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Human T cells recognize polymorphic and non-polymorphic regions of the *Plasmodium falciparum* circumsporozoite protein

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In order to characterize T cell epitopes in the *Plasmodium falciparum* circumsporozoite (CS) protein sequence, we isolated T cell clones, from non-immune donors, which reacted with synthetic peptides corresponding to two predicted CS protein T cell epitopes. Peptide CS.T3 (corresponding to a non-polymorphic region of the CS protein, residues 378-398) was recognized in association with either DR2 or DRw9 restriction elements. T cell clones recognizing CS.T3 also reacted with the sporozoite-derived CS protein. Peptide CS.T2 corresponds to a polymorphic region (residues 325-341) of the CS protein. Unlike the CS.T3-specific clones, the CS.T2-specific clones did not recognize the CS protein. Since the CS.T2 peptide includes residues which are polymorphic in different *P.falciparum* isolates, we investigated whether these residues were critical for recognition of the peptide. We show here that a single amino acid substitution at a position of the CS protein which shows genetic polymorphism affects recognition of the sequence by human T cells. The implications of these data for malaria vaccine development are discussed.

Key words: malaria/vaccine/synthetic peptides/T lymphocyte clones

Introduction

The circumsporozoite (CS) proteins of malaria parasites all have the same basic structure, consisting of a central stretch of repetitive amino acid sequence flanked by two non-repetitive domains, each ~120 amino acids long (Nussenzweig and Nussenzweig, 1985; Miller *et al.*, 1986). In the case of *Plasmodium falciparum*, the central repetitive domain includes a tetrapeptide sequence (Asn-Ala-Asn-Pro or NANP) repeated ~40 times, plus three or four variants (NVDP) of the basic sequence (Dame *et al.*, 1984; Enea *et al.*, 1984). The repetitive region of all species of *Plasmodium* studied is immunodominant for antibody responses. However, the $(NANP)_n$ sequence of the *P.falciparum* CS protein does not represent a dominant epitope for either mouse or human T cells, as shown by the inability of most mouse strains to respond to $(NANP)_{40}$ or related compounds (Del Giudice *et al.*, 1986; Good *et al.*, 1986), and the generally poor anti-$(NANP)_n$ T cell responses of individuals living in malaria-endemic areas (Good *et al.*, 1988; Sinigaglia *et al.*, 1988), or volunteers immunized with vaccine candidates containing the repetitive sequence (Ballou *et al.*, 1987; Herrington *et al.*, 1987; Etlinger *et al.*, 1988).

Although immunity against sporozoite infection can be conferred by antibodies against repetitive sequences in an animal model (Zavala *et al.*, 1987), T lymphocytes play a very important role in anti-sporozoite immunity (Spitalny *et al.*, 1977; Egan *et al.*, 1987; Schofield *et al.*, 1987; Weiss *et al.*, 1988). Recently, two groups have shown that protective immunity to rodent malaria (*P.berghei* or *P.yoelii*) depends on the presence of $CD8^+$ T cells in sporozoite-immunized animals (Schofield *et al.*, 1987; Weiss *et al.*, 1988). These results stress the importance of identifying epitopes on sporozoites which are recognized by T cells. With this aim, we synthesized peptides corresponding to regions of non-repetitive sequence predicted to be T cell epitopes, and tested their ability to stimulate proliferation of peripheral blood mononuclear cells (PBMC) from donors living in a malaria-epidemic area, or from non-immune donors (Sinigaglia *et al.*, 1988). Lymphocytes from both malaria-immune and non-exposed donors proliferate in response to non-repetitive peptides corresponding to residues 103-122, 325-341 and 378-398 of the CS protein sequence described by Dame *et al.* (1984). Since one of these regions (326-343) was reported to be recognized by mouse T helper cells (Good *et al.*, 1987) and more recently also by human T cells (Good *et al.*, 1988; Sinigaglia *et al.*, 1988), but to be genetically polymorphic in different strains of *P.falciparum* (De la Cruz *et al.*, 1987; Del Portillo *et al.*, 1987), we have obtained human T cell clones responding to this sequence, and compared their properties with those of clones recognizing a non-polymorphic CS protein sequence.

Table I. Responses of T cell clones to synthetic peptides and CS protein[a]

Clone	Antigen			
	CS.T2	CS.T3	CS Protein	Medium
MG.D3	0.4 ± 0.0[b]	40.1 ± 0.8	31.4 ± 0.2	0.6 ± 0.0
MG.D14	0.3 ± 0.3	17.5 ± 0.0	4.0 ± 0.3	0.5 ± 0.1
HM.B23	24.2 ± 1.0	1.0 ± 0.0	0.8 ± 0.1	0.8 ± 0.0
HM.B25	34.3 ± 0.7	0.5 ± 0.1	1.0 ± 0.1	0.8 ± 0.1
HM.B27	10.3 ± 0.9	0.9 ± 0.2	0.7 ± 0.0	1.0 ± 0.0
HM.B37	43.5 ± 0.2	0.5 ± 0.0	0.7 ± 0.0	0.6 ± 0.0

[a]Cloned T cells (2×10^4) from donors M.G. or H.M. were stimulated in the presence of autologous irradiated PBMC (10^5), with medium alone, with peptides CS.T2 or CS.T3 (10 μg/ml), or with CS protein purified from 10^6 sporozoites by electrophoresis is a SDS-containing polyacrylamide gel as previously described (Sinigaglia *et al.*, 1988).

[b]Responses are given as mean c.p.m. $\times 10^{-3}$ ± SE [^{3}H]thymidine uptake of triplicate cultures after 48 h. Positive responses are underlined.

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Table II. HLA-DR restriction of CS-peptide-specific T cell clones[a]

APC[b]	DR	MG.D3 (c.p.m. × 10^{-3})	MG.D14 (c.p.m. × 10^{-3})	HM.B37 (c.p.m. × 10^{-3})
H.M.	5,7	0.5 ± 0.1[c]	0.8 ± 0.1	38.7 ± 0.5
M.G.	2,w9	13.8 ± 0.4	25.1 ± 1.2	0.2 ± 0.0
E.D.R.	1	0.8 ± 0.2	0.4 ± 0.3	0.5 ± 0.1
N.O.L.	2	29.6 ± 1.7	0.6 ± 0.1	0.6 ± 0.2
H.A.R.	3	0.4 ± 0.3	0.2 ± 0.0	0.2 ± 0.0
B.S.M.	4	0.6 ± 0.3	0.6 ± 0.2	0.2 ± 0.0
A.T.H.	5	0.5 ± 0.0	0.6 ± 0.0	57.0 ± 2.1
A.P.D.	w6	0.7 ± 0.1	0.2 ± 0.1	0.4 ± 0.0
E.K.R.	7	0.4 ± 0.1	0.3 ± 0.0	0.6 ± 0.1
L.U.Y.	w8	0.4 ± 0.1	0.6 ± 0.2	0.3 ± 0.3
D.K.B.	w9	0.5 ± 0.0	42.3 ± 1.6	0.3 ± 0.0

[a]Cloned T cells (2 × 10^4) from donors M.G. or H.M. were stimulated with peptides CS.T3 or CS.T2 (10 μg/ml) respectively, in the presence of irradiated EBV-B cells (10^4) from M.G., H.M. and a panel of HLA-DR homozygous donors.
[b]HLA-DR phenotypes of EBV-transformed antigen-presenting cells (APC) are shown.
[c]Results are expressed as mean c.p.m. ± SE [^{3}H]thymidine uptake (× 10^{-3}) of triplicate cultures. Positive responses are underlined.

Results and discussion

As previously reported, lymphocytes from immune and non-immune individuals proliferate in the presence of synthetic peptides CS.T2 and CS.T3, corresponding to regions 325–341 and 378–398 of the *P.falciparum* CS protein respectively (Sinigaglia *et al.*, 1988). PBMC from two non-immune individuals (H.M. and M.G.) responded well to CS.T2 and CS.T3 respectively (the relevant stimulation indices were 10 and 9). From the stimulated PBMC, T cell clones responding to CS.T2 and CS.T3 were isolated as previously described (Sinigaglia *et al.*, 1987, 1988). The antigen specificity of representative clones is shown in Table I. All the clones isolated expressed the $CD4^+/CD8^-$ phenotype.

From PBMC of donor M.G., 12 T cell clones recognizing peptide CS.T3 were obtained, each with a probability of clonality >90% as determined by Poisson analysis (Lefkovits and Waldman, 1979). Four out of six tested CS.T3-specific clones responded to the isolated, parasite-derived CS protein (Sinigaglia *et al.*, 1988, Table I). The response of all 12 clones was HLA-DR-restricted, since it depended on the DR type of the antigen-presenting cells used and (at least for all six clones tested) could be inhibited by anti-DR but not anti-DP or anti-DQ antibodies (Table II, unpublished data). Peptide CS.T3 was recognized, at concentrations < 1 μg/ml, in association with either DR2 (eight clones) or DRw9 (four clones).

Ten T cell clones responding to CS.T2 were isolated from PBMC of donor HM. The probability of clonality for each was >90%. The clones were antigen-specific and DR5-restricted (Tables I and II). Although donor HM was typed as HLA-DR 5,7 no clones recognizing CS.T2 in association with DR7 were obtained, suggesting that the DR7 allele may be associated with poor responsiveness to the peptide. Unlike the CS.T3-specific clones, the five CS.T2-specific clones tested did not respond to purified CS protein (Table I). Since the CS.T2 peptide includes residues 333 and 339 which are polymorphic in different *P.falciparum* isolates (De la Cruz *et al.*, 1987; Del Portillo *et al.*, 1987), we investigated whether these residues were critical for recognition of the peptide. A series of peptides with truncated CS.T2 sequences was synthesized. The shortest sequence inducing proliferation of clone HM.B23 comparable to that induced by peptide CS.T2 was included in residues 330–341 (Table III). Deletion of histidine 330 resulted in >50% decrease of T cell proliferation and deletion of residues 340–341 abrogated the T cell response. The responses to two truncated peptides (starting at residues 329 and 330) are higher than the response to the full-length peptide which was used to select the clone, perhaps because these shorter peptides can more easily assume a conformation recognized by the

Table III. Effect of N- and C-terminal truncations of peptide CS.T2 on stimulatory activity for T cell clone HM.B23[a]

CS peptide[b]	Sequence	Response[c] (%)
325–341	EPSDKHIEQYLKKIKNS	100
326–341	P----------------	73
329–341	K----------	130
330–341	H----------	120
331–341	I----------	51
332–341	E----------	37
333–341	Q----------	13
334–341	Y----------	1
325–339	----------K	1

[a]T cells (2 × 10^4) were incubated in 0.2 ml medium with autologous irradiated EBV-B cells (10^4) and peptide (10 μg/ml).
[b]Peptide CS.T2 corresponds to residues 325–341 of the CS protein sequence. Residue numbers and sequences of synthetic truncated peptides are given. Dashes indicate identity to CS.T2.
[c]Results are expressed as percentage of the response ([^{3}H]thymidine uptake) obtained in presence of the full-length CS.T2 peptide (mean c.p.m. ± SE of triplicate cultures = 55 422 ± 1210).

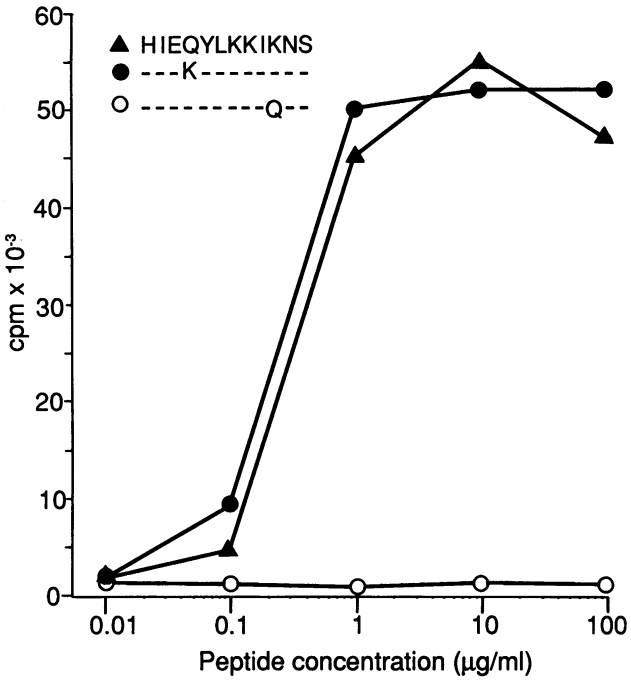

Fig. 1. Response of clone HM.B23 to CS peptide 330–341 (▲) and its analogues with single-amino-acid substitutions at residues 333 (●) and 339 (○). T cells (2 × 10^4) of clone HM.B23 were cultured with irradiated autologous EBV-B cells (10^4) and antigen at the concentrations indicated.

restriction molecule and/or by the T cell receptor. Removal of two carboxy-terminal residues completely abolishes activity, in spite of the fact that the shorter peptide in this case has a carboxy-terminal lysine residue, a property which is shared by many T cell epitopes (Berzofsky *et al.*, 1987). For the seven clones from donor H.M. tested, the minimum length peptide inducing a significant T cell proliferation was 332–341, which has a high amphipathic index (29.2), consistent with proposed properties of T cell epitopes (Berzofsky *et al.*, 1987).

Since the polymorphic residues 333 and 339 were included in the minimum stimulatory sequence, we next synthesized and tested variant peptides in which Gln-333 and Lys-339 were replaced by the naturally occurring variants, Lys and Gln respectively. Figure 1 shows that, for clone HM.B23, the substitution at position 333 has no effect on T cell proliferation, whereas the Gln–Lys replacement at position 339 completely abolishes recognition of the peptide. Similar results were obtained with the four other CS.T2-specific clones tested. These results show that a single amino acid substitution, at a position in the CS protein sequence which shows genetic polymorphism, affects recognition of the sequence by human T cells. Preliminary experiments (J.Kilgus and F.Sinigaglia, unpublished data) suggest that the substitution at position 339 affects binding of the CS.T2 peptide to HLA-DR molecules, since a 200-fold excess of peptide 330–341, Gln-339 had no effect on the presentation of peptide 330–341, Lys-339 to cloned T cells by glutaraldehyde-fixed, antigen-presenting cells.

The CS.T2 sequence is present in the *P.falciparum* isolate IMTM22/7G8 (Dame *et al.*, 1984). However, the recently sequenced NF54 CS protein (P.Caspers *et al.*, in preparation) contains Gln at position 339. These results offer a likely explanation for the lack of recognition of the CS protein of isolate NF54 by T cell clones specific for CS.T2.

It is possible, as suggested by De la Cruz *et al.* (1987) and Good *et al.* (1988), that polymorphism may be advantageous to the parasite by enabling it to escape the T cell-mediated immunity stimulated by genetically different isolates. The recent results of Good *et al.* (1988) provide evidence that immunodominant T cell epitopes occur preferentially in polymorphic regions of the CS protein. However, the CS.T3 peptide sequence, for which there is no evidence of polymorphism (De la Cruz *et al.*, 1987; Del Portillo *et al.*, 1987; P.Caspers *et al.*, in preparation), is also recognized by human T cells in association with at least two different DR alleles. If this or other non-polymorphic T cell epitopes are recognized in association with a wide range of HLA haplotypes, the inclusion of these sequences could improve current malaria vaccine candidates.

Materials and methods

Donors

Blood (20–40 ml) was obtained from two Europeans (laboratory staff) with no history of malaria infection. Both donors had previously travelled in a malaria-endemic region, but had no detectable serum antibodies to either sporozoites or malaria blood stage antigens.

Peptide synthesis

Peptides were synthesized by the solid-phase technique and purified by reverse-phase HPLC as described (Sinigaglia *et al.*, 1988). The amino acid compositions determined for each peptide corresponded to the expected values.

Peptides CS.T2 and CS.T3 correspond to residues 325–341 and 378–398 of the CS protein sequence, except that in CS.T3 the CS protein cysteine residues 384 and 389 are replaced by alanine residues (Sinigaglia *et al.*, 1988).

Lymphocyte proliferation assays and isolation of T cell clones

These were carried out as previously described (Sinigaglia *et al.*, 1987, 1988).

Purification of CS protein

Purification of CS protein from NF-54 isolate sporozoites was carried out as described (Sinigaglia *et al.*, 1988).

HLA-DR-homozygous cell lines

These were obtained from E.Goulmy and J.van Rood (Department of Immunohaematology, University Hospital, Leiden, The Netherlands) and maintained in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 5×10^{-5} M β-mercaptoethanol, 1% non-essential amino acids (100% stock solution; Gibco) 50 U/ml streptomycin and 10% fetal calf cerum. The lines are Epstein–Barr virus-transformed B (EBV-B) cell lines, which were irradiated (5000 rad) before being used as antigen-presenting cells. EBV-B cell lines from donors M.G. and H.G. were prepared as described (Sinigaglia *et al.*, 1987) and maintained as above.

Monoclonal antibodies

The specificities and the sources of the monoclonal antibodies used in the T-cell inhibition assays are as follows: MoAb E.31, monomorphic anti-DR (Trucco *et al.*, 1979); MoAb Tü 22, monomorphic anti-DQ (Ziegler and Milstein, 1979) obtained from A.Ziegler; MoAb B7/21 (23), monomorphic anti-DP obtained from Becton Dickinson, Sunnyvale, CA. The antibodies were added to cultures as a 1/100 dilution of ascites fluid.

Cell surface analysis

T cell surface phenotypes were analysed by direct immunofluorescence on a flow cytometer (FACS Analyser 1, Becton Dickinson) using the 'Simultest T Helper/Suppressor-Kit' purchased from Becton Dickinson. HLA-DR typing of donors M.G. and H.M. was done by Dr S.R.de Cordoba (Department of Immunogenetics, New York Blood Center).

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