Brief Definitive Report

NEUTRALIZATION OF THE INFECTIVITY OF SPOROZOITES OF *PLASMODIUM KNOWLESI* BY ANTIBODIES TO A SYNTHETIC PEPTIDE

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Sterile protective immunity against malaria has been achieved in hosts ranging from birds to humans by vaccination with irradiated sporozoites. The main antigens involved in protective immunity are the circumsporozoite (CS) proteins (reviewed in reference 1). These proteins are a family of surface membrane molecules with similar physicochemical and antigenic properties, and which are shed by the parasite when cross-linked by antibodies (circumsporozoite reaction). The CS molecules seem to be the most immunogenic constituent of sporozoites, and display a unique immunodominant region bearing at least two identical epitopes (2).

The gene that codes for the CS protein of the monkey malaria parasite *P. knowlesi* has been identified (3). The nucleotide sequence codes for 363 amino acids, a large portion of which consist of a repetitive segment of 12 amino acids, QAQGDGANAGQP (4). Several monoclonal antibodies against *P. knowlesi* sporozoites react with this dodecapeptide and neutralize the infectivity of the parasite (5).

These findings raised the possibility that this small peptide could be the basis for developing a synthetic vaccine against *P. knowlesi* malaria. The present investigation was undertaken as a first step in this direction. In this paper we study the properties of rabbit and monkey antibodies to the synthetic peptide, in particular, their reactivity with the *P. knowlesi* CS protein.

Materials and Methods

Peptide Synthesis. A tetraeicosapeptide (24Mer) representing two tandem repeats of the subunit epitope was synthesized by a modification of the Merrifield method (6).

Peptide Conjugation. The 24Mer was conjugated to keyhole limpet hemocyanin (KLH) or to bovine gamma globulin (BGG) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (7). After removal of the small molecular weight materials by filtration through a 2 × 100 cm Sephadex-G50 column equilibrated 1 N acetic acid, the conjugated proteins were lyophilized. The coupling efficiency was estimated by adding a trace amount of

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radiolabeled peptide to the conjugating mixture. We calculated that the 24Mer contributed ~20% of the weight of the conjugates.

Animals and Parasites. New Zealand white rabbits and *Saimiri sciureus* (squirrel) monkeys were used in this study. Sporozoites were dissected from the salivary glands of *Anopheles dirus* mosquitoes fed on *Macaca mulatta* (Rhesus monkeys) infected with the Malaysian H strain of *Plasmodium knowlesi.* These were kindly provided to us by Dr. R. Gwadz, National Institutes of Health.

Immunization. Two rabbits were immunized with 24Mer-BGG and one rabbit received 24Mer-KLH. On day 0 the animals were injected intradermally at several points in the dorsal region with 600 μg of the conjugates incorporated in complete Freund's adjuvant. They were boosted on three occasions with 400 μg of conjugate. On days 28 and 84 the conjugate was dissolved in saline and injected intravenously. On day 49 it was incorporated in incomplete Freund's adjuvant and injected intradermally. A fourth rabbit was injected intravenously with ~5×10^6 X-irradiated *P. knowlesi* sporozoites four times on days 0, 21, 35, and 63. Sera were collected for analysis on day 101.

S. sciureus monkeys were immunized with 24Mer-BGG and 24Mer-KLH, and BGG alone. 200 μg of the conjugate of BGG in Freund's complete adjuvant were injected intradermally at multiple sites on day 0. This was followed by an intravenous injection (400 μg in saline) on day 28 and an intradermal injection (400 μg in saline) on day 42.

Five *Saimiri* monkeys were immunized intradermally with irradiated *P. knowlesi* sporozoites (5×10^6) in Freund's adjuvant on day 0 and four times at 2–3-wk intervals in saline. A sixth monkey received the irradiated sporozoites intravenously four times at 2–3-wk intervals. Sera were obtained for analysis on day 63.

Serological Analysis. Antibody titers in the hyperimmune sera were determined by means of an immunoradiometric assay using as antigen either the 24Mer, or sporozoite extracts (8). Indirect immunofluorescence (IFA) was performed with 0.1% glutaraldehyde-fixed sporozoites. CSP reactions (9), that is, the shedding of the CS protein following incubation of viable parasites with anti-CS protein antibodies, were observed in the light microscope. Western blotting analysis was performed as in (10).

Sporozoite Neutralization. Equal volumes of pooled sera from two rabbits immunized with 24Mer-KLH or 24Mer-BGG were used to neutralize the infectivity of the parasite. The sera were obtained 2 wk after a fourth intradermal injection of antigen. 2×10^5 sporozoites of *P. knowlesi* were incubated at room temperature for 30 min with 1 ml of pooled preimmune or immune sera, and 0.5 ml of each sample injected intravenously into *Saimiri* monkeys. 1 or 2 d after inoculation, the animals were splenectomized. Beginning on day 7, blood smears were examined daily for the presence of blood stage parasites.

Results

The serum from one out of two rabbits immunized intradermally with the 24Mer-BGG, and from the single rabbit immunized with 24Mer-KLH reacted in the IRMA with the 24Mer and with extracts from salivary gland sporozoites (Table I). The IRMA titration curves of each antiserum with both immobilized antigens were almost identical (not shown). Only antibodies to BGG but not to 24Mer were detected in the serum of the other animal injected with 24Mer-BGG. All antisera that reacted with the 24Mer in the IRMA immunoprecipitated the CS protein (Fig. 1), and bound to the sporozoite surface membrane as shown by positive IFA reactions with glutaraldehyde-fixed parasites. The antiserum to 24Mer-KLH gave a CSP reaction when incubated with viable parasites.

Similar results were obtained with the serum of the squirrel monkeys immunized with the conjugates (Table II). The primates produced antibodies that recognized the tetraeicosapeptide and the genuine CS protein in the sporozoite extracts. One antipeptide antiserum, with the highest titer, gave the CSP reaction.

TABLE I

Antibody Response of Rabbits following Immunization with P. knowlesi Sporozoites, with 24Mer-BGG, or with 24Mer-KLH

Antigen used to immunize the rabbits	Log titer of IRMA‡ using as antigen:		CSP reaction
	24Mer	Extracts from salivary gland sporozoites	
Irradiated sporozoites	3.4	Not done*	Pos
24Mer-BGG	2.4, Neg	2.9, Neg	Neg
24Mer-KLH	2.9	2.9	Pos
None (preimmune sera)	All Neg	All Neg	All Neg

* IRMA with sporozoite extracts were not performed in this instance because the results would be uninterpretable. The sera of the rabbits immunized with irradiated salivary gland sporozoites also contained antibodies to the microorganisms present in the salivary glands of noninfected mosquitoes.

‡ Titers are defined as the highest serum dilution giving counts three times above the normal serum background.

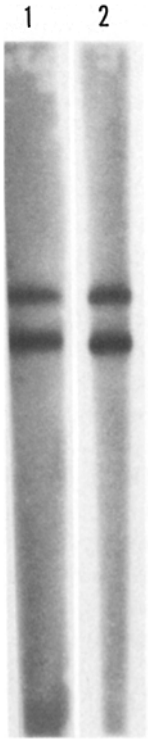

FIGURE 1. Western blot analysis of *P. knowlesi* extracts. Extracts of *P. knowlesi* sporozoites were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and proteins were transferred to nitrocellulose membranes. These were incubated with a rabbit antiserum to 24Mer-KLH (lane *1*) or to *P. knowlesi* sporozoites (lane *2*). The paper strips were washed and reincubated with an excess of affinity-purified, ^{125}I-labeled goat anti-rabbit IgG. The membranes were washed, dried, and an autoradiograph performed. As shown, both antisera recognized PK52 + PK50 (top band) and PK42 (bottom band).

TABLE II

Antibody Response of Saimiri sciureus to Immunization with P. knowlesi Sporozoites, or with 24Mer-BGG or with 24Mer-KLH

No. of animals	Antigen	Properties of the serum antibodies			
		Log titer*‡ of IRMA using as antigen:		Log titer of IFA	CSP reaction
		24Mer	Extracts from salivary gland sporozoites		
6	Irradiated sporozoites	4.3 3.4 3.1 2.8 2.8 2.5	Not done*	Not done	All Neg
3	24Mer-BGG	3.0 2.0 1.0	3.0 2.0 1.0	3.4 2.5 2.2	Pos Neg Neg
2	24Mer-KLH	3.0 Neg	3.0 Neg	2.2 Neg	All Neg
3	BGG (control)	All Neg	All Neg	All Neg	All Neg

*‡ See footnote to Table I.

TABLE III

Neutralization of Plasmodium knowlesi Sporozoite Infectivity by Antibodies to a Synthetic Peptide

Rabbit serum used to neutralize sporozoites	Properties of the neutralizing serum			Day of patency of recipient monkeys	
	Log titer of IRMA performed with:		CSP reaction		
	24Mer	Extracts from salivary gland sporozoites			
Preimmune	Neg	Neg	Neg	8	8
Pooled antisera to 24Mer-BGG and 24Mer-KLH	3	4	Pos	Neg (day 33)	Neg

Conversely, the rabbit and monkey sera obtained following immunization with X-irradiated sporozoites reacted in the IRMA performed using 24Mer as antigen. The titers of antipeptide antibodies in these sera were not significantly different from those in the sera of monkeys or rabbits injected with the 24Mer conjugates.

A pool of rabbit sera with the highest IRMA titers was assayed for the ability to neutralize sporozoites (Table III). Two *Saimiri* monkeys were each injected intravenously with 10^5 sporozoites that had been incubated in vitro for 30 min with preimmune rabbit serum. Asexual parasites of *P. knowlesi* were detected 8 d later in their blood. Two other *Saimiri* monkeys, which were injected with a mixture of 10^5 sporozoites and the pooled rabbit immune sera containing antipeptide antibodies, were not infected.

Discussion

These observations reveal the similarities between the properties of antibodies to a synthetic peptide (24Mer) and antibodies to the repetitive epitopes of the CS protein of *P. knowlesi*, generated by immunization with intact sporozoites. Both types of antibodies react with the synthetic peptide and with the surface membrane of sporozoites, giving the CSP reaction, and, more importantly, they neutralize the infectivity of the parasite.

The present findings support previous observations indicating that antibodies to the repetitive epitope of the CS protein recognize uninterrupted sequences of

amino acids in this molecule. For example, all monoclonal or polyclonal antibodies to this epitope react with the CS protein heated at 100°C for 30 min, or subjected to complete denaturation by treatment with 6 M guanidine and 1% β-mercaptoethanol (reference 4, and unpublished observations). Other studies show that most of the monoclonal antibodies to *P. knowlesi* recognized an epitope around the sequence GDGANAGQ (11). The experiments represented in Tables I and II show, in addition, that the reactivities of the polyclonal antipeptide antibodies with the 24Mer, or with the native CS protein, were quite similar, judging from the almost superimposable IRMA titration curves obtained with the corresponding immobilized antigens (not shown).

These observations should be contrasted with the findings of other investigators who have shown that antibodies to proteins usually do not recognize primary sequences, but rather three-dimensional structures. For example, antisera against influenza virus hemagglutinin do not react with any of 20 synthetic peptides covering 75% of the polypeptide sequence, although antipeptide antibodies bind specifically to the isolated protein and to the intact virus (12).

The simplest explanation for the unusual immunological properties of the repetitive epitope of the CS protein of *P. knowlesi* may be that the corresponding sequences are extended in the native molecule. A computer analysis of the amino acid sequence of the repetitive domain of the CS protein based on the Chou-Fasman method (13) does not predict the formation of α-helixes, or β-pleated sheaths. Moreover, each repeat contains three glycines and one proline, residues that tend to disrupt regular structures. If indeed the repetitive subunits of the CS molecule have little or no secondary structure, this portion of the molecule could be rather faithfully reproduced in a synthetic peptide, and used as a vaccine. Experiments are now in progress to evaluate the effectiveness of such a vaccine in protecting a very sensitive host, Rhesus monkeys, against infection with *P. knowlesi.* Preliminary results (Barnwell et al., in preparation) show that the degree of protection is as good as that afforded by vaccination with γ-irradiated sporozoites (14).

Also of interest is the finding that the 24Mer and the parasite extracts seemed equally effective in the IRMA assay as antigens to detect antibodies to CS proteins. It is likely, therefore, that the corresponding synthetic repeats from the human malaria parasites could be used to measure levels of anti-sporozoite antibodies in endemic areas.

Summary

Antibodies against a synthetic peptide representing the repetitive epitope of the circumsporozoite protein (CS) of *Plasmodium knowlesi* have properties similar to those of antibodies against the native protein. Either antibody reacts with the synthetic peptide, cross-links the CS protein on the membrane of the parasite giving the CSP reaction, and neutralizes the infectivity of sporozoites.

The synthetic peptide and sporozoite extracts were equally effective when used in an immunoradiometric assay as antigens to detect antibodies to CS proteins. It is likely that the corresponding synthetic repeats from the human malaria parasites could be used to measure levels of anti-sporozoite antibodies in endemic areas, or to evaluate the humoral response to anti-sporozoite vaccines.

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