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Immune response in mouse and malaria-exposed humans to peptides derived from Pf11-1, a highly repetitive megadalton protein of *Plasmodium falciparum**

We have investigated the immune response against the *Plasmodium falciparum* gametocyte-specific antigen Pf11-1. This megadalton parasite molecule has been implicated in the process of erythrocyte rupture during gametogenesis. The molecule is composed in great part of degenerated nonapeptide motifs which are tandemly repeated several hundred times. A computer algorithm searching for T sites predicted that the entire repeat region of the Pf11-1 represents potential T cell antigenic major histocompatibility complex class II-binding sites. To test this hypothesis, synthetic peptides corresponding to two nonamer subtype repeats, differing only at two amino acid positions, were used to immunize congenic mouse strains. Both peptides were shown to contain both B and T cell epitopes. The immune response is restricted to the H-2^d and H-2^k haplotypes. The T cell response against the peptides appeared to be highly specific, clearly discriminating between the two similar nonamer repeat sequences, whereas the humoral response produced cross-reacting antibodies. We also investigated the humoral and T cell reactivities of *P. falciparum*-primed individuals in West Africa against the synthetic Pf11-1 peptides. Among 51 individuals 35 had antibodies to at least one of the two peptides and a majority of them (28) had antibodies reacting with both peptides. The cellular response was analyzed by [^{3}H] thymidine incorporation or interferon-γ release. There was considerable variation in the response to the two peptides. Among the human samples 36% responded to one repeat subtype, while only 13% responded to the second subtype. Interestingly, in individual donors the T cell response to both peptides are associated, suggesting that, as shown for mice, the response is restricted by a genetic element. The data obtained on the two subtypes of the nonamer repeat region suggest that the entire Pf11-1 molecule might induce an unusually heterogenous B and T cell response during natural infection in man.

1 Introduction

The search for the development of a vaccine against the human malaria parasite *Plasmodium falciparum* has led to the isolation and characterization of a large number of plasmodial genes that encode molecules recognized by the immune system during natural infections in man (for review see [1]). Surprisingly, most of the isolated *P. falciparum* genes contain tandemly repeated motifs, which in general represent immunodominant protein regions. The repeats vary in number and length and can be very distinct among different proteins. On the other hand, several gene families coding for immunologically related proteins of *P. falciparum*, such as the glutamic acid (Glu)-rich gene family [2–4], have been described (for review see [5]).

It has been proposed that certain repeated regions function as ligands for parasite-host interaction [6]. Two alternative theories suggest that repeats are directly implicated in the immunomodulation of the immune response. Anders [7] proposed that repeats serve to suppress the development of high-affinity "protective" antibodies through a strategy of serological cross-reaction of related but degenerated repeat epitopes. Schofield and Uadia, on the other hand, suggested that under conditions of natural exposure, malaria repetitive antigens induce a T cell-independent B cell response, an event that has presently been shown only for the circumsporozoïte protein (CSP) [8].

We have been studying a peculiar malaria antigen, termed Pf11-1, expressed during the bloodstage of *P. falciparum*, [9]. Recently, Pf11-1 has been identified as a giant (approximately 10^6 Da) gametocyte-specific protein that has been implicated in a critical part of the parasite evasion-process of the infected red blood cell, namely, the rupture of the erythrocyte [10]. In addition, a monoclonal antibody directed against the Pf11-1 molecule has been shown to inhibit the transmission of *P. falciparum* to the mosquito vector [11]. Analysis of the 30 kb Pf11-1 locus showed that the major part of the molecule consists of approximately 700 nine-amino acid repeats. Although the length of the repeated unit is very conserved, many variations occur in certain amino acid positions resulting in an unprecedented large number of related repeat sequences. The degeneracy

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of the nonamer repeat contrasts with the moderate or high conversation of the repeats reported for almost any other plasmodial antigen. The repeat consensus sequence is (P-E-E-X-X-E-E-X-X), where X is generally a hydrophobic amino acid such as valine, leucine or isoleucine [12].

In this report we analyzed the immune response to the highly repetitive megadalton protein Pf11-1 in different H-2 congenic mouse strains and in *P. falciparum*-primed individuals living in West Africa. The specificity of the B cell response to two distinct nonamer repeat motifs, called P9A and P9B, was analyzed. Prediction of T cell antigenic sites of the Pf11-1 repeat region using various computer algorithms [13] suggested a high propensity for T cell epitopes for the entire nonamer repeat region. We demonstrate that the repeat motifs P9A and P9B contain T and B cell epitopes in Pf11-1 immunized mice and human individuals naturally exposed to *P. falciparum*. The T cell response to both types of repeats is limited to the H-2^d and H-2^k haplotype in mice and the peptides induce a proliferative T cell response in 36% of the humans analyzed. We discuss the possible implication of the Pf11-1 molecule in the immunomodulation of the host immune response.

2 Materials and methods

2.1 Study area and population

The population of the village of Dielmo (Senegal, West-Africa) has been followed since June 1990 in a prospective longitudinal study. This study concerns epidemiological and immunological criteria of *P. falciparum* malaria as already described [14]. Located in South Senegal, near the frontier of The Gambia, Dielmo was selected on the basis of a high level of malaria prevalence and little uncontrolled use of antimalarial drugs. Malaria transmission is perennial with moderate seasonal variations. The 250 inhabitants agreed to participate in the study, which follows all of the rules edicted by the "Perfectionnement Council" joining the Pasteur Institute of Dakar and the Senegal authorities.

2.2 Collection of blood samples in the field

In May-June, (just before the rainy season, *i.e.* the period of high transmission) veinous samples were taken from every individual, except children less than 2 years old, for PBMC preparation, plasma collection and malaria parasite analysis. Fifty-three individuals, without any symptoms of malaria attack or other infectious diseases, were selected for this study, resulting in 23 donors representative of the age group 2 to 20 years old, and 30 adults over 20 years of age. Veinous blood (20 ml) was collected between 8 a.m. and 10 a.m. (to avoid nycthemeral variations) in two siliconized, dry evacuated tubes containing 0.1 ml of heparin (2500 UI/ml, Liquemine Roche). The tubes were transported to the Pasteur Institute in Dakar by car within 5 h in isotherm boxes to maintain the temperature between 20 °C to 30 °C. Blood (5 ml) was also collected in EDTA-containing tubes for hematological studies. The plasma and PBMC were prepared from heparinized tubes as previously described [14].

2.3 Detection of Pf11-1 peptide-specific T cells in humans

2.3.1 Mitogens and control antigens

Leukoagglutinin (LeuA, Sigma Chemicals, St. Louis, MO) and PPD (Statens Serum-Institute, Copenhagen, Denmark) were used at a final concentration of 10 μg/ml to check the quality of the culture. Culture conditions: PBMC were incubated with the different stimuli at 2×10^5 cells per well in round-bottom 96-well plates (Costar), in 0.2 ml of complete culture medium: RPMI 1640 supplemented with 3.7 g/l sodium bicarbonate, 2 mM glutamine, 1% sodium pyruvate, 1% nonessential amino acids, 10 mM Hepes, 100 U/ml penicillin and streptomycin (Flow Laboratories), 10% heat-inactivated human serum AB (Jacques Boy-France). The different stimuli were added at appropriate concentrations to quadruplicate wells, and the plates incubated for 6 days at 37 °C in humidified 5% CO_2-containing air.

2.3.2 Proliferation assays and expression of results

To assess the proliferative responses of cells to the various stimuli, 1 μCi of [^{3}H] thymidine/well was added and incorporation evaluated on a LKB Betaplate apparatus after 14 to 16 h.

Stimulation indices (SI) were calculated from geometric means of quadruplicates as follows: SI was defined as the ratio of the geometric mean cpm of quadruplicate test cultures to the geometric mean cpm of quadruplicate control cultures. A positive proliferative response was attributed to samples for which the SI was > 2 and the cpm difference between geometric means of test cultures and control cultures was superior to 1000 cpm. The maximum proliferation was obtained at day 6 for the different parasite antigens and derived peptides. The T cell response was assessed by checking [^{3}H] thymidine incorporation at day 6.

2.3.3 IFN-γ assay and expression of results

The IFN-γ concentration in pooled supernatants from the quadruplicate wells was assessed by the capture ELISA performed in duplicate. Briefly, wells were coated with 50 μl of anti-IFN-γ mAb RU 40.2 at 5 μg/ml in PBS and PBS-BSA (3%) was used to block further nonspecific binding. Undiluted supernatant (50 μl) was added for 16 to 18 h at room temperature, and the wells were then washed three times for 10 min with PBS-Tween 0.1%. A second anti-IFN-γ mAb, RU 308, coupled to peroxidase, diluted in RPMI 1640 containing 50 mM Hepes, was added in the wells at 0.8 μg/ml and left for 3 h at room temperature. The wells were again washed three times and the activity was revealed after a 15-min incubation at room temperature with 100 μl of reaction substrate (OPD 1 mg/ml, H_2O_2 30% 1 μl/ml in citrate buffer). The reaction was stopped by adding 50 μl/well of 3 N HCl and the absorbance read at 450 nm on a Dynatech reader. The IFN-γ content of supernatants was calculated from standard curves performed on culture medium containing known amount of IFN-γ, included on each plate. Readings were compared with the international

human NIH IFN-γ standard Gg23-901-530. This test is able to detect 0.25 IU/ml. Thus, we consider as positive samples for which the quantity of IFN-γ secreted in the presence of the antigen was more than 1 IU/ml. We never found detectable levels of IFN-γ in control wells (PBMC in absence of antigen). Preliminary studies showed that the optimal IFN-γ production after stimulation with *P. falciparum* schizont lysate was obtained around days 3 or 4 but no detectable decrease in concentration was observed at day 6. Thus, culture supernatants (100 µl/well) were harvested in the evening of the day 5, frozen at minus 80 °C until use, and replaced by 100 µl of fresh medium containing the [^{3}H] thymidine for assessing proliferation. Anti-human IFN-γ monoclonal antibodies were kindly given by Mrs. Cousin, Roussel Uclaf, France.

2.4 Generation of mouse T cell lines specific for peptides P9A and P9B

The strategy described by Taylor et al. [15] was used to establish T cell lines specific for the peptides P9A and P9B. Synthetic peptide (50 µg) was injected in Freund's complete adjuvant (Difco) at the base of the tail (50 µl volume). Eight days later the inguinal and periaortic lymph nodes were removed and cultured (2×10^6 cells/ml) in the presence of 50 µg/ml peptide P9A or P9B for 4 days. Cells were harvested and viable cells were resuspended at 2×10^5 cells/ml in the presence of irradiated spleen cells (2×10^6 cells/ml) and 50 µg/ml peptide (feed period) After 4 days the viable cells were harvested and recultured with irradiated syngenic spleen cells without antigen for 7 days (starve period). After that the T cells were maintained for two further "feed" and "starving" cycles before the T cell proliferation assay. The cellular proliferation of the P9A/P9B T cell lines (4×10^5/ml) was induced, after a starving period, with various amounts of peptide (0.03–30 µg/ml) in the presence of APC (4×10^6/ml). Triplicates of 200 µl were cultured in 96-well flat-bottom microtiter plates at 37 °C. After 3 days 25 µl of [^{3}H] thymidine (20 µCi/ml, Amersham) was added. Six hours later the cells were harvested onto glass fibre filter paper (Whatman) and the radioactivity counted in vials containing non-aqueous scintillation fluid.

2.5 Antibody detection

Mouse or human antibodies against the Pf11-1 nonamer repeat peptides were analyzed by ELISA as follows: Luxlon ELISA plates were coated with 250 ng/well of the P9A or P9B peptides in PBS overnight at 4 °C. After saturation (PBS-0.1% Tween 5%-milk for 1 h at 37 °C), the wells were washed in PBS 0.1% Tween and incubated for 1 h at 37 °C with dilutions of test and control plasmas. The wells were washed and peroxydase-conjugated goat anti-human or goat anti-mouse IgG antibodies (KPL, Gaithersburg, MD) were added for 1 h at 37 °C. After incubation the wells were washed and developed with TMB substrate (KPL) used according to the supplier instructions. The reaction was stopped with 1 M H_3PO_4 and the absorbance read at 450 nm. The mouse sera were tested in duplicate at the dilution of 1/100. The human plasma were tested in duplicate at the dilution 1/2500. The data from different ELISA plates were standardized from a standard curve derived from a known positive plasma included on each plate. To quantify the level of anti-peptide antibody a value of 100 arbitrary units (AU) was ascribed to the reference plasma diluted to 1/100. The level of antibody in all plasma tested was expressed in AU compared to the reference plasma.

2.6 Synthetic peptides and recombinant polypeptides

The synthetic peptides: RESA 3′: Y-(E-E-N-V-E-H-D-A)$_2$ and Pf332: Y-(S-V-T-E-E-I-A-E-E-D-K)$_2$ have been described elsewhere [4]. Synthetic peptides corresponding to the amino acid repeats of Pf11-1, Y-(P-E-E-V-V-E-E-V-V)$_2$-P, P9A and Y-(P-E-E-L-V-E-E-V-I)$_2$-P, P9B, were synthesized by Appligene (Strasbourg, France). The peptide P9A was > 86% and the P9B > 96% pure as tested by HPLC. The recombinant β-galactosidase fusion protein pPf11.1 has been described [9]. The fusion protein as well as *E. coli* β-galactosidase was purified by affinity chromatography columns as described [16] and was > 95% pure.

3 Results

3.1 The B cell response to the Pf11-1 nonamer repeats P9A and P9B in mice

To search for potential T cell epitopes able to bind MHC class II molecules in the Pf11-1 molecule we applied several computer algorithms incorporated into one protein sequence analysis package called "T sites" [13]. Surprisingly, the entire nine amino acid repeat-region displayed an unexpected high prediction for T cell epitopes using the algorithms for amphipathic α helices [17] as well as the Rothbard/Taylor sequence motifs [18]. In contrast, no antigenic site was predicted for the mouse H-2^d haplotype

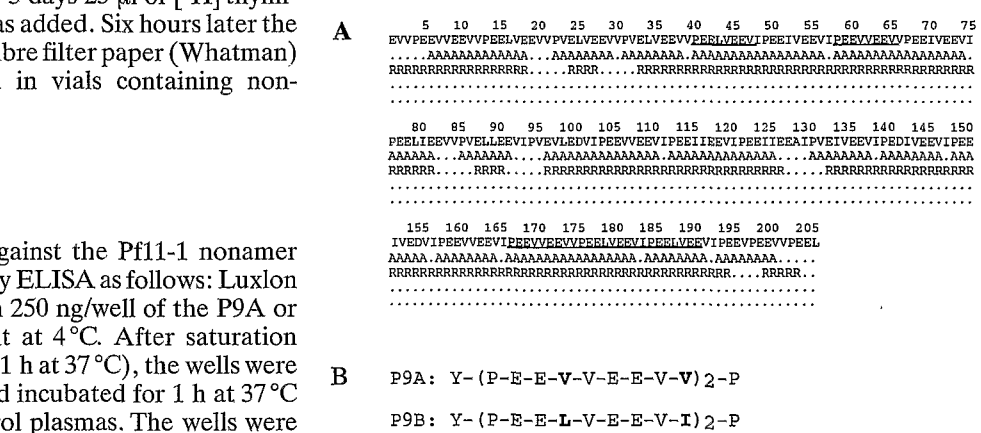

Figure 1. A. Prediction of the antigenic T sites of the nonamer repeats of the Pf11-1 antigen using the computer program "T sites" [13]. The protein sequence encoded by the recombinant clone pPf11-1 [9] is shown in the top line. Second line: A = AMPHI mid points of blocks of the AMPHI algorithm [17] (window size: 11) Third line: R = residues matching the Rothbard/Taylor motif [18]. Fourth line: D = Residues matching the IA^d motif and fifth line: d = residues matching the IE^d motif. B. Sequence of synthetic peptides P9A and P9B corresponding to repeat motifs found in the nonamer tandem repeat region of Pf11-1.

based on the model of Sette [19]. In Fig. 1 A, an example of the T site analysis of a polypeptide encoded by clone pPf11-1 [9] is shown.

To study the immune response to the potential immunogenic repeat region of the Pf11-1 antigen, we choose two synthetic peptides which represent two distinct repeat motifs termed P9A and P9B (Fig. 1 B). The two peptides differ in position 4 and 9 by functionally similar amino acids. The P9A and P9B repeat motifs are present many times in the nonamer repeat-region of the Pf11-1 protein and exist several times in the sequence of clone pPf11-1 (see Fig. 1 A).

The specificity of the immune response was first analyzed in H-2-congenic strains of inbred mice. Of five H-2 haplotypes, two, H-2^d and H-2^k, induced strong antibody responses after immunization with carrier-free P9A or P9B dimeric peptides, respectively (Fig. 2 A). However, nonresponder mouse strains, for example strains representing H-2^b haplotype, could make antibodies when the P9A was covalently coupled to the carrier molecule KLH (data not shown). We were interested in the fine specificity of the antibody response to the peptides P9A and P9B and the impact of amino acid exchanges in position 4 and 9 of the nonamer repeat on the cross-reactivity to related Glu-rich malaria-derived synthetic peptides. Inhibition of P9A or P9B antibody binding in the ELISA to the homologous peptide was analyzed by mixing various concentrations of the synthetic peptides P9A and P9B as well as two other immunologically cross-reactive peptides RESA 3′ and Pf332 [4] to a fixed concentration of sera. The most efficient inhibition of antibody binding was obtained with the homologous peptides (Fig. 2 B and C). The heterologous Pf11-1 peptide showed a significantly lower inhibition capacity and was not able, even at concentrations of 200 μg/ml, to inhibit totally the antibody binding. The RESA peptide inhibited significantly the binding of P9A antibodies (55% at 200 μg/ml) but much less that of the P9B antibodies (20% at 200 μg/ml), whereas the Pf332 peptide had the lowest inhibiting capacities to the Pf11-1 antibodies. In conclusion, the immune response to the individual peptides P9A and P9B in mice produced nonamer subtype-specific antibodies as well as antibodies with a broader specificity that are able to cross-react with distinct repeats of the Pf11-1 molecule or, to a lower extent, with related Glu-rich epitopes from other antigens of *P. falciparum*.

3.2 P9A- and P9B-specific T cell lines do not cross-react with heterologous nonamer repeats

To analyze the T cell response to the nonamer repeats T cell lines derived from BALB/c (H-2^d) mice were established for the peptides P9A and P9B. Eight days after injection of the peptides emulsified in Freund's complete adjuvant into mice, lymph node cells were cultured in presence of antigen-presenting cells and P9A or P9B peptides. Only a weak proliferation (SI ≤ 2) was obtained directly from lymph node cells. Therefore, specific T cell lines were obtained after three rounds of stimulation with the peptides P9A and P9B. Fig. 3 shows a dose-response curve of [^{3}H] thymidine uptake of a P9A- or P9B-specific T cell line stimulated with various peptides or polypeptides. Surprisingly, the T cell response to the two nonamer repeats clearly

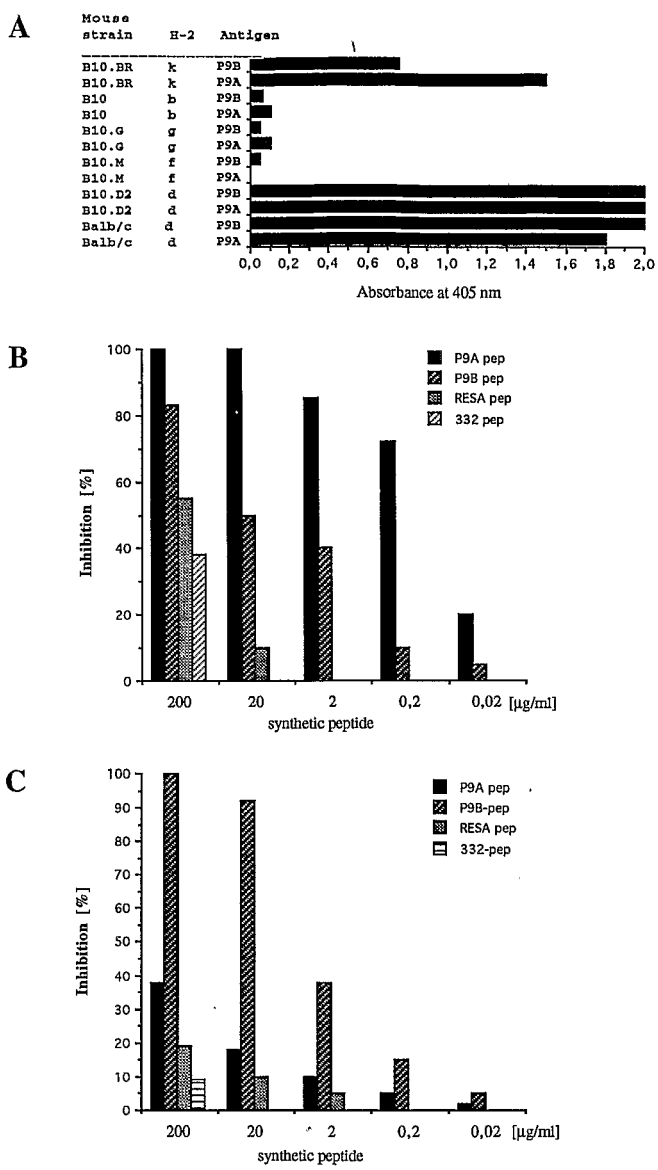

Figure 2. The B cell response to P9A and P9B in mice is H-2^d and H-2^k restricted and generates both repeat-specific as well as cross-reactive antibodies. A. IgG response directed against the peptides P9A and P9B of six mice strains representing five different H-2 haplotypes. Five mice/group were immunized with 50 μg peptide (without carrier). Sera were taken 7 days after the first boost, diluted 1 : 100 and tested by ELISA employing the peptides P9A or P9B, respectively. Solid bars represent the average absorbance obtained for each group of mice. B. The inhibition of the anti-P9A antibodies or C. anti-P9B antibodies binding in ELISA to its homologous peptide was analyzed by mixing various concentrations of synthetic peptides to 1 : 100 diluted mouse anti-P9A or P9B serum. The percentage of inhibition was calculated from absorbance values obtained in the presence and absence of synthetic peptides. The values report the arithmetic mean of duplicates.

distinguishes between the two types of Pf11-1 repeat even though they differ by only very limited amino acid exchanges (valine is replaced by isoleucine and leucine). The homologous peptide induced a strong dose-dependent T cell proliferation while the heterologous peptide showed no significant capacity to induce proliferation (Fig. 3 A and B). When the recombinant polypeptide pPf11-1 (see

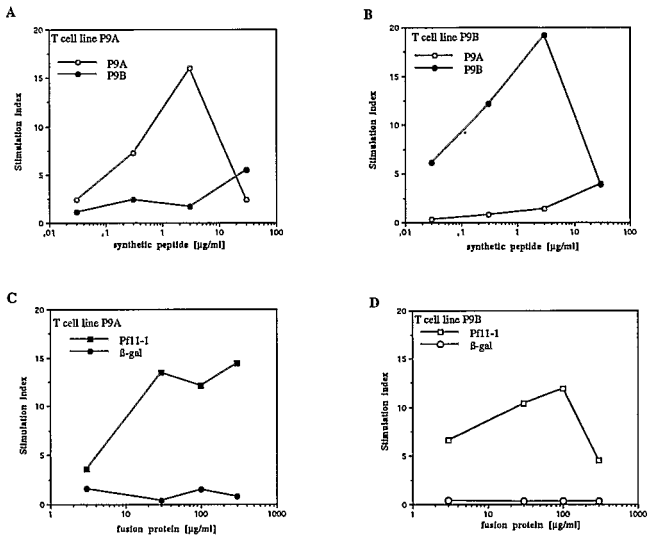

Figure 3. Dose-response curve of [^{3}H] thymidine uptake of a P9A- or P9B-specific T cell line. The values represent the arithmetic mean of triplicates. The proliferation is expressed as SI: ratio of cpm in cultures given antigen and APC divided by the response to APC alone. A. Proliferative response of T cell line P9A with the peptide P9A and P9B, B. of T cell line P9B with the peptide P9A and P9B, C. of T cell line P9A with the recombinant pPf11-1-β-galactosidase fusion protein and β-galactosidase and D. of T cell line P9B with pPf11-1-β-galactosidase and β-galactosidase.

Fig. 1 A) fused to β-galactosidase was used, significant T cell proliferation was obtained with both the P9A and P9B lines (Fig. 3 C and D), suggesting that the antigen-presenting cells are able to process and present the P9A and P9B sequences within the pPf11-1 nonamer repeat region to the T cell lines in a manner similar to that observed with the synthetic repeat peptides.

3.3 Antibody responses to the P9A and P9B peptides in humans

The anti-Pf11-1 antibody activity from 51 individuals was assessed by ELISA test. A standard curve was determined on peptide P9B on each ELISA plate by using five serial dilutions of a reference serum. Sera from 15 non-exposed, caucasian individuals were analyzed using the same assay, their antibody levels ranged in a Gaussian distribution, with an arithmetic mean of 11.70 ± 1.20 AU on peptide P9A and 11.90 ± 2.5 AU on P9B. Positive antibody content was attributed to samples containing more than the mean of control levels + 2 SD, *i.e.* 15 AU for P9A and 17 AU for P9B. Using those criteria, 16 (31.3%) samples from exposed individuals were negative on both peptides, 32 of the 51 samples (62.75%) were positive on peptide P9A while 31 (60.8%) were positive on P9B and 28 of these samples were positive on both peptides (Fig. 4 and Table 1). The level of antibodies specific of the two peptides appeared to be strongly correlated within the 28 samples (Spearman rank correlation test, $p = 0.0001$). In contrast, when the same samples were analyzed for their antibody activity for another *P. falciparum* antigen, PfHsp70, the antibody levels did not correlate with the anti-P9A nor P9B antibody level ($p = 0.93$ and $p = 0.33$, respectively) (data not shown).

3.4 Human T cell reactivities on the peptides P9A and P9B

The cellular response was performed in the same cohort as analyzed for the humoral response. Cells from all 53 individuals analyzed responded to the positive mitogen control LeuA; 90% proliferated in response to PPD, demonstrating that all the PBMC preparations contained viable, responding T cells (data not shown).

The proliferative responses of the cells from the 53 individuals to the peptides are summarized in Table 1. Peptide P9B induced a positive response in 19/53 individuals (35.85%), whereas only 7/53 (13.20%) samples were positive when stimulated with peptide P9A (Table 1). It is noteworthy that all samples positive for P9A were also positive for P9B, suggesting that the populations responding to the two peptides are associated ($\chi^2 = 11.39$, $p = 0.0007$). The SI ranged from 2.87 to 27.8 for P9A, and from 2.03 to 28.54 for P9B. PBL from 20 caucasian never

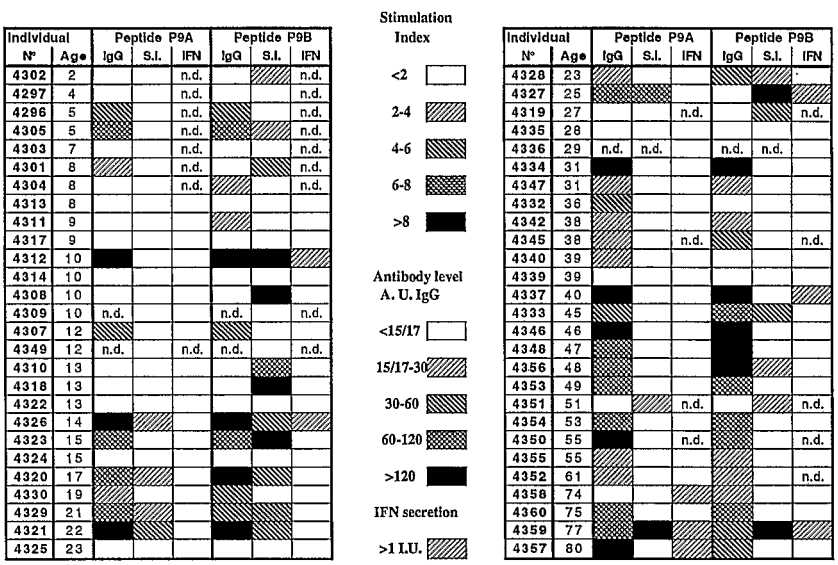

Figure 4. Humoral and cellular response to the peptides P9A and P9B in individuals living in an malaria endemic area.

Table 1. Human immune response to the nonamer repeats P9A and P9B

	Humoral responses		
	Total	Positive P9A	Negative P9A
Total	51	32 (62.7%)	19 (37.2%)
Positive P9B	31 (60.8%)	28 (54.9%)	3 (5.9%)
Negative P9B	20 (39.2%)	4 (7.8%)	16 (31.3%)

	Cellular responses		
	Total	Positive P9A	Negative P9A
Total	53	7 (13%)	46 (87%)
Positive P9B	19 (36%)	7 (13%)	12 (22.5%)
Negative P9B	34 (64%)	0	34 (64%)

exposed to malaria were tested in the same conditions. In all cases the SI was < 2 (data not shown). In contrast to the humoral response, the levels of the P9A-specific and P9B-specific proliferative responses in PBMC samples responding to both peptides were not correlated (Spearman rank correlation test, $p = 0.2$).

IFN-γ production was detected in the supernatants of PBMC stimulated with P9A or P9B in only few samples (3 out of 42 for P9A and 5 out 40 for P9B) (Fig. 4). Moreover, the IFN-γ levels detected were low (no more than 5 IU/ml), compared to the high levels (from 1 to 140 IU) detected during the same experiment after stimulation of PBMC from the same individuals with PPD, or with other *P. falciparum* antigens (data not shown).

4 Discussion

In the present report we investigated the immune response to the product of the Pf11-1 gene, a giant parasite protein being composed of tandemly repeated nine-amino acid units. To this end, we dissected the B and T cell response to the very large nine-amino acid repeat region by using synthetic peptides corresponding to two distinct nonamer repeat motifs (Fig. 1B). Our data clearly demonstrate that the dimeric nonamer repeat peptides tested are able to induce a humoral and cellular response in mice. In addition they are recognized by antibodies and T cells from humans naturally infected with *P. falciparum*.

The B cell response to the individual peptides, P9A and P9B, which was shown to be associated with the H-2^d and H-2^k haplotypes, displays an interesting feature. The specificity of the B cell response is dependent on those hydrophobic amino acids which differ in both synthetic peptides (positions 4 and 9; see Fig. 1B). It appears that each subtype repeat induces in mice high-affinity antibodies to the homologous peptide, which are still cross-reactive, though to a lesser degree, with the heterologous nonamer repeat peptide and to a much lower degree with other Glu-rich peptides, such as RESA and Pf332 (Fig. 2). The cross-reactivity is probably due to the presence of pairs of glutamic acids also found in other members of the *P. falciparum* Glu-rich antigen family. This idea is supported by recent work by Ahlborg et al., [20] who studied a cross-reacting monoclonal antibody that cross-reacts with numerous members of the Glu-rich antigen family. By analyzing the specificity of the epitope recognized by this monoclonal antibody 33G2, the authors have shown that two pairs of glutamic acids separated by hydrophobic amino acids are essential for the recognition site.

In contrast to the B cell response in mice, T cells clearly discriminate between the nonamer subtypes P9A and P9B. When the heterologous nonamer repeat peptide was used to stimulate P9A or P9B specific T cell lines, no cross stimulation was observed (Fig. 3A and B). This finding suggests that the hydrophobic amino acids are recognized by the T cell receptor, whereas the glutamic acid residue(s) could interact with the MHC molecules as well as with the T cell receptor. This interpretation is consistant with the observation that the immune response to Glu-rich peptides of the RESA molecule, such as $(EENV)_4$ or $(EENVEHDH)_2$, are also restricted to class II molecules of H-2^d and H-2^k haplotypes [21]. Thus, pairs of glutamic acids spaced by hydrophobic amino acids could represent a sequence motif for the binding of H-2^d and H-2^k MHC molecules. In summary, the results obtained with mice suggest strongly that each nonamer repeat contains at least one B and T cell antigenic determinant. Thus, the Pf11-1 protein could contain up to several hundred determinants with a different immunogenicity.

To determine whether the results found in mice represent the events in humans, the reactivities to the peptides P9A and P9B were examined in individuals naturally sensitized to *P. falciparum* living in an endemic area in West Africa. This group has been followed since 1990 in the context of an epidemiological survey on the natural acquisition of immunity against *P. falciparum* malaria [14]. The analysis of the humoral response to the Pf11-1-derived peptides showed that 70% of the tested individuals have significant antibody levels to both P9A and P9B peptides. Moreover, we found that the IgG antibody reacting specifically with P9A and P9B were significantly correlated which could be explained by cross-reactivities of antibodies directed against either P9A or P9B. This interpretation is in agreement with the results showing that the humoral response of mice immunized with either synthetic Pf11-1 peptide cross-reacts with the other. Since Pf11-1 is a member of the family of immunologically related antigens including blood-stage antigens such as RESA, Pf332 and FIRA, the antibodies detected in human sera by ELISA tests on P9A and P9B could have been induced not only by Pf11-1, but also by other blood-stage immunogens.

When analyzing cellular responses to the Pf11-1 derived peptides, we found a significant proportion of samples with a stimulatory response, tested positive either by proliferation or IFN-γ production. Therefore both peptides contain at least one T cell epitope for man. It is interesting to note that the frequency of responses to P9B was nearly twice as high than to P9A and that all responders for P9A responded also to P9B. Based on the results obtained in congenic strains of mice, this correlation could be due to identical individual genetic elements, such as the MHC locus, regulating the immune response. We are presently typing all the individuals living in the village to determine the HLA alleles. In contrast to the humoral response, no correlation

was found between proliferation levels in response to the two peptides. This result is also in agreement with the results obtained in mice.

The most frequent amino acids in the approximately 145 repeats presently sequenced are: P_1-E_2-E_3-L_4-V_5-E_6-E_7-V_8-I_9. Although positions 1, 2, 3, 6 and 7 are relatively conserved (85–92%) compared to the residues at positions 4, 5, 8 and 9 (40–70%), almost any type of amino acid replacement is observed (Fig. 5). To illustrate the enormous diversity, 15 out of 22 repeats of a cloned Pf11-1 genomic fragment are distinct from each other by at least one amino acid [9]. Considering the large number of similar but distinct nine amino acid repeats occurring in the Pf11-1 molecule, it is probable that each of the approximately 700 degenerated nonamer repeats might represent a B cell epitope, resulting in a very diverse B cell response. Thus, the Glu-rich antigen network could participate in the polyclonal B cell activation program of the parasite that, as proposed by Anders [7], acts as a smokescreen to reduce antibody maturation against critical "protective" epitopes. Furthermore, our studies show the exceptional propensity of the nonamer repeats to act as T sites. Based on the results with mice, the degenerate repeats could also be involved in the stimulation of an unusually diversified T cell response by the Pf11-1 molecule. For example, it has been reported recently that a synthetic tripeptide polymer can induce a very heterogenous T cell response in mice [22].

In summary, the data obtained on the two subtypes of the nonamer repeat region suggest that the entire Pf11-1 molecule might induce an unusually heterogenous B and T cell response during natural infection in man. Importantly, a recent study showed that a monoclonal antibody directed against the nonamer repeat of the Pf11-1 molecule is able to inhibit the transmission of *P. falciparum* gametocytes to the mosquito vector [11]. Consequently, the nonamer repeats are potentially important for the development of a transmission-blocking vaccine. It is noteworthy to emphasize that circulating gametocytes expressing the Pf11-1 should be able to boost the immune response to the Pf11-1 antigen, a situation that is not true for gamete-specific antigens. Among the individuals tested in this study, 30% showed on thick smear examination the presence of *P. falciparum* gametocytes (data not shown). It will be of interest to study more precisely the correlation between the level of anti-Pf11-1 antibodies and the presence of circulating gametocytes. However, such a study will have to be performed on a larger number of individuals, in order to improve the statistical significance of the data and to evaluate the possible effects of the anti-Pf11-1 antibody content on malaria transmission in a longitudinal epidemiological study.

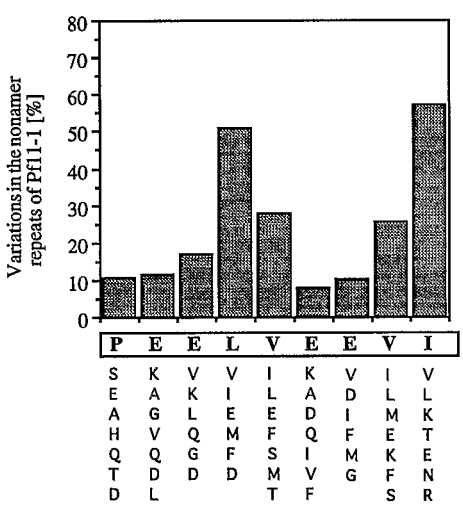

Figure 5. Diversity in the nonamer repeats of the Pf11-1 molecule. The consensus amino acid sequence derived from approximately 145 aligned repeats is shown in the box and the relative variation from the consensus is shown by the bar.

Our study also shows the limits of synthetic peptides for vaccination of large populations. However, genetic restriction and/or low B and T cell response might be overcome by using a polymer containing different nonamer repeats, as for example found in the recombinant molecule pPf11-1 (Fig. 1). This could be very helpful for the development of a transmission-blocking vaccine against *P. falciparum*.

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