Structure of an Immunodominant Epitope of the Circumsporozoite Surface Protein of *Plasmodium knowlesi*†

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ABSTRACT: Previous studies have shown that the immunodominant region of the circumsporozoite surface (CS) protein of *Plasmodium knowlesi* is contained within a tandemly repeated dodecapeptide: Gln-Ala-Gln-Gly-Asp-Gly-Ala-Asn-Ala-Gly-Gln-Pro. We show here that the CS protein epitopes reacting with six monoclonal antibodies raised against the intact parasite are represented in a synthetic tandem repeat of this dodecapeptide. The specificity of four of these antibodies was studied further by preparing synthetic peptides corresponding to overlapping regions of the repeats and measuring their ability to inhibit the specific interaction between the antibodies and CS proteins. We find that three antibodies have very similar patterns of reactivity with this series of peptides and that they define an epitope of eight amino acids (Gly-Asp-Gly-Ala-Asn-Ala-Gly-Gln) within the dodecapeptide. The remaining antibody probably recognizes a configurational epitope formed by a tandem repeat of the dodecapeptide.

Sporozoites are the infective stage of *Plasmodium* protozoan parasites. The membrane of sporozoites is covered with a protein [circumsporozoite surface (CS)[1] protein; reviewed in Nussenzweig & Nussenweig (1984)] which is synthesized in relatively large amounts when the parasite matures inside the salivary glands of the invertebrate host, *Anopheles* mosquitoes. Fab fragments of monoclonal antibodies to CS proteins neutralize the infectivity of the sporozoites and prevent their attachment to the target cells in vitro (Hollingdale et al., 1982). For these reasons, it has been suggested that the CS protein plays a role in the process of invasion of the host liver cells where the sporozoites continue their life cycle and transform into the next developmental stage.

Recently the gene that codes for CS protein of the monkey malaria parasite *Plasmodium knowlesi* has been isolated (Ellis et al., 1983) and its structure determined (Ozaki et al., 1983). This protein has the unique 12 amino acid segment Gln-Ala-Gln-Gly-Asp-Gly-Ala-Asn-Ala-Gly-Gln-Pro repeated in tandem 12 times. This dodecapeptide has been shown to contain an immunoreactive region of the protein (Godson et al., 1983). All monoclonal and most of the polyclonal antibodies raised against sporozoites of various species are directed against a domain of the CS molecule (Zavala et al., 1983).

The present studies are aimed at elucidating the structure of the epitopes which react with the various monoclonal antibodies of *P. knowlesi*.

We were particularly interested in verifying whether the epitopes were represented within an uninterrupted sequence of amino acids or whether they were configurational, i.e., whether they were formed by residues apart in the primary structure and brought together only after folding of the polypeptide chain. Since several of these monoclonal antibodies neutralized the infectivity of sporozoites of *P. knowlesi*, we reasoned that if the epitopes were formed by relatively short peptides it might be possible to develop synthetic vaccines.

Materials and Methods

Synthetic Method. Derivatized amino acids were of the L configuration and were purchased from Bachem. Syntheses were carried out by using a benzhydrylamine resin (0.654 mequiv/g) (Beckman Instruments) on a Vega Model 250C synthesizer controlled by a Motorola computer with a program based on a modification of the Merrifield method (Merrifield, 1962, 1963). A 0.5–3-g sample of the benzhydrylamine resin was suspended and washed 3 times with methylene chloride, 3 times with ethanol, and 3 times with CH_2Cl_2 in the synthesizer. The resin was washed (2 min) and then treated with 50% trifluoroacetic acid containing 10% anisole in CH_2Cl_2 for 30 min, washed 10 times with CH_2Cl_2, and neutralized by washing twice with 10% diisopropylethylamine in CH_2Cl_2. The first Boc-amino acid was coupled for 1 h to the benzhydrylamine resin by using dicyclohexylcarbodiimide in the presence of a 3 molar excess of CH_2Cl_2 and hydroxybenzotriazole. Then another aliquot of hydroxybenzotriazole and diisopropylethylamine was added at a 2-fold molar excess for an additional 1 h. Following coupling, the resin was washed with CH_2Cl_2 (three washes), absolute ethanol (three washes), and CH_2Cl_2 (three washes), and an aliquot of the mixture was then tested by using the Kaiser ninhydrin procedure (Kaiser et al., 1970) to test for completion of coupling of the Boc-amino acid to the growing peptide chain.

Cleavage and Extraction of Peptide from the Benzhydrylamine Resin. Cleavage of each of the peptide resins (2 g of each) was performed in a Peninsula HF apparatus in the presence of anisole (1.2 mL/mg of resin) and methyl ethyl sulfide (1 mL/mg) at 0 °C for 1 h after which the mixture was thoroughly dried under high vacuum. The mixture was then washed with cold anhydrous ether, extracted with alternate washes of water and glacial acetic acid, and then lyophilized.

Purification of Crude Synthetic Product. Crude synthetic peptides were desalted and purified by gel filtration on a column of Sephadex G-25 (120 × 2.0 cm) equilibrated with

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[1] Abbreviations: CS protein, circumsporozoite surface protein; Boc, *tert*-butyloxycarbonyl; HF, hydrofluoric acid; PBS, phosphate-buffered saline; PTH, phenylthiohydantoin; BSA, bovine serum albumin; BHA, 3-*tert*-butyl-4-hydroxyanisole; OBz, benzoxy; NPE, nitrophenyl ester.

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0.1 NH_4HCO_3, pH 8.0. Column effluent was monitored by the UV absorbance at 254 and 206 nm with an LKB UV-Cord III monitor.

Characterization and Criteria of Purity: Amino Acid Analysis. Samples were hydrolyzed in 5.7 N HCl for 22 h at 110 °C, dried, reconstituted in 0.2 N sodium citrate, pH 2.2, and applied to the amino acid analyzer (Liquimat III) according to the method of Spackman et al. (1958).

Amino Acid Sequence Analysis. At selected steps during synthesis, aliquots of the peptide resin were removed from the reaction vessel of the synthesizer, mixed wiith glass beads, and subjected to automated solid phase Edman degradation (Laursen, 1971) on a Sequemat Mini 15 peptide sequencer. Following cleavage of the peptide from the resin, crude and purified synthetic peptides were subjected to automated liquid phase sequence analysis (Edman & Begg, 1967) on a Beckman (Model 890) sequencer using a "DMAA" (dimethylallylamine) peptide program to detect the presence of failure sequence and of side chain protecting groups on the peptide not removed by HF cleavage. Error peptides and side chain protecting groups were assessed by sequence analysis followed by identification and quantification of the PTH-amino acids by high-performance liquid chromatography (Simmons & Schlesinger, 1980; Schlesinger, 1983). For a thorough discussion of "preview" analysis, see Niall et al. (1972), Tregear (1974), and Tregear et al. (1977).

Monoclonal Antibodies. Monoclonal antibodies against surface antigens of sporozoites of the simian malaria parasite *Plasmodium knowlesi* were produced by fusion of NS-1 plasmacytoma cells with spleen cells of a mouse immunized with the parasite as previously described by Cochrane et al. (1982). The monoclonal antibodies were partially purified from ascites fluids of mice bearing hybridomas by 50% ammonium sulfate precipitation followed by molecular sieve chromatography on Sephadex G-200, (Sephadex G-200, Pharmacia Fine Chemicals, Piscataway, NJ).

Radioimmunoassay. The immunoreactivity of the synthetic peptides was evaluated by measuring their ability to inhibit the reaction between monoclonal antibodies and the antigen, that is, CS proteins of *P. knowlesi.* The assays were performed as described below:

(*A*) *Preparation of Antigen.* A total of 3000 purified *P. knowlesi* sporozoites (Vanderberg et al., 1968) in 50 μL of phosphate-buffered saline (PBS) containing a mixture of protease inhibitors (Yoshida et al., 1980) were delivered to the bottom of polystyrene (Falcon 3911 plate, Becton-Dickinson and Co., Oxnard, CA) microtiter plates, which were sealed with Parafilm and frozen at −70 °C for at least 10 min. The plates were then allowed to defrost slowly at room temperature. This procedure was repeated 6 times. Then the plates were incubated at 4 °C overnight and at 56 °C for 5 min. Previous experiments showed that this procedure ensured optimal extraction and binding of the CS protein to the plastic (Zavala et al., 1982). The *P. knowlesi* extract was then removed from the wells, and these were washed 3 times with PBS containing 1% bovine serum albumin (BSA) and 0.1% NaN_3. The wells were then filled with PBS–BSA–NaN_3 and incubated for a few hours at room temperature.

(*B*) *Preparation of Peptides and Assay.* All synthetic peptides were dissoved in PBS at a concentration of 10 mM and subsequently diluted serially in PBS-containing 1% BSA and 0.001% NaN_3. Twenty-five-microliter dilutions of each peptide were added to the wells coated with antigen. Control wells received only the diluent. Then, 25 μL of the ^{125}I-labeled monoclonal antibody (about 5–10 ng, 10^5 cpm) was added to the wells. The microtiter plates were incubated at 4 °C for 18 h and washed 5 times with PBS–BSA–NaN_3. The wells were then counted in a γ counter (LKB 1260) Multi-gamma; LKB instruments, Inc., Gaithersburg, MD).

Radiolabeling. The antibodies were labeled with ^{125}I by using Iodogen (Pierce Chemical Co., Rockford, IL). Glass tubes were coated with Iodogen according to the instructions of the manufacturer. Twenty-five microliters protein (1 mg/mL in PBS) was added. After incubation for 20 min at room temperature, the protein was dialyzed extensively against PBS. The synthetic peptides were labeled with the Bolton–Hunter reagent (Amersham Corp., Arlington Heights, IL). The peptide (5 μg) in 10 μL of 0.1 M borate buffer, pH 8.5, was added to the dried iodinated ester. After 15 min at 0 °C, the labeled peptide was isolated by filtration through a Sephadex G-25 column. In each case, 1 mCi of ^{125}I was used.

Results

Peptide Synthesis. The dodecapeptide (1–12) was assembled on 3 g of the benzhydrylamine resin. The following peptide was synthesized by using protected amino acids with a 3-fold molar excess as described under Materials and Methods: Boc-Gln(NPE)-Ala-Gln(NPE)-Gly-Asp(OBz)-Gly-Ala-Asn(NPE)-Ala-Gly-Gln(NPE)-Pro-CO-BHA.

At this point in the synthesis, 50% of the protected peptide resin was removed and saved for HF cleavage and purification. The tandem repeat, i.e., the tetraeicosapeptide (1–24), was then assembled by the sequential addition of protected amino acids in the same order as for the dodecapeptide.

Following synthesis, 2.0 g each of the (1–12)- and (1–24)-protected peptide resins was subjected to treatment with HF as described above. The cleaved and deprotected peptides were washed separately with anhydrous ether and extracted with alternate washes of glacial acetic acid and water. The crude peptides were then desalted on Sephadex G-25 in 200-mg aliquots.

Four groups of peptide analogues of the dodecapeptide were synthesized, each possessing as the C-terminal amino acid either Pro, Gln, Gly, or Ala, which correspond to positions 12, 11, 10, and 9, respectively, of the dodecamer (Table I). The group of peptide analogues possessing Pro at their C-terminus was synthesized by removing dried protected peptide resin (approximately 10% at each step) at positions 10, 8, 7, 6, 5, 4, 3, and 2 during the synthesis of the dodecapeptide. This procedure yielded nine protected peptide resins.

Similarly, two peptides were synthesized containing the C-terminal amino acid Gln, i.e., peptides comprising residues in positions 5–11 and 4–11 of the dodecamer. Only one peptide containing Gly at its C-terminus, corresponding to positions 4–10 of the dodecamer, was synthesized. Finally, four peptides possessing Ala at their C-terminus (positions 7–9, 6–9, 5–9, and 4–9 of the dodecapeptide) were synthesized in analogous fashion. One peptide which bridges parts of two epitopes was synthesized, i.e., Ala_7-Asn_8-Ala_9-Gly_{10}-Gln_{11}-Pro_{12}-Gln_1-Ala_2-Gln_3-Gly_4-Asp_5, to determine if the single monoclonal antibody which did not recognize the dodecapeptide might be directed against a sequence containing parts of two tandem repeats.

These protected peptide resins were cleaved and deprotected with HF, desalted on Sephadex G-25, and characterized by amino acid composition and sequencing preview analysis (Table I). The peptides were used in imunological studies without further purification.

Reaction of Monoclonal Antibodies with the Tetraeicosapeptide. All monoclonal antibodies prepared against *P. knowlesi*, selected for reactivity with the surface membrane

Table I: Amino Acid Composition and Preview Analysis of Synthetic Peptide Segments of the CS Protein *P. knowlesi*

peptide segment	composition					preview (%)
	Asp	Pro	Glu	Gly	Ala	
(1–12)	1.84 (2)	0.95 (1)	3.00 (3)	3.00 (3)	2.97 (3)	1.4
(2–12)	2.21 (2)	0.85 (1)	2.15 (2)	3.00 (3)	3.02 (3)	NR
(3–12)	2.18 (2)	1.07 (1)	2.00 (2)	2.77 (3)	1.88 (2)	NR
(4–12)	2.00 (2)	1.10 (1)	0.88 (1)	2.84 (3)	1.97 (2)	NR
(5–12)	2.18 (2)	1.17 (1)	1.00 (1)	2.00 (2)	1.93 (2)	NR
(6–12)	0.93 (1)	1.00 (1)	0.97 (1)	2.13 (2)	2.16 (2)	NR
(7–12)	0.90 (1)	1.14 (1)	1.03 (1)	1.00 (1)	2.00 (2)	NR
(8–12)	0.92 (1)	1.05 (1)	1.01 (1)	1.00 (1)	1.00 (1)	NR
(10–12)		1.10 (1)	1.01 (1)	1.00 (1)		NR
(1–24)	4.15 (4)	1.97 (2)	6.14 (6)	6.05 (6)	5.95 (6)	2.1
(4–11)	2.16 (2)		0.88 (1)	3.40 (3)	2.00 (2)	NR
(5–11)	2.06 (2)		0.95 (1)	2.21 (2)	2.01 (2)	NR
(4–10)	1.88 (2)			3.00 (3)	1.75 (2)	NR
(4–9)	2.00 (2)			2.18 (2)	1.97 (2)	NR
(5–9)	2.00 (2)			0.81 (1)	2.14 (2)	NR
(6–9)	1.00 (1)			0.75 (1)	2.15 (2)	NR
(7–9)	1.00 (1)				2.21 (2)	NR
(7–6)[a]	1.94 (2)	0.89 (1)	3.06 (3)	2.95 (3)	2.91 (3)	NR

[a] Peptide bridging two epitopes, i.e., Ala_7-Asn_8-Ala_9-Gly_{10}-Gln_{11}-Pro_{12}-Gln_1-Ala_2-Gln_3-Gly_4-Asp_5-Gly_6.

Table II: Binding of ^{125}I-Labeled Tetraeicosapeptide to Different Monoclonal Antibodies Raised against the Sporozoite Surface Protein of *P. knowlesi*[a]

beads bearing monoclonal antibodies against surface protein	cpm (% of input) of radiolabeled tetraeicosapeptide bound to beads suspended in	
	PBS–BSA	PBS–BSA + excess of cold tetraeicosapeptide
2G3	2922 (25.8)	25 (0.2)
5H8	2332 (19.7)	20 (0.2)
8B8	3155 (27.8)	20 (0.2)
8A8	1092 (10.3)	25 (0.2)
control[b]	25 (0.2)	0

[a] Five milligrams of several purified monoclonal antibodies was bound to 1 mL of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) according to instructions of the manufacturer. The 24 amino acid peptide was radiolabeled with ^{125}I with the Bolton–Hunter reagent (Amersham Corp., Arlington Heights, IL) to a specific activity of approximately 10^7 cpm/μg. Twenty microliters of beads bearing the different idiotypes was incubated for 60 min at room temperature in the presence of 100 μL of PBS–BSA containing either 1 μg of the radiolabeled peptide alone or radiolabeled peptide in the presence of 200 μg of cold tetraeicosapeptide. Then the beads were washed by centrifugation in PBS–BSA and counted in a γ counter. [b] Beads bearing a monoclonal antibody (3D11) against the surface protein of *P. berghei*.

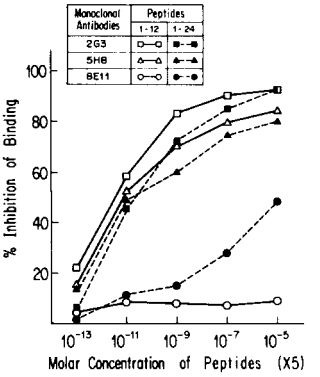

FIGURE 1: Inhibitory activity of peptides (1–12) and (1–24) on the binding of monoclonal antibodies 2G3, 5H8, and 8E11 to the *P. knowlesi* CS protein. The peptide (1–24) contains a tandem repeat of peptide (1–12). As shown, the activities of (1–12) and (1–24) were almost identical with regard to the binding of antibodies 2G3 and 5H8. However, only (1–24) effectively inhibited the interaction between the antigen and the radiolabeled antibody 8E11.

Table III: Inhibitory Effect of Synthetic Peptides on the Binding of Radiolabeled Monoclonal Antibodies to the CS Protein of *P. knowlesi*

peptide	molar concn of peptide (×5) necessary for 50% inhibition of binding of monoclonal antibody[a]		
	8B8	2G3	5H8
(1–12)	10^{-12}	10^{-13}	10^{-12}
(2–12)	10^{-12}	10^{-12}	10^{-11}
(3–12)	10^{-12}	10^{-12}	10^{-11}
(4–12)	10^{-12}	10^{-11}	10^{-12}
(5–12)	10^{-12}	10^{-11}	10^{-11}
(6–12)	10^{-10}	10^{-10}	10^{-9}
(7–12)	10^{-7}	10^{-6}	10^{-6}
(4–10)	10^{-10}	10^{-5}	10^{-7}
(4–11)	10^{-12}	10^{-12}	10^{-10}

[a] Data calculated from comparison of the results shown in Figures 1–3 with the results in controls in which the monoclonal antibodies were incubated with *P. knowlesi* CS protein in the absence of synthetic peptides (or in the presence of irrelevant peptides).

of the parasite (Cochrane et al., 1982), reacted with the tetraeicosapeptide. In Table II, we show that the radiolabeled (1–24) bound specifically to monoclonal antibodies 2G3, 5H8, 8B8, and 8A8 coupled to Sepharose beads. As expected, the binding was totally inhibited by adding an excess of cold (1–24) to the incubation mixture. Identical results were obtained in other experiments using monoclonal antibodies 8E11 and 6B8 (not shown).

Localization of the Epitope within the Tetraeicosapeptide. Further studies aimed at a definition of the epitope were performed only with monoclonal antibodies 2G3, 5H8, 8B8, and 8E11. We initially compared the inhibiting activity of (1–12) and (1–24) on the binding of the radiolabeled antibodies to the *P. knowlesi* sporozoite extracts immobilized on the microtiter wells. The results of Figure 1 demonstrate that for two out of three monoclonal antibodies (2G3, and 5H8) the epitope must be contained within (1–12) since the inhibitory activities of (1–12) and (1–24) were almost identical. Similar results were obtained with 8B8 (see below). However, this was not true in the case of antibody 8E11, whose binding to antigen was only inhibited by (1–24).

Epitope Recognized by Monoclonal Antibodies 2G3, 5H8, and 8B8. The series of shorter peptides and one peptide overlapping two epitopes shown in Table I was used to analyze the specificity of the 2G3, 5H8, and 8B8 antibodies by using

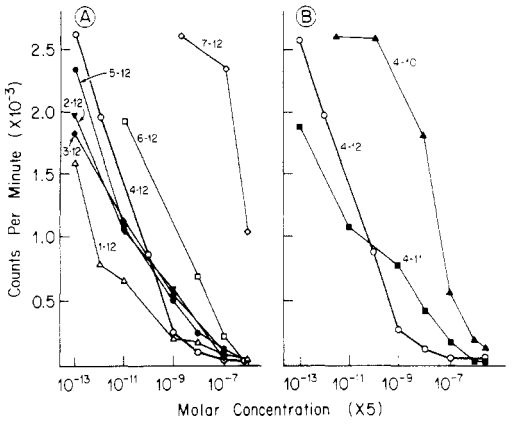

FIGURE 2: Inhibition of binding of radiolabeled monoclonal antibody 2G3 to *P. knowlesi* CS protein in the presence of increasing concentrations of various peptides, designated by their position within the dodecapeptide Gln_1-Ala_2-Gln_3-Gly_4-Asp_5-Gly_6-Ala_7-Asn_8-Ala_9-Gly_{10}-Gln_{11}-Pro_{12}. The results are shown in two separate panels. The peptides examined in panel A have an identical C-terminal amino acid, while those in panel B have an identical N-terminal amino acid.

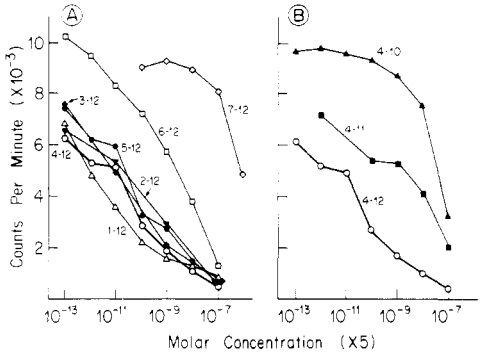

FIGURE 3: Inhibition of binding of radiolabeled monoclonal antibody 5H8 to *P. knowlesi* CS protein in the presence of increasing concentrations of various peptides, designated by their position within the dodecapeptide Gln_1-Ala_2-Gln_3-Gly_4-Asp_5-Gly_6-Ala_7-Asn_8-Ala_9-Gly_{10}-Gln_{11}-Pro_{12}. The results are shown in two separate panels. The peptides examined in panel A have an identical C-terminal amino acid, while those in panel B have an identical N-terminal amino acid.

the inhibition immunoassays referred to above. As shown in Figures 2–4 and summarized in Table III, the patterns of reactivity of these antibodies were strikingly similar. The peptides (1–12), (2–12), (3–12), (4–12), and (5–12) were strongly inhibitory and as effective as (1–24). On a molar basis, their activities were similar although in some instances the larger peptides appeared to be slightly better inhibitors. However, profound drops in activity were observed in (6–12) and (7–12) as compared to (5–12). These results suggested the presence of an immunodominant epitope around the segment Asp_5-Gly-Ala-Asn-Ala-Gly-Pro_{12}. To determine the participation of the C-terminal amino acids, the activity of (4–10) and (4–11) as compared to (4–12) was studied. As also shown in Figures 2–4, the removal of proline (peptide 4–11) had little effect on the reactivity with monoclonal antibodies 2G3 and 8B8, while removal of both glutamine and proline (4–10) diminished markedly the inhibitory capacity for all three antibodies. The shorter peptides (4–9), (5–9), (6–9), (7–9), (8–12), (9–12), and (10–12) were virtually inactive.

These experiments localized the epitope recognized by the three monoclonal antibodies around the (4–11) peptide segment. The reactivity of (5–11) with the three antibodies was much lower than that of (4–11) or (5–12).

Epitope Recognized by Monoclonal Antibody 8E11. As mentioned above, the reaction of this antibody with the CS

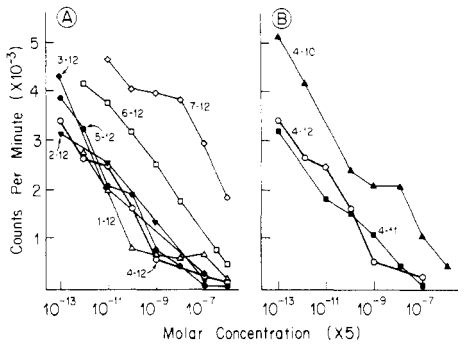

FIGURE 4: Inhibition of binding of radiolabeled monoclonal antibody 8B8 to *P. knowlesi* CS protein in the presence of increasing concentrations of various peptides, designated by their position within the dodecapeptide Gln_1-Ala_2-Gln_3-Gly_4-Asp_5-Gly_6-Ala_7-Asn_8-Ala_9-Gly_{10}-Gln_{11}-Pro_{12}. The results are shown in two separate panels. The peptides examined in panel A have an identical C-terminal amino acid, while those in panel B have an identical N-terminal amino acid.

protein of *P. knowlesi* was inhibited by (1–24). About 50% inhibition of binding of radiolabeled 8E11 to the CS protein was observed with a concentration of (1–24) of 10^{-5} M. In contrast, (1–12) had no effect at a concentration of 10^{-2} M. To determine whether the epitope recognized by 8E11 overlapped between consecutive repeats of (1–12), the peptide Ala_7-Asn_8-Ala_9-Gly_{10}-Gln_{11}-Pro_{12}-Gln_1-Ala_2-Gln_3-Asp_5-Gly_6 was synthesized. This was assayed as before and found to be inactive. We concluded that the monoclonal antibody 8E11 most likely recognized an epitope originating from the juxtaposition of two or more repeats of the (1–12) monomer.

Discussion

In this paper, the specificity of six hybridomas selected from the spleen of a mouse immunized with sporozoites of *P. knowlesi* is reported. Although the single criterion used in the selection procedure was the reactivity of culture supernatants with the surface membrane of the parasite (Cochrane et al., 1982), all of the monoclonal antibodies reacted with a single molecule, the CS protein. This is a stage- and species-specific protein (Vanderberg et al., 1969; Cochrane et al., 1976) distributed uniformly over the entire surface of the parasite and is shed when cross-linked by antibodies (Potocnjak et al., 1980). Evidence that CS proteins of different malaria species are part of a family of structurally related molecules was presented elsewhere (Santoro et al., 1982).

The previously reported results of immunoglical assays indicated that only one area of the CS molecule of *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium knowlesi*, and *Plasmodium berghei* was recognized by all homologous monoclonal antibodies and that this immunodominant region was multivalent with regard to the expression of a single epitope (Zavala et al., 1983). DNA sequence analysis showed that the immunogenic region of the *P. knowlesi* CS protein consisted of a tandem repeat by 12 subunits of 12 amino acids. Moreover, the epitope recognized by one monoclonal antibody (2G3) directed against the CS protein of *P. knowlesi* was included within a single subunit. This conclusion was based on the observation that a synthetic dodecapeptide very effectively inhibited the binding of the antibody to the native CS protein, while the tetraeicosapeptide interacted simultaneously with two molecules of 2G3.

The results of the present paper demonstrate that the epitopes reacting with five other monoclonal antibodies are also represented within the tetraeicosapeptide. The specificity of four monoclonal antibodies was studied further by synthesizing peptide segments corresponding to overlapping regions of their

repeats and measuring their ability to inhibit the binding of radiolabeled monoclonal antibodies to the CS protein. Despite the complexity of the intersecting curves (Figures 2–4) it is clear that the overall patterns of reactivity of three monoclonal antibodies with the various synthetic peptides were very similar.

As summarized in Table III, the dodecapeptide NH_2Gln-Ala-Gln-Gly-Asp-Gly-Ala-Asn-Ala-Gly-Gln-Pro-$CONH_2$, (1–12) and also the peptides (2–12), (3–12), (4–12), and (5–12) were very efficient inhibitors. However, following removal of Asp or Asp and Gly (positions 5 and 6), the degree of reactivity was considerably lower. The role of amino acids from the C-terminal end of (1–12) was also examined. As shown in panel B of Figure 3, removal of Pro (position 12) or Gln (position 11) from the dodecapeptide had a considerable effect in its reactivity with the monoclonal antibody 5H8. However, for monoclonals 2G3 and 8B8, the reactivity with (4–12) or (4–11) was almost identical, while (4–10) was much less reactive (panel B, Figures 3 and 4). It is interesting that the dodecapeptide contains only one proline (at the C-terminus) and that this residue was not essential for reactivity with two monoclonal antibodies. This reaction however, may play a structural role in providing a β turn in the tandem repeats. The multiple folding of the dodecamer may then lend itself to intermolecular interactions as well. It is conceivable that this 144 amino acid segment (the epitope repeated 12 times) contributes to the formation of a sheath on the surface of the protein, which may protect the parasite against the defense mechanisms of the vertebrate host and/or participate in the invasion of the vertebrate liver cells (Hollingdale et al., 1982).

Although the present data are insufficient to precisely define the structure of the epitope recognized by these three monoclonal antibodies, it is clear that they react with a sequence of amino acids around residues (4–11). In figure 2, we showed that the specificity of 8E11 differed markedly from that of the other monoclonal antibodies, in that the interaction of 8E11 with the CS protein was only inhibited by (1–24) and not by (1–12). To examine the possibility that 8E11 recognized a sequence overlapping between two tandem dodecapeptides of (1–12), several other peptides were synthesized and assayed for inhibitory activity, with negative results. We conclude that 8E11 probably reacted with a configurational or topographic epitope formed by joining two dodecamers.

Interestingly enough, differences in specificity between 8E11 and the other three antibodies had been previously reported. For example, 2G3, 8B8, and 5H8, but not 8E11, cross-reacted with sporozoites of *Plasmodium cynomolgi* (Cochrane et al., 1982). Also, 8E11 was exceptional in that it did not give a CS protein reaction when incubated with viable sporozoites (Cochrane et al., 1982), a phenomenon which depends on the cross-linking of CS molecules on the surface of the parasite (Potocnjak et al., 1980). Perhaps the configurational epitopes recognized by 8E11 in neighboring CS polypeptides of the sporozoite membrane are sufficiently far apart to prevent their reaction with single antibody molecules.

The size and shape of epitopes found in carbohydrate antigens were extensively studied in the past, but less is known about the structure of epitopes from protein molecules. Recently, some epitopes of protein antigens have been better defined at the level of their three-dimensional structure. Interestingly enough, in every instance the epitopes were formed not by the primary sequences alone but by the apposition of residues brought together by the folding of the polypeptide chain(s) of the native molecule. For example, monoclonal antibodies to sperm whale myoglobin did not react with any of the three CNBr cleavage fragments which collectively encompass the whole sequence of hemoglobin (Berzofsky et al., 1982; East et al., 1982). The epitope of a monoclonal antibody to lysozyme included a region containing the Arg-68–Arg-45 complex which borders the catalytic site (Smith-Gill et al., 1982). Monoclonal antibodies which recognized the A-chain loop (A 8–10) of insulin failed to bind to isolated A chains, or to synthetic peptides (Shroer et al., 1983).

The present findings contrast with these previous reports. Three out of four monoclonal antibodies to CS proteins reacted with a peptide consisting of eight consecutive amino acids. Because the epitope was repeated twice within a sequence of 24 consecutive amino acids, it is not likely that the combining sites recognized secondary or tertiary structures of the polypeptide chain. The striking immunogenicity of this epitope most likely is a reflection of the unusual structure of the CS protein, half of which consists of tandem repeats of 12 amino acids, each repeat containing a potential epitope. It is noteworthy that the streptococcal M protein type 24 also has a repeated peptide subunit, which contains the immunodominant epitope (Beachey et al., 1980). Perhaps the presence of repetitive structures within an antigen facilitates its interaction with immunocompetent cells and enhances the immune response.

Finally, it should be pointed out that monoclonal antibodies 2G3 and 8E11 (or the corresponding Fab fragments) not only bound to the *P. knowlesi* CS protein but also neutralized the infectivity of the sporozoites (Cochrane et al., 1982). In light of the present results, it seemed possible that polyclonal antibodies to synthetic peptides representing the repetitive epitope of the CS of *P. knowlesi* protein could have similar biological activities. Indeed, rabbits and mice have been immunized with the tetraeicosapeptide (1–24) conjugated to a carrier protein. Several animals made antibodies that reacted with the membrane of sporozoites and immune-precipitated the CS protein. In addition, sporozoites lost their infectivity when incubated in the serum from one of the immunized rabbits (J. Gysin et al., unpublished results). This observation raises hopes that if equivalent peptides from the CS proteins of human malaria parasites are found to be immunogenic in vivo, they may be used as vaccines.

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Registry No. Dodecapeptide (1–12), 92125-73-0; dodecapeptide (2–12), 92125-74-1; dodecapeptide (3–12), 92125-75-2; dodecapeptide (4–12), 92125-76-3; dodecapeptide (5–12), 92125-77-4; dodecapeptide (6–12), 92125-78-5; dodecapeptide (7–12), 92125-79-6; dodecapeptide (8–12), 92125-80-9; dodecapeptide (10–12), 92125-81-0; dodecapeptide (1–24), 92125-82-1; dodecapeptide (4–11), 92125-83-2; dodecapeptide (5–11), 92125-84-3; dodecapeptide (4–10), 92125-85-4; dodecapeptide (4–9), 92125-86-5; dodecapeptide (5–9), 92125-87-6; dodecapeptide (6–9), 92125-88-7; dodecapeptide (7–9), 92125-89-8; dodecapeptide (7–6), 92125-90-1.

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