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Improved Detection of Human Antibodies to a *Plasmodium* Antigen Using a Peptide Modified with Aib Residues

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Abstract: A 17-mer sequence was selected as a model to study the influence of modifications of terminal ends both on the conformation of a peptide and on its antigenicity towards naturally developing antibodies. This sequence corresponded to a tandemly repeated motif, found in a long repetitive region, with high helical propensity, of a *Plasmodium falciparum* liver-stage antigen (LSA-1), immunogenic in man. Our model peptide was synthesized with ionizable or non-ionizable ends, or modified in both extremities by introduction of the helix-promoting residue α-aminoisobutyric acid (Aib). Helical contribution, absent in the 17 amino-acid sequence possessing ionizable ends, was detectable when non-ionizable ends were introduced, and dramatically increased in the Aib-modified analogue. The presence of ionizable ends totally abolished reactivity towards human sera, otherwise detectable with the peptide possessing non-ionizable ends. While modification by Aib residues was neither detrimental nor beneficial to antigenicity in solution, it clearly resulted in an improved sensitivity of the specific antibody detection when used as solid-phase antigen in ELISA.

Keywords: peptide; conformation; antigenicity; α,α-disubstituted residues; ELISA

INTRODUCTION

Synthetic peptides as solid-phase antigens in a new generation of serological 'site-directed' ELISAs offer the advantage of being easier to standardize than purified proteins, and are thus more reliable and more reproducible. These peptides [1, 2] usually selected after a detailed epitope mapping of the parent proteins, are presumably able to mimic only 'continuous' epitopes [3] in which the residues responsible for the antibody contact are displayed sequentially in the primary structure. Such epitopes are usually associated with protruding regions, such as turns, or relatively flexible loops.

However, some peptides are liable to present conformational elements which may contribute to their cross-reactivity with anti-protein antibodies. Recognition by antibodies elicited during a natural infection of synthetic peptides possessing a preferential secondary structure has been documented by several examples: peptides deriving from Epstein–Barr nuclear antigen [4], α-helical peptides derived from fibrillar proteins of *Streptococcus pyogenes* [5] or from parasite antigens such as *Echinococcus granulosus* [6] or *Plasmodium falciparum* [7].

The repetitive nature of the LSA-1 antigen offered an opportunity to evaluate the role of conformation on the antigenicity of a peptide towards human antisera. To this end, we have selected one of the possible definitions of the repeat unit (KEKLQEQQS-

Abbreviations: Aib, α-aminoisobutyric acid; 2-ClZ, chlorobenzyl; BOC, tertio butyloxycarbonyl; DIEA, diisopropylethylamine; FAB, fast atom bombardment; NMP, *N*-methyl pyrolidinone; OPD, ortho phenyldiamine; PBS, phosphate buffer saline; TFE, trifluoroethanol.

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DLERLA: sequence 13–29). This peptide was likely to contain some epitopes expressed by the native antigen in which it is found tandemly repeated, but in a context relatively unfavourable to helical conformation: the positively charged side chain of the N-terminal lysine residue was likely to destabilize this conformation by introducing a repulsive interaction with the positive end of the helix dipole [8, 9].

In this paper, we describe the consequences of terminal-end modifications of the sequence 13–29, by ionizable or non-ionizable groups, or by the introduction of the helix-promoting residue α-aminoisobutyric acid (Aib) [10, 11] on its conformation and on its antigenicity towards naturally developing antibodies in solution, as inhibiting antigens, or in ELISA, as solid-phase antigens.

MATERIALS AND METHODS

Peptide Synthesis

All protected amino acids were purchased from Propeptide (Vert-Le-Petit, France), except Boc-α-aminoisobutyric acid (Boc-Aib, Novabiochem, Meudon, France). *p*-Methylbenzhydrylamine resin (0.77 mmole/g) and *t*-Boc-Ala-OCH2-PAM resin were from Applied Biosystems (Foster City, CA).

All peptides except 13–29{Aib} were prepared in an Applied Biosystems model 430A peptide synthesizer by stepwise solid-phase synthesis according to the Boc-TFA scheme [12], on a benzhydrylamine resin (peptides Ac-1–17-NH_2, Ac-13–29-NH_2 and LSA-41), or on Boc-Ala-OCH2-PAM resin (peptide H-13–29-OH).

Trifunctional amino acids were protected as follows: Arg(Tos), Asp(OBzl), Glu(OBzl), Lys(2-ClZ), Ser(OBzl). Amino acids were introduced using symmetrical anhydride activation in DMF (single coupling), except for Gln, which was introduced using the DCC/HOBt activation protocol. At the end of the synthesis of the acetylated peptides, the Boc group was removed, and the N-terminal function was acetylated with acetic anhydride.

The peptide 13–29{Aib} was manually synthesized: the two first Aib residues, followed by the Ala found in the C-terminal position of the 13–29 sequence were introduced on the benzydrylamine resin (0.77 mmole/g:1·3 g:1 mmole) using 2.5 mmole of Boc-protected amino acid and the BOP/HOBt/DIEA activation [13] in 20 ml NMP for 60 min; then DMSO (2.5 ml) was introduced and the reaction proceeded for 40 min. Introduction of the first Boc-Aib residue was monitored by ninhydrine, and appeared complete after five coupling steps. After capping with acetic anhydride, and introduction of Boc-Ala (two coupling steps followed by a capping step), the Boc group was removed and the free amino groups were quantified using picric acid (Gisin method) [14]: 0.15 mmole (15% of the initial charge) was present. The next amino acids were introduced in the automated peptide synthesizer as above, except for the N-terminal Aib-Ala-Aib, which was synthesized manually using the same BOP/HOBt/DIEA activation scheme (two coupling steps per residue).

Final deprotection and cleavage of the peptidyl resins were by the high HF procedure, for 1 h at 0 °C. The cleaved deprotected peptides were precipitated with cold diethylether, collected by low-speed centrifugation, washed with cold diethylether and then dissolved in 5% acetic acid and lyophilized. The cleavage yields were 70–75% except for the peptide 13–29{Aib}: a yield of 35% was obtained after this step; an additional 2 h of cleavage was performed for this peptidyl-resin, with a yield of approximately 11%.

The shortest peptides (Ac-18–29-NH_2 and Ac-23–34-NH_2) were purified by RP-HPLC on a 8.2 × 500 mm column (Nucleosil 100 Å, 5 μm C18, Macherey Nagel), eluted with an acetonitrile/water/0.05% TFA gradient solvent system; purification yields were 50–55%. Other peptides were purified by gel filtration (TSK HW40S, Merck), followed by ionic exchange chromatography on a sulphoethyl aspartimide SCX column (Nest group) using a gradient buffer system 0.5 M CaCl2/ acetonitrile 12.5%–phosphate 20 mM pH 2.3. Collected fractions were controlled on a 4.6 × 250 mm RP-HPLC column (Nucleosil 100 Å, 5 μm C18, Macherey Nagel). Pure fractions were loaded on the RP-HPLC column in HCl pH2, and eluted as hydrochloride (> 98% pure), using a step gradient to 60% acetonitrile in HCl pH2. Final purification yields were 5–8%.

Peptides were checked for identity by amino acid analysis. Peptide Ac-18–29-NH_2: Asp 0.99 (1); Ser 0.66 (1); Glx 5.93 (6); Ala 0.97 (1); Leu 1.89 (2); Arg 0.94 (1). Peptide H-23–34-NH_2: Glx 5.95 (6); Ala 0.97 (1); Leu 2.93 (3); Arg 0.94 (1); Lys 1.97 (2). Peptide H-13–29-OH: Asp 0.99 (1); Ser 0.57 (1); Glx 8.16 (8); Ala 0.97 (1); Leu 2.99 (3); Lys 1.97 (2); Arg 0.94 (1). Peptide Ac-13–29-NH_2: Asp 1.01 (1); Ser 0.69 (1); Glx 7.8 (8); Ala 1.07 (1); Leu 3.18 (3); Lys 1.95 (2); Arg 0.94 (1). Peptide 13–29{Aib}: Asp 1.04 (1); Ser 0; 72 (1); Glx 8.15 (8); Ala 1.90 (2); Leu 2.94 (3); Lys 2.01 (2); Arg 0.95 (1); Aib n.d. (4). Peptide LSA 41: Asp 2.02 (2); ser 1.89 (2); Ser 1.89 (2); Glx 17.09

(17); Gly 1.06 (1); Ala 2.98 (3); Leu 7.66 (8); Lys 5.61 (6); Arg 1.59 (2).

Molecular Mass Determination of Peptide 13–29{Aib}

The fast atom bombardment mass spectra were obtained with a Kratos concept II HH mass spectrometer fitted with a saddle-field FAB gun. Xenon was used as a FAB gas. The gun was operated at 8 kV, at a 1 mA discharge current. The ion-accelerating voltage was 8 kV. The sample was loaded onto the probe by dissolution in a few μl of glycerol acidified by 0.1% trifluoroacetic acid. The observed $[M+H]^+$ species was 2483 (calculated 2483.8).

Circular Dichroism Studies

CD spectra were recorded on a Roussel Jouan 185 Model II at room temperature. Peptide concentrations were adjusted from titration of stock solutions by quantitative amino acid analysis after total acid hydrolysis. CD studies were performed on peptide hydrochlorides, 15 mM in 100 mM NaCl in cell path of 0.1 mm or 3 mM in 90% TFE in cell path of 1 mm. Circular dichroism results were reported in terms of mean residue ellipticity $[\theta]$ expressed in deg/dmol/cm. Helix contents were calculated from the CD spectra, taking $[\theta]_{222} = -35{,}700$ deg . cm^2/dmol for 100% helicity [15], and taking into account the number of peptide amide bonds (22 for peptide 13–29{Aib}, 16 for peptide 13–29, 18 for peptide Ac-13–29-NH_2).

Human Sera

Selected human sera were obtained from individuals living in a malaria endemic area with high antibody titres to liver-stage specific antigens ($>1/2000$ by immunofluorescence assay). Sera were diluted in PBS-Tween 0.05%-non-fat milk (Regilait) 1.25% at the level of 50% of the OD maximum obtained when tested in direct ELISA against peptide LSA-41.

ELISA Studies

For inhibition tests, microtitre plates (Nunc, Maxisorp, Rocksilde, Denmark) were incubated overnight with 100 μl per well of a 10 μg/ml solution of peptide LSA-41 in 0.1 M PBS, pH 7.2. To block nonspecific antibody binding, the wells were saturated with a low-fat powdered milk solution (Regilait 2.5% W/V in PBS pH 7.2). The inhibiting antigens were incubated at the concentrations indicated with the diluted sera. After 1 h at 20°C and 1 h at 4°C, the sera were put in triplicate wells (100 μl per well): after incubation for 80 min at 20°C followed by washings, the presence of antibodies was revealed by adding a 1/1000 dilution of peroxydase-conjugated goat anti-human IgG (Biosys). The plates were incubated again for 50 min, washed, and substrate solution (citrate buffer/H_2O_2/OPD) was added. The enzymatic reaction was allowed to proceed for 30 min. Readings were performed at 450 nm in a Titertech multiscan ELISA reader (Flow Laboratories).

For direct assays, the coating procedure was similar: each test peptide was coated at 10 μg/ml, and sera were tested (in the absence of competing peptide) at 1/100 dilution. Results were expressed as a ratio of the OD read out (mean of three wells) compared with the mean OD from normal healthy blood donor sera augmented by twice the standard deviation value.

RESULTS AND DISCUSSION

We studied a peptide model corresponding to a repeated motif of a liver-stage antigen of *Plasmodium falciparum* (LSA-1). In the native antigen, the repeating fragment of the molecule represents more than 77% of the whole molecule. This antigen is highly immunogenic under natural conditions of exposure: a 65-amino acid fragment was initially identified after gene-cloning and recognition by human sera from individuals living in an endemic area [16]. A 41-mer synthetic peptide (LAKEKLQE QQSDLEQERLAKEKLQEQQSDLEQERLAKEKLQ = LSA-41) was found to mimic the antigenicity of the recombinant fragment, and is used as the reference antigen in this paper.

Computer analysis of the sequence of LSA-1 predicts that the LSA repeats may assume an α-helical conformation presumably associated with a fibrillar structure in the native antigen. Partial helical conformation was observed in LSA-41 (Figure 1).

In order to evaluate the contribution of the helical configuration on the antigenicity, we selected the 13–29 sequence since this 17 amino-acid sequence corresponds to a possible definition of the repeated unit. Two partially overlapping dodecapeptides were also selected, corresponding to the sequences 18–29 and 23–34. These three peptides contained all the

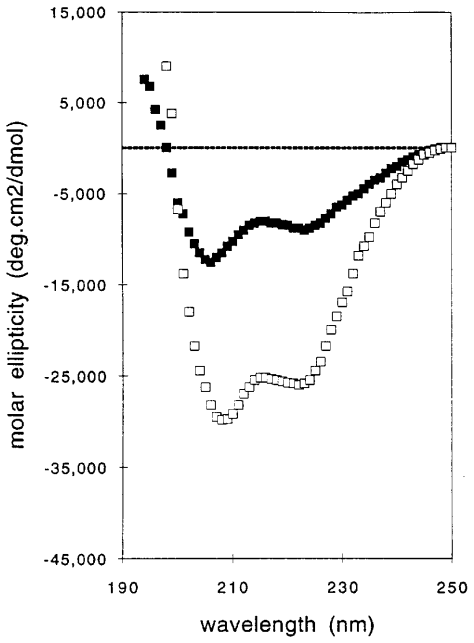

Figure 1 CD spectra of peptide LSA-41 (hydrochloride) at 25 °C. ■ 15 mM in aqueous solution; □ 3 M in 90% TFE. These spectra are characteristic of helical formation, with minima occurring at 206 and 222 nm. Helix content was estimated from the mean residue ellipticity [θ] at 222 nm, and was 25% in water medium, and 73% in TFE. These results are in agreement with the hypothesis of an helical organization in the native antigen.

sequential determinants expressed by two consecutive repeats (sequence 1–34; Table 1).

We selected as a definition of the repeat sequence a peptide with an unfavourable display of the charged side-chains with regard to the helix dipole [8, 9], in order to obtain a sequence in which the helix conformation would be possible, but strongly dependent on flanking modifications. We then designed three chemically defined analogues of this peptide, in which modulation of the helical conformation was expected from modifications of peptide extremities, without altering the internal part of the molecule (Table 2). This sequence was first synthesized with acetyl N-terminal and carboxamide C-terminal non-ionizable ends (peptide Ac-13–29-NH_2). Helix-destabilizing features were introduced with ionizable α-amino N-terminal and carboxylic C-terminal ends (peptide H-13–29-OH). In a third homologue, helix-promoting α-aminoisobutyryl residues (Aib) were introduced at both ends: two consecutive Aib in the C-terminal and an Aib-Ala-Aib sequence in the N-terminal position (peptide 13–29{Aib}). Our idea was to stabilize the α-helical conformation by introducing a turn of α-helix or 3_{10} helix at both ends of the peptide [10].

The CD spectra of the peptide Ac-13–29-NH_2 (Figure 2(a)) showed a partially ordered structure in water. A weak helical contribution (8%) was detected in 90% TFE. In peptide H-13–29-OH (Figure

Table 1 Amino Acid Sequence of the Different Peptides Used in this Study

Numeration	Sequence
[1–34][a]	EQQSDLEQERLAKEKLQEQQSDLEQERLAKEKLQ
[18–29]=[1–12][b]	EQQSDLEQERLA EQQSDLEQERLAKEKLQ
[23–34]=[6–17][b]	LEQERLAKEKLQ LEQERLAKEKLQ
[13–29]	KEKLQEQQSDLEQERLA

[a]Two consecutive repeat units of the liver-stage antigen LSA-1 of *Plasmodium falciparum*.
[b]Owing to the repeating nature of this antigen, two numerations are possible.

Table 2 Sequence of the Three 13–29 Peptide Analogues

Peptide		Sequence
H-13–29-OH	H-	KEKLQEQQSDLEQERLA-OH
Ac-13–29-NH_2	CH_3CO-	KEKLQEQQSDLEQERLA-NH_2
13-29{Aib}	H-UAU	KEKLQEQQSDLEQERLA UU-NH_2

U corresponds to the conventional one-letter code used for the α-amino isobutyric acid (Aib).

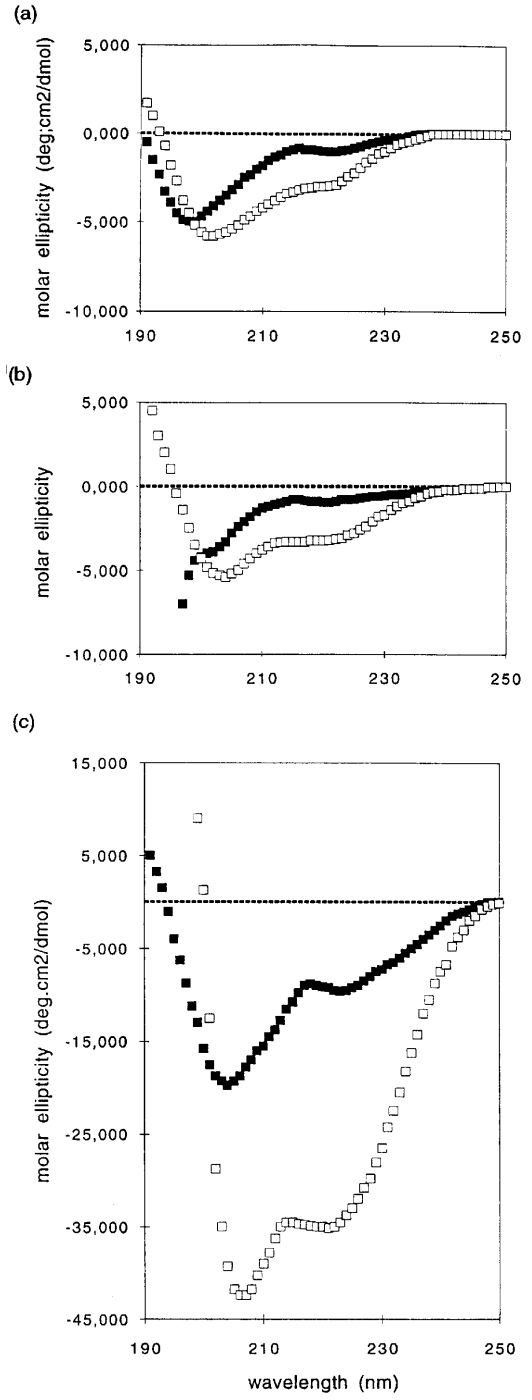

Figure 2 CD spectra of the 13–29 peptide analogues (hydrochlorides) at 25 °C. ■ 15 mM in aqueous solution; □ 3 mM in 90% TFE. (a) Peptide Ac-13–29-NH_2; (b) peptide H-13–29-OH; (c) peptide 13–29{Aib}.

2(b)) the side chain of the first lysine residue, together with the charged N- and C-terminals were likely to destabilize an α-helical conformation. As expected, this peptide was devoid of any detectable conformation in water but the helix-promoting solvent TFE was able to induce a helical contribution similar to the one observed with the Ac-13–29-NH_2 analogue.

Introduction of conformationally restricted α-aminoisobutyric acid in the third analogue (peptide 13–29{Aib}) resulted in a dramatic increase in helical content (Figure 2(c)): 37% helix was observed in aqueous solution and in 90% TFE the helix formation was complete (98%).

In order to evaluate the relations between the different levels of ordered structure and antigenicity of the peptides, we first tested their binding towards antibodies found in human sera in ELISA competition assays. A panel of seven sera were incubated in solution with the varius 13–29 peptide analogues, and then added to a microtitre plate coated with the peptide LSA-41 (Figure 3)

Under these conditions the repeat unit defined by the H-13–29-OH analogue was devoid of any detectable inhibiting capacities towards all the sera, even at the highest concentration tested (100 μM). Obviously, the ionizable ends hampered the antibody-binding to the recognition sites present in the sequence, either by a direct mechanism (the terminal charges inserted in positions where an amide bond was found in the native structure introduced repulsive interactions towards the antibodies), or by an indirect mechanism (the terminal charges destabilize the helical configuration through repulsive interactions with the helix macrodipole).

Detectable inhibiting capacities, with IC50 in the 10 μmole range in four of the seven sera, were observed with the two other 13–29 analogues. No significant differences were observed between the inhibiting capacities of the peptide Ac-13–29-NH_2 and 13–29{Aib}, or even the shorter peptides Ac-18–29-NH_2 and Ac-23–34-NH_2. The increased order due to the introduction of the α,α-disubstituted amino acids, especially in TFE, did not correlate with an improved reactivity in solution.

Nevertheless, in the context of a serodiagnostic test the synthetic peptide is used as a solid-phase antigen. In order to appreciate the consequences of the modifications of the 13–29 sequence found in its different analogues, we tested the capacities of these peptides to detect antibodies to the LSA-1 in the same human sera (Figure 4).

No reactivity was observed using the H-13–29-OH as solid-phase antigen (not shown). A positive signal was obtained with three sera (SHI 5, 1 and 7) when using the Ac-13–29-NH_2 analogue. A much more efficient detection of the LSA-reacting antibodies

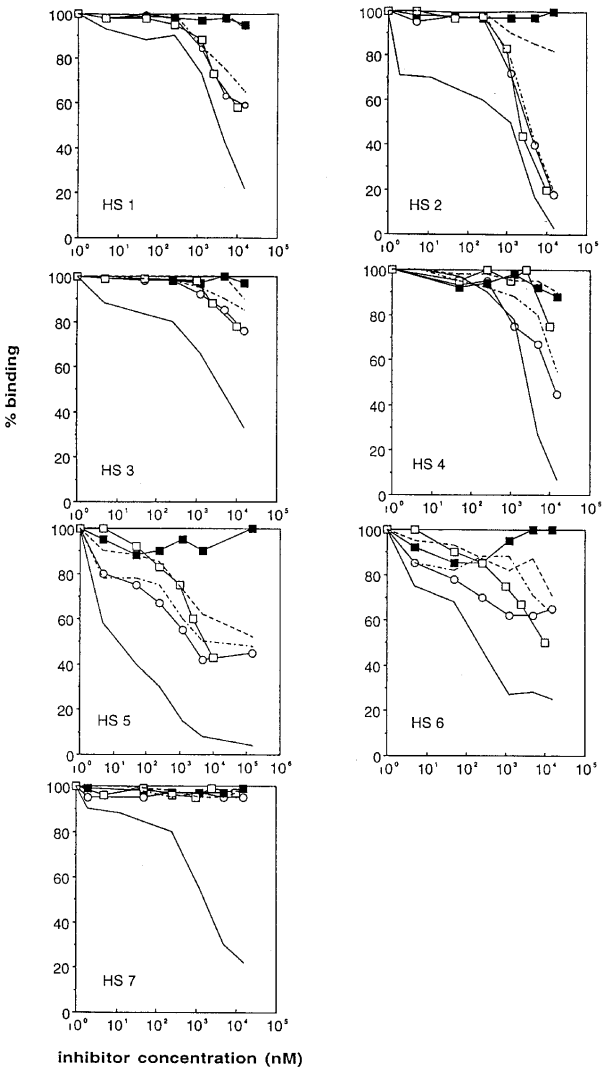

Figure 3 ELISA inhibition experiments. A panel of seven human sera (HS 1–7) were selected for their ability to recognize the LSA-1 antigen. Serum dilutions were 1/200 (HS 1–4), 1/100 (HS 6 and 7), 1/400 (HS 5). Inhibiting antigens were incubated at the indicated concentrations with the diluted sera, before incubation with the solid-phase antigen (peptide LSA-41). Inhibiting antigens were: O peptide Ac-13–29-NH_2; ■ peptide H-13–29-OH; □ peptide 13–29{Aib}; — LSA 41; - - - - Ac-18–29-NH2; –·–·– Ac-23–34-NH_2

was observed with the 13–29{Aib} analogue. In this case six out of seven sera gave positive signals with ELISA ratios between 1.25 to 12.2.

The increased hydrophobicity of the 13–29{Aib} analogue probably leads to a better coating capacity of the peptide antigen, which could account for a better signal. However, while the coating capacities of the different peptide solutions can be considered

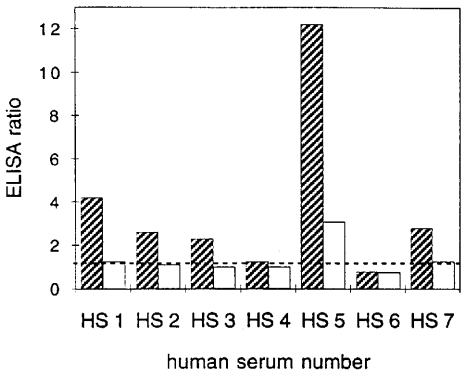

Figure 4 Direct ELISA. Each test peptide was coated at 10 μg/ml, and sera were tested (in the absence of competing peptide) at 1/100 dilution. Results were expressed as the ratio of the OD read-out, compared with the mean OD from 10 normal, healthy, blood donor sera + two standard deviations. Solid phase antigens were: white bars, peptide Ac-13–29-NH_2; grey bars, peptide 13–29{Aib}. SH 1–7: human serum number. Signals were considered positive when the ratio was above 1.2 (dotted line).

as constant, the ratio of the signal observed after recognition of the peptide Ac-13–29-NH_2 to the signal observed after recognition of the peptide 13–29{Aib} was different according to each individual serum: this ratio varied from 1.25 (HS 4) to 3.94 (HS 5). In addition to hydrophobicity, another factor thus contributed to the better reactivity of the peptide 13–29{Aib}. As no difference was observed when reacting in solution, a direct recognition of the exotic Aib residues replacing the residues found in the natural sequence can be ruled out. It is highly probable that a conformational component was involved, varying from one serum to another. The increased helical content observed in TFE with peptide 13–29{Aib} correlates with a better antigenicity of the plastic-coated peptide suggesting that the peptide adopts, in this context, a conformation that mimics the organization found in the native antigen.

CONCLUSIONS

Our results demonstrate that introduction of Aib residues at both ends of a 17-mer peptide with helical propensity, without alteration of the internal part of the sequence, stabilizes the helical conformation. Although this was not sufficient to confer a better antibody-binding activity in water, with reference to a peptide possessing non-ionizable acetyl and carboxamide terminal ends, a clear advantage

linked to this modification was observed when the peptide was used as a solid-phase antigen in a direct immunoassay. The signals observed were more intense, allowing a more efficient detection of specific antibodies to a liver-stage antigen of *Plasmodium falciparum*, found in the sera of malaria-infected individuals.

The improved sensitivity of the direct ELISA test was most probably related to a better detection of antibodies to 'conformational' epitopes of this fibrillar antigen. The helix-promoting activities of the α,α-disubstituted amino-acids, together with the interaction with the plastic surface, stabilized in the peptide a conformation which efficiently mimics the configuration found in the native antigen.

Thus, applications of site-directed serology are not limited to continuous determinants. This approach could also be used for the detection of conformational antibodies to helical parts of proteins, at the cost of a minimal modification of the synthetic antigens.

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