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Immunology

Vaccine T-cell epitope selection by a peptide competition assay

(*Plasmodium falciparum*/human T-cell clones)

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ABSTRACT **The binding of several peptides derived from the *Plasmodium falciparum* circumsporozoite protein (CS protein) to the human major histocompatibility complex class II proteins HLA-DR5 and -DRw6 was examined in a competition assay. Fixed antigen-presenting cells (APCs) were incubated with various concentrations of each peptide and suboptimal concentrations of stimulator peptides. The binding of the CS peptides to DR5 or DRw6 proteins was then determined in a proliferation assay using two established DR5 or DRw6-restricted T-cell clones with specificity for the stimulator peptides as responder cells. One of five CS peptides, comprising together about 50% of the CS protein sequence, was found to compete with the binding of the stimulator peptides to DR5 and DRw6. The CS peptide CS-(378–398), binding to DR5 and DRw6, was then shown to be able to induce primary *in vitro* responses of T cells from donors with DR5 and DRw6 haplotypes. CS-(378–398)-induced T-cell clones responded not only to the homologous peptide but also to the native CS protein in the presence of appropriate APCs. The strategy we have applied is of considerable general interest for the engineering of vaccines against any pathogen, since it greatly facilitates the selection of appropriate T-cell epitopes to be incorporated in the vaccine.**

Although vaccination with attenuated pathogens is the most successful area of applied immunology, some widespread pathogens, such as parasites causing malaria, cannot be generated in sufficient quantities for attenuated vaccine preparation. In these cases, it is necessary to engineer vaccines that are able to induce protective immunity. The engineering of vaccines has been greatly facilitated by recent advances in gene technology and our increased understanding of how T cells recognize antigens and how they collaborate with B cells. While the mechanisms that mediate protective immunity against malaria parasites are not yet entirely clear, it is likely that class II (1) and class I major histocompatibility complex (MHC)-restricted T cells (2, 3), as well as B cells (4), are involved. In our previous work, we have identified several regions of the circumsporozoite protein (CS protein) of *Plasmodium falciparum* as epitopes recognized by human T cells (5, 6). Since, in general, any given T-cell epitope is only recognized in association with one or a few MHC restriction elements (7, 8), we wished to know the spectrum of HLA antigens with which each T-cell epitope-containing peptide could associate. Therefore, we developed a competition assay that allows us to examine many different peptides for their capacity to bind to different HLA class II proteins. In a second step we examined a peptide selected by the competition assay for its ability to induce primary T-cell responses and to induce T-cell clones able to respond to the native protein in the presence of appropriate antigen-presenting cells (APCs). In the present study, we used these methods to extend our previous work, which had already suggested that only one of four CS peptides tested is a good candidate for incorporation into a vaccine as a T-cell epitope (5). The results shown here suggest that the strategy we have used can be applied on a much larger scale for the selection of T-cell epitopes to be incorporated in any vaccine.

MATERIALS AND METHODS

Antigens. Peptides were synthesized by the solid-phase technique (9). Purity was assessed by HPLC and amino acid analysis. Peptide CS-(378–398) corresponds to residues 378–398 of the CS protein sequence, except that the CS protein cysteine residues 384 and 389 were replaced by alanine residues (5). The peptide CS-(23–43), insoluble in culture medium, was dissolved as a stock solution in 6 M urea. The sequences of the "stimulating" peptides used in the studies reported here are described below:

CS-(325–341): Glu-Pro-Ser-Asp-Lys-His-Ile-Glu-Gln-Tyr-Leu-Lys-Lys-Ile-Lys-Asn-Ser.
CS-(378–398): Asp-Ile-Glu-Lys-Lys-Ile-Ala-Lys-Met-Glu-Lys-Ala-Ser-Ser-Val-Phe-Asn-Val-Val-Asn-Ser.
p190-(260–273): Leu-Asp-Asn-Ile-Lys-Asp-Asn-Val-Gly-Lys-Met-Glu-Asp-Tyr.

Recombinant proteins dihydrofolate reductase (DHFR)-CSVI-$(His)_6$ and DHFR-CS1A-$(His)_6$ were expressed and purified as described (10). Briefly, DNA fragments CSVI and CS1A encoding amino acids 309–364 and 362–408 of the CS protein (11), respectively, were integrated into the unique *Bgl* II restriction site of plasmid pDHFR-$(His)_6$ (10). The resulting plasmids pDHFR-CSVI-$(His)_6$ and pDHFR-CS1A-$(His)_6$ were subsequently used for expression of protein DHFR-CSVI-$(His)_6$ and DHFR-CS1A-$(His)_6$ in *Escherichia coli* M15 cells harboring plasmid pDMI.1 (12). The proteins were extracted with 6 M guanidine hydrochloride/0.1 M NaH_2PO_4, pH 8.0, from the *E. coli* cell paste and purified according to Hochuli *et al.* (10) by immobilized metal ion affinity chromatography. The crude extract was directly loaded onto the metal chelate adsorbent. The column was washed first with extraction buffer and then with 8 M urea/0.1 M NaH_2PO_4/0.01 M Tris·HCl, at pH 8.0, 6.5, and 6.0. Finally, DHFR-CSIV-$(His)_6$ and DHFR-CS1A-$(His)_6$ were eluted with a purity of ≈70% using urea buffer at pH 4.0.

Purification of CS protein from sporozoites was carried out as described (5).

Cells. The derivation and characterization of T-cell clones HM.37 and AC.129 have been described elsewhere (6, 13). Isolation of T-cell clones and lymphocyte proliferation assays

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Abbreviations: CS, circumsporozoite; APC, antigen-presenting cell; MHC, major histocompatibility complex; EBV-B, Epstein–Barr virus-transformed B cells; PBMC, peripheral blood mononuclear cell; DHFR, dihydrofolate reductase; mAb, monoclonal antibody.
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were carried out as described (5, 14). Briefly, peripheral blood mononuclear cells (PBMCs) from two donors with no history of malaria infection [SD(DR5, -7) and BR(DR4, -w6)] were stimulated *in vitro* with peptide CS-(378–398) (10 μM), expanded in interleukin 2-containing medium, and cloned by limiting dilution. To test the specificity of the clones in a proliferation assay, cloned T cells (2×10^4) were cocultured in triplicate with 10^4 APCs [irradiated autologous or DR homozygous Epstein–Barr virus-transformed B (EBV-B) cells] in 0.2 ml of complete medium (14) with CS-(378–398) (10 μM) or without antigen. [^{3}H]Thymidine incorporation was measured 72 hr later. Homozygous cell lines were maintained in RPMI 1640 medium (GIBCO, Paisley, Scotland) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 1% nonessential amino acids (100% stock solution; GIBCO), streptomycin (50 units/ml), and 10% fetal calf serum. The lines are EBV-B-cell lines, which were irradiated (5000 rad; 1 rad = 0.01 Gy) before being used as APCs. EBV-B-cell lines from donors SD and BR were prepared as described in ref. 14 and maintained as described above.

According to the new HLA nomenclature, the ATH cell line is DRw11[5] and the BEC II line is DR14[w6].

Monoclonal Antibodies (mAbs). The mAbs used and their specificities are as follows: mAb E.31, monomorphic anti-DR (15); mAb Tü 22, monomorphic anti-DQ (16) obtained from A. Ziegler (University of Marburg, F.R.G.); mAb B7/21 (17), monomorphic anti-DP obtained from Becton Dickinson, Sunnyvale, CA. All antibodies were in ascites fluid, which was added to cultures to a final dilution of 1:100.

Competition for Antigen Presentation. Competition for antigen presentation was performed with fixed EBV-B-cell lines as APCs. Cells were fixed by resuspending them in 0.05% glutaraldehyde for 90 sec. The reaction was stopped by addition of 0.2 M glycine and the cells were then resuspended in a mixture of RPMI 1640 medium containing phenylmethylsulfonyl fluoride (final concentration, 1 mM), aprotinin (100 units/ml), leupeptin (10 μg/ml), aminocaproic acid (10 μl per ml of a saturated solution), N^{α}-(*p*-tosyl)-L-lysine chloromethyl ketone (1 mM), and 10% human serum. Fixed APCs (5×10^4 cells per well) were incubated with various concentrations of the competitor peptide (0.1–100 μM) and suboptimal concentrations (1–10 μM) of the stimulator peptides that are recognized by the test clones. After incubation for 20 hr at 37°C, the cells were washed three times and cultured with 4×10^4 T cells from the test clones. The cultures were pulsed with 1 μCi of [^{3}H]thymidine (1 Ci = 37 GBq) at day 2 and the incorporation of labeled nucleotide was determined after another 16 hr.

RESULTS

Interaction of CS-(378–398) with DR5 and DRw6 Molecules. To investigate the interaction of CS peptides with HLA class II molecules, we established a competition assay. In this assay, we used glutaraldehyde-fixed EBV-B cells as APCs and two T-cell test clones restricted to either DR5 or DRw6, each specific for a different malaria peptide (Fig. 1). Clone HM.37 recognizes peptide 325–341 of the CS protein in association with DR5 molecules (6), whereas clone AC.129 is specific for peptide 260–273 of the *P. falciparum* blood stage p190 protein (18) and DRw6 molecules (Fig. 1). The proliferative response of the clones HM.37 and AC.129 was used as a read-out system for the inhibitory capacity of various peptides. A typical competition experiment is shown in Fig. 2. Peptide 378–398 competes very efficiently with the stimulator peptides for interaction with both DR5 and DRw6 molecules. The other peptides comprising ≈50% of the CS protein sequence did not exhibit discernible competition in the concentration range used (up to 100 μM).

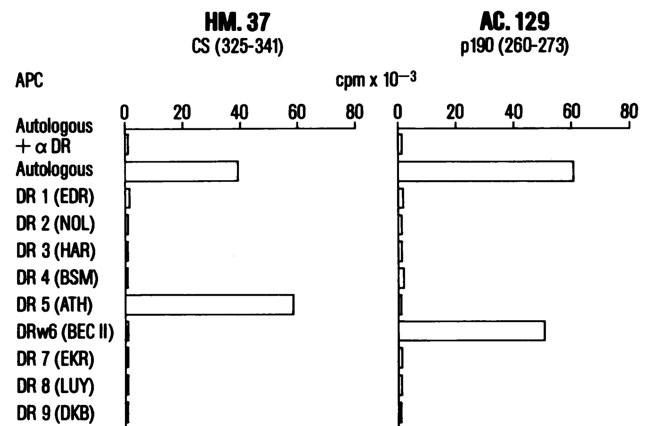

FIG. 1. Antigen specificity and MHC restriction of the T-cell clones used in the competition assay. T cells (2×10^4 cells per well) were cocultured in triplicate wells with irradiated autologous or DR homozygous EBV-B cells (10^4 cells per well) in the presence of 10 μM CS-(325–341) or 10 μM p190-(260–273) peptide. [^{3}H]Thymidine incorporation was measured 72 hr later. Results are mean values of cpm. Anti-DR mAb E.31 was added as a 1:100 dilution of ascites fluid.

Correlation Between Peptide Binding and Peptide Immunogenicity. The above results indicate that the CS-(378–398) peptide is capable of binding to DR5 and DRw6 class II molecules. To assess the immunological relevance of this binding, we used CS-(378–398) to immunize *in vitro* PBMCs from nonimmune donors with *DR5*$^+$ (SD) and *DRw6*$^+$ (BR) haplotypes. From the *in vitro* primed PBMCs of these two donors we derived, by limiting dilution, a total of 17 and 10 T-cell clones, respectively. All 27 T-cell clones responded well to CS-(378–398) but not at all to a control peptide, CS-(325–341) (data not shown). The MHC restriction of these clones was assessed by testing the effect of anti-MHC class II antibodies on the proliferative response. All 27 clones were DR restricted, since their response was inhibited by anti-DR antibodies but by neither anti-DP nor by anti-DQ antibodies. The restricting *DR* allele was determined for each clone by using HLA-DR homozygous EBV-B cells as APCs (Fig. 3). The 17 clones obtained from donor SD(DR5, -7) were DR5 restricted (4 clones) or DR7 restricted (13 clones), whereas the clones from donor BR(DR4, -w6) were either DR4 restricted (5 clones) or DRw6 restricted (5 clones).

***In Vitro* Induced Antipeptide T-Cell Clones Recognize the *P. falciparum* CS Protein.** The T-cell clones generated from primary *in vitro* stimulation were assessed for their ability to recognize the parasite-derived CS protein as well as a recombinant polypeptide containing the 378–398 sequence with the native protein's cysteine residues at positions 384 and 389. As shown in Fig. 4, both the native as well as the recombinant CS sequence were clearly recognized, although not as efficiently as the 378–398 peptide. Three of six of the BR T-cell clones tested failed to respond to the native parasite sequence (data not shown).

Quantitative Aspects of the Competition Assay. Peptides binding to the same MHC class II molecule are capable of competing with each other (8, 19). Peptide CS-(378–398) competes with peptide CS-(325–341) for interaction with DR5 molecules (Fig. 2). Conversely, peptide CS-(325–341) should compete with CS-(378–398) for the same restriction molecule. Indeed, this is the case (Fig. 5), but the affinity of the interaction of these two peptides for the DR5 molecule is clearly different. Peptide CS-(378–398) is a very strong competitor since 1 μM peptide already inhibits 50% of the response to 10 μM CS-(325–341). In contrast, CS-(325–341) is a weak inhibitor, since 50% inhibition of the response to

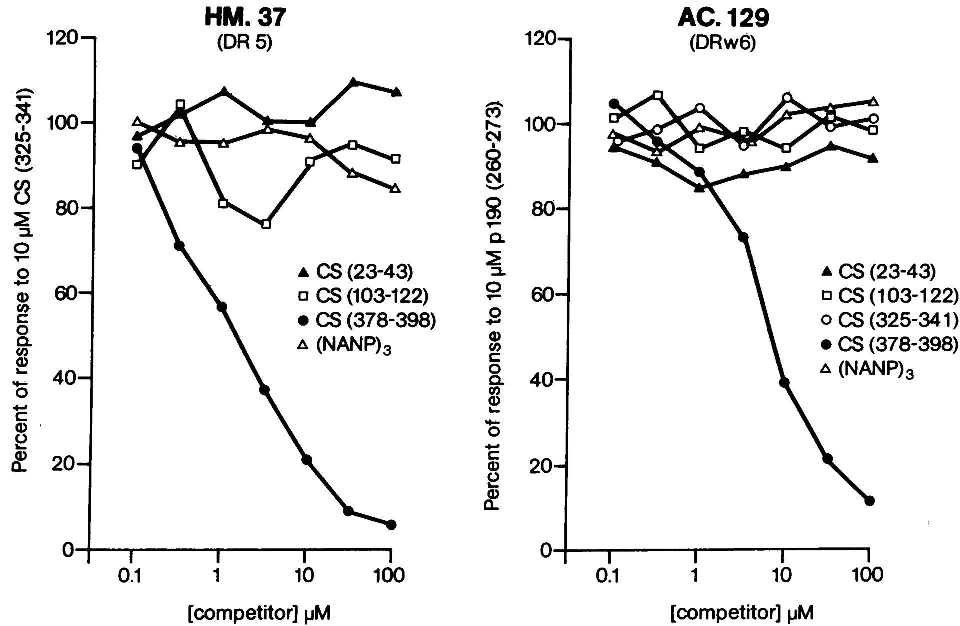

FIG. 2. *In vitro* competition between CS peptides for interaction with DR5 and DRw6. Inhibition of antigen presentation was determined by incubating glutaraldehyde-fixed EBV-B cells (5×10^4 cells per well) with 10 μM peptide CS-(325–341) (clone HM.37) or peptide p190-(260–273) (clone AC.129) and 1–100 μM competitor peptides for 18 hr. APCs were then washed and HM.37 or AC.129 T cells (4×10^4 cells per well) were added. After 48 hr, the cultures were pulsed with [^{3}H]thymidine. Data are presented as percentage [^{3}H]thymidine incorporation obtained in response to 10 μM stimulator peptide. Responses (mean of triplicates) in the presence or absence of stimulator peptide were 43,512 and 738 cpm, respectively, for clone HM.37 and 51,830 and 521 cpm, respectively, for clone AC.129.

CS-(378–398) is attained only by 100-fold excess of competitor.

DISCUSSION

Previous studies by ourselves and others have identified T-cell epitopes in different regions of the CS protein of *P. falciparum* (5, 6, 20, 21). We have established several human T-cell clones that recognize synthetic peptides corresponding to these regions. The aim of the present study was to establish simple methods that would allow us to determine for each peptide the spectrum of HLA-encoded immune response genes. In general, the products of responder alleles differ from those of nonresponder alleles by the fact that they can bind the peptide in question. The binding can be analyzed directly *in vitro* by using a cell-free system. Using a direct binding test, of the five CS peptides tested (Fig. 2) only CS-(378–398) was found to be able to bind to DR1 proteins (T. Jardetzky, personal communication). The direct binding test requires purified MHC class II proteins and is not readily applicable to screening the capacity of many peptides to bind to many MHC proteins. Therefore, we established an indirect functional assay for peptide binding.

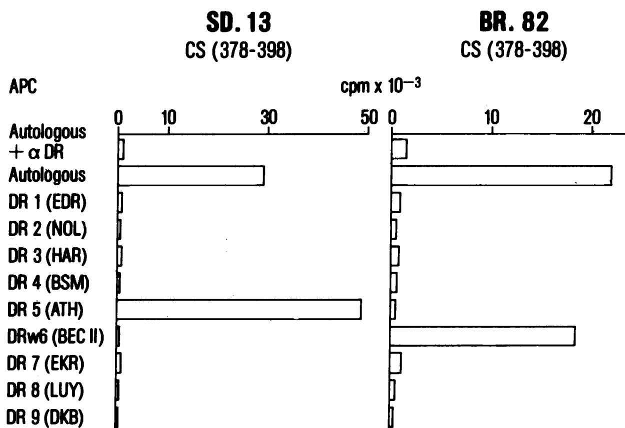

FIG. 3. Restriction specificity of CS-(378–398)-specific T-cell clones. Cloned T cells (2×10^4cells) from donor SD(DR5, -7) or BR(DR4, -w6) were cocultured in triplicate with irradiated autologous or DR homozygous EBV-B cells (10^4 cells) in 0.2 ml of complete medium with CS-(378–398) (10 μM) peptide. [^{3}H]Thymidine incorporation was measured 72 hr later. Results of representative clones are mean values of cpm. Anti-DR mAb E.31 was added as a 1:100 dilution of ascites fluid.

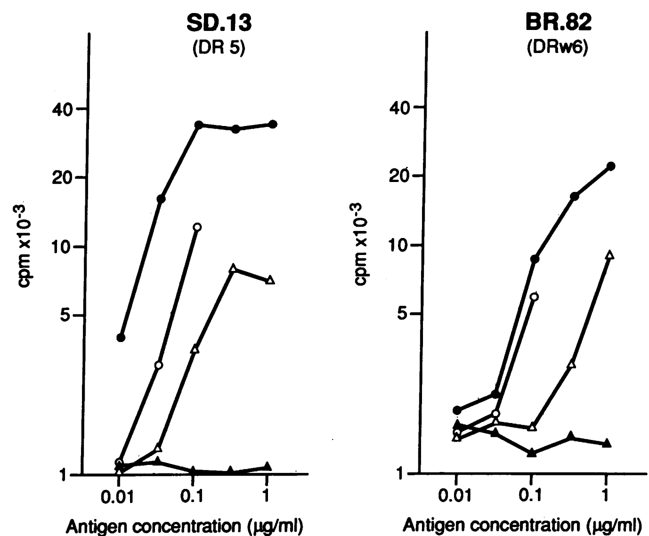

FIG. 4. Proliferative response of T-cell clones SD.13 and BR.82 to CS-(378–398) peptide and CS protein. Cloned T cells (2×10^4 cells) were stimulated in the presence of autologous irradiated PBMCs (10^5 cells), with peptide CS-(378–398) (●), CS protein (○) purified from sporozoites as described (5), and purified recombinant DHFR-CSVI-(His)$_6$ (▲) or DHFR-CS1A-(His)$_6$ (△). Responses in the presence of medium alone were 918 and 1615 cpm for clone SD.13 and BR.82, respectively.

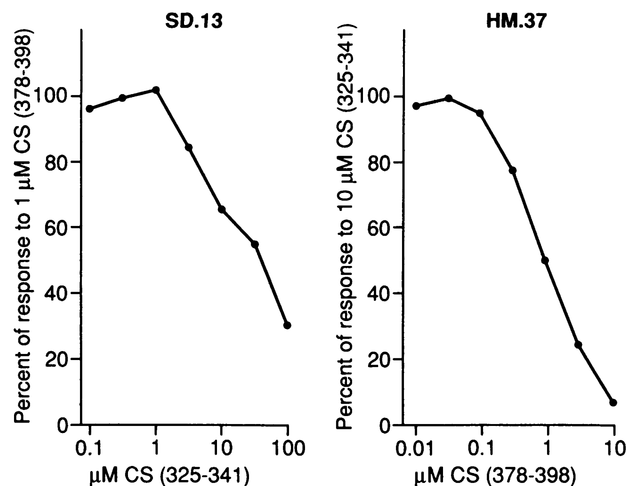

FIG. 5. Cross-competition between peptides binding the same MHC molecule. Responses of clone SD.13, to DR5 APCs pulsed with stimulator peptide CS-(378–398) (1 μM) and increasing doses of CS-(325–341), and clone HM.37 to APCs pulsed with stimulator peptide CS-(325–341) (10 μM) and increasing doses of CS-(378–398) as competitor peptide. Results of competition assays are shown as percentages of the proliferative responses to the stimulator peptide. Responses of clone SD.13 (mean of triplicates) in the presence or absence of peptide CS-(378–398) were 13,168 and 395, respectively; responses of clone HM.37 in the presence or absence of peptide CS-(325–341) were 36,810 and 412, respectively.

The assay is based on the fact that different peptides that are able to bind the same MHC protein compete with each other for binding (8, 19). The tools required for this assay are a panel of cell lines that can present defined peptides to a panel of T-cell clones with different restriction specificities. In the first step of the competition assay, fixed APCs are pulsed with suboptimal concentrations of stimulator peptide in the presence of various concentrations of peptides to be examined for binding. In the second step, the amount of stimulator peptide that is present on the surface of the APC is monitored in a proliferation assay with the appropriate T-cell test clones used as responder cells.

Using this competition assay, five different CS peptides, which compose ≈50% of the CS protein, were tested for their capacity to bind to DR5 and DRw6 proteins. While CS-(378–398) bound to both DR proteins, no binding was detectable with any of the other peptides.

One could argue that we have detected CS-(378–398) binding only because of its extraordinary strength and that we have missed the binding of other peptides because it could have been much weaker than that of the stimulator peptide. This, however, is clearly not the case. In the cross-competition experiment, the use of CS-(378–398) as a very strongly binding stimulator peptide did not prevent the detection of the much weaker binding of peptide CS-(325–341). The competition data can be used not only to identify the spectrum of MHC proteins that bind a peptide but also allow a semiquantitative estimate of the relative strength of the binding in each case. Strongly binding peptides are more suitable as T-cell epitopes in vaccines than weakly binding peptides, which are more sensitive to antigenic competition.

CS-(378–398) has been found previously, using different assay systems, to be recognized by T cells in association with several murine and human MHC class II proteins (22). Including the data presented here, at least seven DR proteins are now known to bind CS-(378–398). Although many peptides are known to bind to more than one allotypic form of a MHC class II protein, to our knowledge no peptide has been reported yet to bind to as many as seven HLA class II proteins. The structural basis for this broad binding capacity of CS-(378–398) is unclear. One possibility is that CS-(378–398) binding is largely if not exclusively dependent on the DR α chain, which shows very little polymorphism (23). If true, this would be analogous to the binding of some antigens to certain mAbs that involves the light-chain variable region only with little or no contribution of the heavy chain (24). Binding of peptides to MHC class II molecules is a condition necessary, but not sufficient, for the activation of T-cell clones (25). In some instances, peptide–MHC complexes do not activate T cells, presumably because of the absence of T cells carrying the appropriate T-cell receptor (25). However, "holes" in the T-cell repertoire seem to represent a relatively unfrequent event in T-cell recognition (26). Therefore, in most cases, as exemplified by the present results, peptides binding to MHC class II molecules should also be able to induce T-cell activation.

In the present study, we have been able to establish DR5- and DRw6-restricted T-cell clones specific for the peptide that was previously shown to compete with the binding of test peptides to DR5 and DRw6 proteins. These clones were established from PBMCs of donors who had not been in contact with *P. falciparum*. In other experiments, we have been able to establish T-cell clones specific for peptides from *P. falciparum* p190 (13) and from the CS protein (5, 6) by using the blood of unprimed donors or even cord blood lymphocytes (F.S. *et al.*, unpublished data). Recently, Carbone *et al.* (27) have been able to induce primary responses of class I MHC-restricted murine T cells to peptides. Most of the clones derived from such cultures were unable to respond to the native protein from which the peptides were derived. In contrast to these findings, most of the human class II MHC-restricted clones that we have derived from peptide-stimulated cultures of cells from unprimed donors were able to respond not only to the peptides but also to the native protein in the presence of appropriate APCs. The primary T-cell responses to the peptide we selected by using the competition assay therefore indicate the ability of the peptide to induce memory T cells that can be boosted by subsequent infections with the pathogen. We believe that the strategy used in the present study can be applied on a much larger scale to select T-cell epitopes to be incorporated into vaccines against any human pathogen. Although in this initial study we used only two sets of test cells in the competition assay, the results encourage us to establish a larger panel of test cells, which include enough HLA class II restriction specificities to cover the vast majority of the human population.

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