$ , but not CIDR $\alpha$ , is greater in exposed donors.** The CD4 T lymphocyte responses of 27 exposed adults, 27 children, and 25 unexposed adult donors to the 3 recombinant PfEMP-1 fragments were determined by flow cytometry. The magnitude of the CD4 T cell responses (no. of divided CD4 T cells) in each group was variable, with considerable overlap between the groups. The ranges of the number of divided CD4 T cell responses to each of the protein fragments were 0–1395, 0–3898, and 0–997 for exon 2; 0–13,486, 0–2949, and 0–3065 for CIDR $\alpha$ ; and 0–9804, 0–3065, and 0–493 for DBL $\alpha$  in the Kenyan malaria-exposed adults and children and the UK non-malaria-exposed adults, respectively. Despite this variation, the CD4 T cell response to exon 2 was significantly greater in the malaria-exposed donors (i.e., medians of 147 and 98 for adults and children, respectively) than in the unexposed donors (i.e., median of 27;  $P = .045$  and  $.0085$  for malaria-exposed adults and children, respectively). However, the magnitudes of response to CIDR $\alpha$  (i.e., medians of 253, 124, and 64 for malaria-exposed adults and children and non-malaria-exposed adults, respectively) and DBL $\alpha$  (i.e., medians of 15, 9, and 7 for malaria-exposed adults and children and non-malaria-exposed adults, respectively) were not different when comparisons were made between the malaria-exposed and -unexposed donors ( $P = .441$  and  $.5278$  for adults and children, respectively, with CIDR $\alpha$  and  $.428$  and  $.312$  for adults and children, respectively, with DBL $\alpha$ ).

CD4 T cell responses with a stimulation index of  $\geq 2$  were observed in all groups (figure 3). The proportion of individuals who responded to all 3 protein fragments of PfEMP-1 was greater in the malaria-exposed groups than in the control group (52%



**Figure 3.** CD4 T cell responses to 3 fragments of *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1; exon 2, cysteine-rich interdomain region [CIDR $\alpha$ ], and duffy binding-like region [DBL $\alpha$ ]) in 2 malaria-exposed population groups (adults and children from Kilifi, Kenya) and in a malaria-naive control group (control). The Y-axis is shown as a log<sub>10</sub> scale. All responses to PfEMP-1 of  $\geq 2$  stimulation indices (SI; no. of divided CD4 T cells in cultures containing antigen/no. of divided CD4 T cells in cultures without antigen) were defined as positive. Horizontal line represents 2 SI.





**Figure 4.** Interferon (IFN)- $\gamma$  and interleukin (IL)-10 produced *in vitro* in response to 3 different *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) fragments (exon 2, duffy binding-like region [DBL $\alpha$ ], and cysteine-rich interdomain region [CIDR $\alpha$ ]) in 2 malaria-exposed population groups (children [C] and adults [A] from Kilifi, Kenya) and 1 malaria-naive control group (Co). Each symbol represents an individual donor, and the values shown are the net amounts of cytokine after subtraction of the background levels of cytokine from the control cultures without antigen.

and 48% to exon 2, compared with 12% of controls; 70% and 67% to CIDR $\alpha$ , compared with 44% of controls; and 30% to DBL $\alpha$ , compared with 4% of controls). Of interest, both the prevalence of responders and the magnitude of the responses to the CIDR $\alpha$  region were higher in all groups than those to exon 2 or DBL $\alpha$ , regardless of a history of previous malaria exposure.

Not surprisingly, because samples were taken at the end of the dry season, only a small proportion of the malaria-exposed donors were parasite positive (6 of 54, distributed equally between children and adults). The CD4 T cell responses of parasite-positive donors did not appear to differ from those of donors who were parasite negative. The lack of response to recombinant PfEMP-1 fragments in a number of donors in all the groups was not due to loss of viability in culture, because cells from all donors responded vigorously to PHA (data not shown). The CD8 T cell responses were negligible in all cases. The number of dividing cells and proportion of responders obtained when the CD3 T cell population was used were similar to those obtained when CD4 T cell numbers were used (data not shown).

*CIDR $\alpha$  elicits the greatest levels of IFN- $\gamma$  and IL-10 in all population groups.* IFN- $\gamma$  and IL-10 levels were measured in the supernatants after 7 days of culture with the 3 PfEMP-1 fragments (figure 4). The amount of both cytokines produced by the

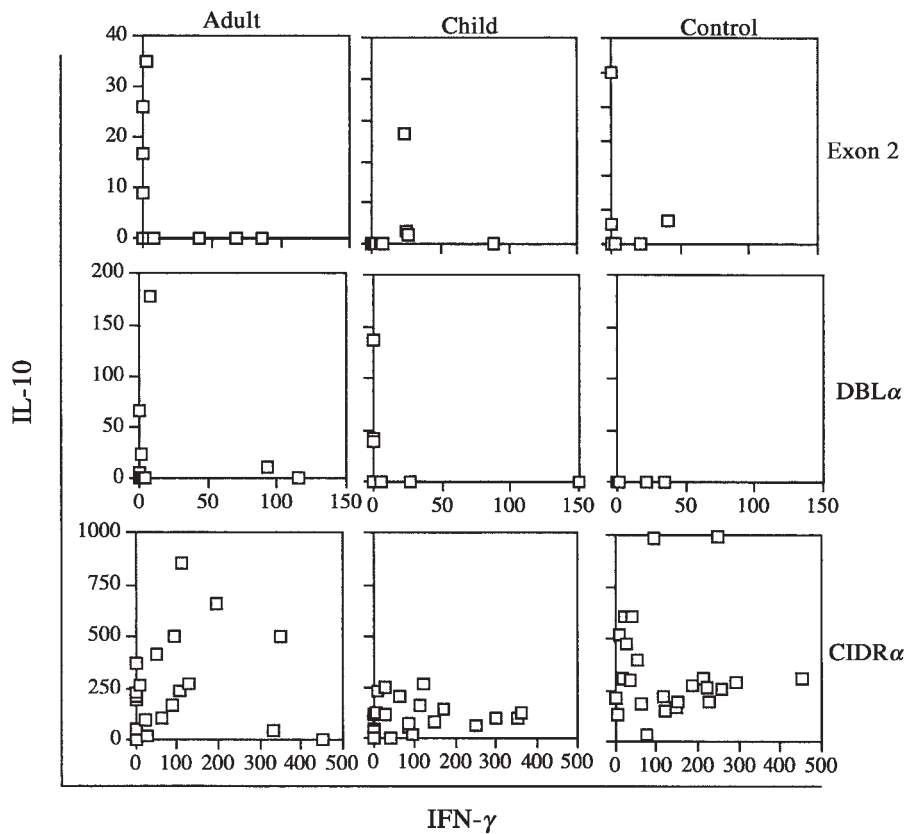
donors within each population group varied widely. Strikingly, only the CIDR $\alpha$  fragment elicited significant levels of IFN- $\gamma$  and IL-10 from a large fraction of all donors. Both exposed and unexposed donors responded. The median IFN- $\gamma$  responses of malaria-exposed adults and children and unexposed controls to exon 2 and DBL $\alpha$  were 7, 22, and 8 and 4, 27, and 11.5 pg/mL, respectively. The median IFN- $\gamma$  responses to CIDR $\alpha$  by adults, children, and control donors were of similar magnitude in all groups (i.e., 109, 94, and 105, respectively). The amount of IL-10 produced by control donors in response to CIDR $\alpha$  (median, 575 pg/mL) was significantly greater than that produced by either exposed adults (median, 219 pg/mL) or children (median, 262 pg/mL) when a nonparametric 2-tailed Mann-Whitney *U* test was used to compare medians of exposed adults and control donors ( $P = .0004$ ) and medians of exposed children and control donors ( $P < .0001$ ). The median IL-10 responses of malaria-exposed adults and children and unexposed control donors to exon 2 and DBL $\alpha$  were 26, 7, and 3 and 42, 73, and 0 pg/mL, respectively.

A large proportion of donors in all 3 groups had cytokine responses to the CIDR $\alpha$  fragment that were significantly above background (32%, 48%, and 50% for IFN- $\gamma$  and 46%, 33%, and 53% for IL-10 in malaria-exposed adults and children and unexposed control donors, respectively).

Although IL-10 and IFN- $\gamma$  were detected in the cultures of PBMC with exon 2 or DBL $\alpha$  from a small number of donors, none of the IL-10 responses to either antigen were significantly above the background production by PBMC cultured with medium. In a very small proportion of donors, IFN- $\gamma$  production in response to exon 2 was significantly above background, but there was no difference in prevalence between exposed and unexposed adults (prevalence of positive IFN- $\gamma$  responses was 12%, 0%, and 8% for malaria-exposed adults and children and unexposed donors, respectively). There was no obvious positive association of IFN- $\gamma$  or IL-10 response with proliferation, and some donors made positive cytokine responses in the absence of CD4 T cell proliferation, regardless of the antigen used (data not shown).

The relationship between IL-10 and IFN- $\gamma$  produced by PBMC from exposed and unexposed donors is shown in figure 5. In the response to exon 2 and DBL $\alpha$ , it appears that, when IFN- $\gamma$  was present in the cultures, no IL-10 was produced and vice versa. The pattern of the response to CIDR $\alpha$  is not as clearcut. There is, however, a clear dichotomy of IL-10 and IFN- $\gamma$  production in the control donors who responded to this fragment.

*Adult plasma recognizes a greater diversity of isolates of P. falciparum-infected red blood cells than plasma of children.* Plasma samples from each group (taken from the same blood sample as the PBMC) were tested for the ability to recognize 9 different isolates of *P. falciparum*, using a flow cytometric method [28]. In agreement with earlier studies, the plasma of exposed children recognized fewer isolates than the plasma of adults; 4 (median) parasite isolates were recognized by each child's plasma,



**Figure 5.** Relationship between interleukin (IL)–10 and interferon (IFN)– $\gamma$  production by peripheral blood mononuclear cells of each donor in response to recombinant *Plasmodium falciparum* erythrocyte membrane protein–1 fragments (exon 2, duffy binding–like region [DBL $\alpha$ ], and cysteine-rich interdomain region [CIDR $\alpha$ ]). Each symbol represents the net concentration of cytokine (in pg/mL), after subtraction of the background cytokine level in the absence of antigen, produced by an individual malaria-exposed adult or child or malaria-naive control donor.

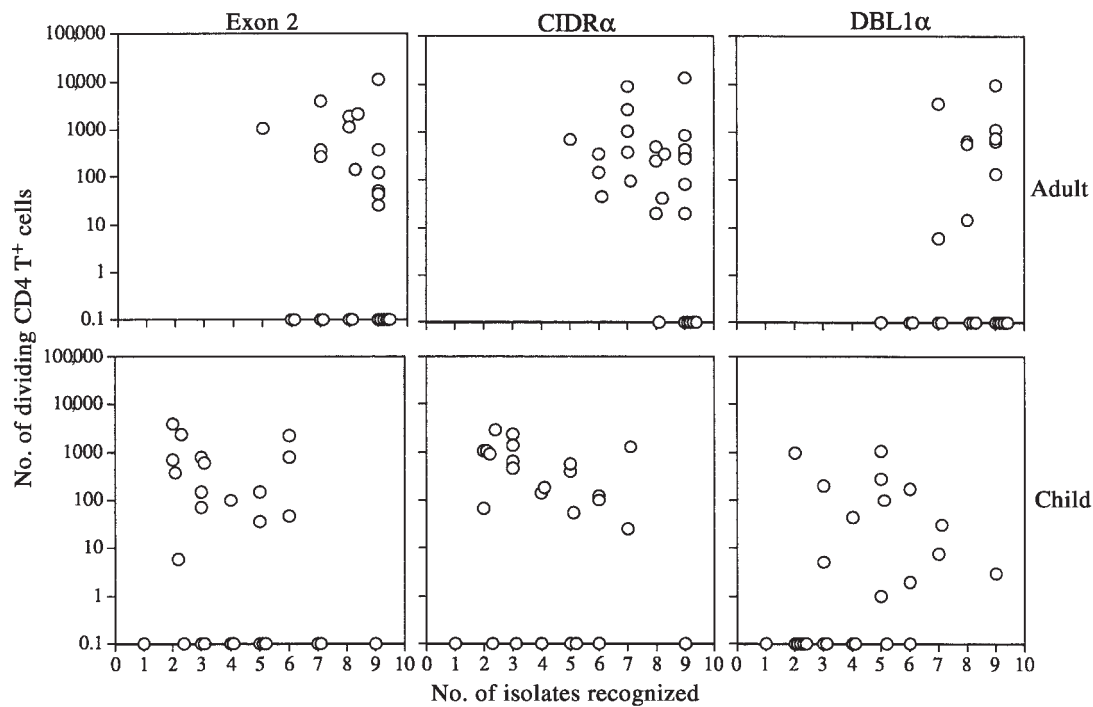
compared with 8 (median) recognized by adult plasma (figure 6). Plasma from noninfected control individuals did not react with any parasite-infected red blood cells (data not shown). The magnitude of the T cell response did not increase with the increase in the number of variants recognized (figure 6). Because there were only 2 exposed donors (both children) who did not recognize any of the 9 isolates tested, it was not possible to determine whether T cell responses to these regions of PfEMP-1 can occur in exposed donors in the absence of antibody.

**Discussion**

In these studies, we have determined the CD4 T cell response in adults and children to 3 different regions of the variant antigen PfEMP-1 of *P. falciparum*, in an area of endemic malaria transmission. Recombinant protein fragments from a DBL $\alpha$  (332 aa) and CIDR $\alpha$  region (234 aa) of the extracellular domains and a 239-aa fragment from exon 2 within the relatively conserved intracellular part of PfEMP-1 were used as antigens (figure 1). Although exon 2 is thought not to be on the surface of infected erythrocytes and therefore cannot be a target for relevant antibodies, it could contain T cell epitopes.

All 3 fragments stimulated a CD4 T cell response in a fraction of malaria-exposed and unexposed individuals. For exon 2 and DBL $\alpha$ , the size of the response and number of responders (defined by the no. of divided CD4 T cells in the culture) reflected a history of previous exposure to *P. falciparum* infection; the prevalence of responders and the magnitude of the response were greater in exposed adults and children than in *P. falciparum*–naive donors. To our knowledge, there are no studies of T cell responses to PfEMP-1 with which to compare these data. However, adults from this same area of coastal Kenya (Kilifi) [2, 11], in an upland region of Kenya, and also in another part of Africa, Gabon [12], have antibody responses to a number of *P. falciparum* isolates. This probably reflects responses to different variants gained over a period of years of exposure to many different isolates. In agreement with this, we also show here that plasma from our adult donors recognized a larger number of isolates than the plasma of the younger donors.

There are several aspects of the T cell response to the PfEMP-1 regions that are intriguing. First, despite the fact that all but 2 of the plasma samples of the exposed donors had antibodies to molecules on the surface of parasite-infected erythrocytes (presumably some of these are specific for PfEMP-1), only



**Figure 6.** Relationship between no. of different *Plasmodium falciparum* isolates recognized by plasma from malaria-exposed adults and children and no. of CD4 T cells that have divided in vitro in response to each of the different fragments of *P. falciparum* erythrocyte membrane protein-1 (exon 2, cysteine-rich interdomain region [CIDR $\alpha$ ], and duffy binding-like region [DBL $\alpha$ ]) in peripheral blood mononuclear cells from the same samples. An antibody response to a given isolate was considered to be positive when >5% of the parasitized red blood cells were positively stained, as assessed by flow cytometry. No. of dividing CD4 T cells was calculated as described in Materials and Methods.

half of the exposed donors responded to exon 2 or DBL $\alpha$ . Second, when there was a T cell response, it was generally low in magnitude. Third, a large fraction of unexposed donors responded, particularly to the CIDR $\alpha$  region, although they had no antibodies to recognize infected erythrocytes.

Low T cell responses in the field have been described elsewhere for malaria antigens. This may be due to a low frequency of specific T cells in the blood at the time of sampling [29–31]. Repeated samples from individuals at different times during and after the transmission seasons would address this. One of the major problems with studying T cell responses to this variant antigen is that we have no knowledge of the nature or extent of the repertoire of variants that are currently present or have been present in the parasite population, in our or any other study area. Because fragments were selected from individual PfEMP-1 molecules, it is highly possible that these are not represented or are rare variants, and, thus, there is a probability that the donors have not carried these variants. This would be particularly true for the DBL $\alpha$  and CIDR $\alpha$  fragments, in which there is more diversity within the stretches of amino acids chosen. Exon 2, although relatively conserved, is also variable. Because it is not yet known which variants are expressed in different areas or whether the T cell response is directed toward shared or common sequences, cross-reactive regions, or sequences unique to different variants, these variables will be a limitation

of the study. Until it is possible to clone a single *var* gene into a parasite and fix its expression, it will be very difficult to look at effects of T cell responses and relate them to the antigen-specific antibody response that may occur to the same part of the molecule.

The response to CIDR $\alpha$  was clearly different from the responses to exon 2 and DBL $\alpha$ . There was a higher frequency of responders, both for CD4 T cell division and for cytokine production to CIDR $\alpha$ . In addition, greater amounts of IFN- $\gamma$  and IL-10 were produced in response to this fragment. There was no evidence of increasing response with length of exposure and the PBMC of unexposed individuals responded as well as, or better than, those of exposed donors, by both proliferation and cytokine production. The responses cannot be explained by contaminated bacterial material, because the expression system and purification steps for the CIDR $\alpha$  fragment were the same as those used for the exon 2 fragment. It is possible that the CIDR $\alpha$  region contains sequences shared by a common pathogen or antigen to which most individuals are primed or sequences that act as a type of superantigen. A more detailed analysis of the memory or naive status of the responding CD4 cells would address this. It is unlikely that the CIDR $\alpha$  fragment itself is mitogenic, because it does not elicit a response in every single individual tested, and the responses are several orders of magnitude smaller than the responses to PHA (data not shown).

The CIDR $\alpha$  region of PfEMP-1 is responsible for binding to CD36 [32–34]. Ligation of CD36 on dendritic cells can modulate T cell activation and cytokine production [7]. Therefore, it would be of great interest to determine whether this CIDR $\alpha$  region of PfEMP-1 has the correct conformation to bind CD36 and whether it could have similar modulatory effects. Because this region of the molecule may be considered to be a possible candidate for a vaccine based on PfEMP-1 [14], it is important to investigate this further.

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