Structure of the Gene Encoding the Circumsporozoite Protein of Plasmodium yoelii

A RODENT MODEL FOR EXAMINING ANTIMALARIAL SPOROZOITE VACCINES*

(Received for publication, September 22, 1986)

Altaf A. Lal‡§, Vidal F. de la Cruz, Judith A. Welsh‡, Yupin Charoenvit‡, W. Lee Maloy†, and Thomas F. McCutchan‡

From the §Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892 and ¶Infectious Diseases Program, Center, Naval Medical Research Institute, Bethesda, Maryland 20814-5055.

The gene encoding the circumsporozoite protein (CSP) from the rodent malaria parasite, Plasmodium yoelii, has been cloned and the nucleotide sequence has been determined. The gene encodes a protein of 367 amino acids as deduced from the nucleotide sequence. This gene is structurally similar to other Plasmodium spp. CSP genes in that it contains putative hydrophobic signal and anchor sequences at the NH₂ and COOH termini, respectively, two small regions (Regions I and II) that are conserved in all CSP genes analyzed to date, and a central region containing the immunodominant repeating peptide sequence. Unlike other CSP genes, however, the immunodominant repeat region of the gene is composed of two distinctly different types of tandem repeats. One repeating unit is six amino acids (Gln-Gly-Pro-Gly-Ala-Pro) in length while the other is only four (Gln-Gln-Pro-Pro) residues long. A second synthetic peptide, Gln-Gly-Pro-Gly-Ala-Pro, strongly inhibits the binding of anti-CSP monoclonal antibody to sporozoite antigens while another peptide, Gln-Gln-Pro-Pro × 4, weakly inhibits the binding of this same antibody to sporozoite antigens. This work should allow the construction of a mouse model system to parallel human vaccine trials.

Protective immunity to malaria can be obtained by immunization with irradiated sporozoites and it has been shown that the host antibody response to the sporozoite is mainly directed against the circumsporozoite protein (CSP) (1–3). The gene encoding this protein has been cloned from two human malaria parasites, Plasmodium falciparum (4, 5) and Plasmodium vivax (6, 7). Information obtained from the sequence of these molecules has been used to construct antigens for use in antimalarial vaccine trials (8–10). Although some questions relating to the effectiveness of a CSP-derived vaccine should be tested in human volunteers, many questions would be better approached using a rodent malaria model system. For example, studies directed toward understanding the involvement of T-cells in the host response can be best approached using a mouse model. As the host response to various CSP-derived vaccine constructs has been shown to be major histocompatibility complex-restricted (11), we have initiated an effort to design an animal model system to parallel human vaccine trials. We describe here the cloning of the CSP gene from the rodent malaria Plasmodium yoelii and show that a repeat peptide encoded by this gene is reactive with monoclonal antibodies encoded against P. yoelii sporozoites.

MATERIALS AND METHODS

Parasites—The P. yoelii 17× lethal (L) and nonlethal (NL) strains were grown in BALB/c mice. Asexual parasites used for DNA preparations were depleted of leukocytes and platelets and were saponin-lysed as previously described (12). P. yoelii sporozoites were isolated from infected Anopheles stephensi mosquitoes.

Gene Isolation and Nucleotide Sequence Determination—P. yoelii genomic DNA (L strain) was isolated and restricted in the presence of DraI endonuclease (Pharmacia F. L. Biochemicals). Fragments 1500 to 1800 base pairs in length were isolated after agarose gel electrophoresis, and ligated into the Smal site of plasmid pUC13. Recombinant molecules were used to transform the E. coli strain JM83. Colonies were transferred to nitrocellulose and probed with an oligonucleotide mixture, CCATCGAGTGTGAAATGGT, as described (7). A positive colony, pPy1, was identified. The pPy1 insert fragment was then subcloned into M13mp bacteriophage vectors and the nucleotide sequence was determined using the dideoxy procedure (13) as shown in Fig. 1.

Monoclonal antibody (mAb) was derived from BALB/c mice immunized with sporozoites of the 17× NL strain of P. yoelii. One mAb, NYS1 (Navy yoelii sporozoite mAb 1), gave a positive reaction in the circumsporozoite precipitin test with P. yoelii sporozoites, identified proteins of 96, 64, 75, 84 kDa in an immunoblot assay with sodium dodecyl sulfate-solubilized P. yoelii sporozoites, and neutralized sporozoite infectivity in C57Bl mice.2

Enzyme-linked Immunosorbent Assays—P. yoelii sporozoites were extracted in PBS containing 1% sodium dodecyl sulfate, and the extract was used to coat wells of Immunolon II plates (Dynatech) at 1000 sporozoite eq/well for 18 h at room temperature. Wels were then emptied and washed three times with PBS containing 0.05% Tween-20. Wells were further incubated for 1 h with the above buffer containing 1% bovine serum albumin. Peptides prepared by the solid-phase synthesis method (14) were serially diluted in the same buffer and incubated with horseradish peroxidase-labeled mAb for 1 h at room temperature. Each peptide/mAb mixture was then placed into microtiter wells and further incubated for 1 h at room temperature. The wells were then washed and incubated with the same antibody bound as described (4).

RESULTS

The DNA fragment that contains the CSP gene of P. yoelii was detected using an oligonucleotide probe whose sequence (Region II) is conserved in all CSP genes analyzed to date.

2 Charoenvit, Y., Leef, M. F., Sedegah, M., and Beaudoin, R. L. (1987) Infect. Immun., in press.
DNA sequence homology between the oligonucleotide probe and *P. yoelii* DNA was first shown by Southern blot analysis. Genomic DNA digested with the restriction nuclease *Dra*I was shown to contain a 1.6-kilobase fragment which hybridized to the Region II probe (data not shown). *Dra*I fragments of this size were isolated by agarose gel electrophoresis and ligated into the *Smal* site of the plasmid pUC13. Plasmids were transformed into the *E. coli* strain JM83 and the resultant colonies were probed with the Region II oligonucleotide. One recombinant plasmid, pPy1, was identified.

The sequencing strategy of the fragment is shown in Fig. 1. The complete sequence of the fragment is shown in Fig. 2. Like other CSP genes, there are hydrophobic stretches of amino acids at the NH$_2$ and COOH termini which probably represent signal and anchor sequences, respectively. Comparison of the amino acid sequences composing the putative signal sequences from the various CSP protein genes indicates that this area of the gene is not highly conserved among *Plasmodium* species, yet the hydrophilic character of this region is retained. A similar comparison of the putative anchor sequences at the COOH terminus of *Plasmodium* CSP gene sequences shows conservation of this region. For example, 17 of the last 21 amino acids at the COOH terminus of the *P. yoelii* and *P. vivax* genes are the same. In this portion of the molecule the *P. yoelii* gene seems to be more similar to the *P. vivax* (6) and *P. knowlesi* genes (16) than to the *P. falciparum* gene (4). Between the putative signal sequence and the Region I sequence (Fig. 1) is a set of five 27-base pair repeats, which will henceforth be referred to as the pre-Region I repeat (Fig. 1). A similar set of pre-Region I repeats has been described in the CSP gene of one isolate of *P. falciparum* (17). The pre-Region I repeats of *P. yoelii* and *P. falciparum* occur in the same relative position in the CSP gene, but no significant sequence homology is evident between the two.

In all CSP sequences analyzed to date, on either side of the central repeating domain that is the immunodominant segment of the protein, there are two regions that are well-conserved. These areas are referred to as Region I and Region II.

---

**Fig. 2. The nucleotide sequence of the *P. yoelii* CSP gene.** The one-letter amino acid codes are shown below the corresponding codons. Regions of the molecule are indicated in the figure.
II (Figs. 1 and 2) (4) and are also conserved in the P. yoelii CSP gene.

The repeating central region of the P. yoelii CSP gene is unusual. It consists of two different sets of tandem repeats. The set of repeats in the 5′ section of the region is made up of an 18-base pair sequence reiterated 15 times. The encoded six amino acids, Gln-Gly-Pro-Gly-Ala-Pro, are invariant in all of the repeats. The 18-nucleotide repeat is followed by a 12-nucleotide repeat unit reiterated eight times. The first and last unit of the 12-nucleotide repeat encode a variant set of four amino acids but the central six units encode the sequence Gln-Gln-Pro-Pro.

To verify that the repeat sequence deduced from the genomic clone was the same as that displayed on the surface of P. yoelii 17X NL sporozoites, we compared the effect of various peptides on the binding of monoclonal antibody to sporozoites. Enzyme-linked immunosorbent assays were conducted by binding P. yoelii sporozoite antigens to microritet wells followed by incubation with a mixture of a sporozoite-directed horseradish peroxidase-conjugated monoclonal antibody (NYS1) and various concentrations of peptide. The degree to which peptide inhibited binding of the antibody to sporozoites was assayed as described (15). Results shown in Fig. 3 show that the six-amino acid repeat, Gln-Gly-Pro-Gly-Ala-Pro × 3, inhibited binding of monoclonal antibody to sporozoite antigens much more strongly than did the four-amino acid repeat, Gln-Gln-Pro-Pro × 4. The control peptide, a 9-amino acid repeat, Gly-Gln-Pro-Ala-Gly-Asp-Arg-Ala-Asp × 2, derived from the immunodominant repeat of the P. vivax CSP sequence, had no effect on binding. The monoclonal antibody, NYS1, therefore seems to be specifically directed toward the Gln-Gly-Pro-Gly-Ala-Pro sequence.

**DISCUSSION**

Circumsporozoite protein-derived antimalarial vaccines have been the subject of intense investigation over the last several years. It is known that antibodies to the CS protein block parasite infection in vivo. There are several unknown factors, however, regarding the efficacy of an anti-sporozoite vaccine that could best be approached in an animal system. A mouse model system would allow us to investigate possible effects of cell-mediated immunity on protection against malaria. For example, a study of T-cell involvement in immunity should help investigators to interpret results of human vaccine trials as well as to design better vaccines. A model system would also allow one to assess the potential of immune pressure as a force in selecting for variant sporozoites with altered CSP genes. This of course is a critical factor in assessing vaccine potential. Even if parasite variants arise at only a very low rate in nature, which they do in the monkey malaria Plasmodium knowlesi (18), their presence, if actively selected for, would greatly reduce the overall effectiveness of a vaccine.

There are several mouse malaria species on which an animal model might be based, and certainly each one offers various advantages over the others. We have selected P. yoelii as a model for several reasons: 1) it is more genetically diverse than species like Plasmodium berghei (19); 2) cloned lines of parasites are available which are infective to mosquitoes (20); and 3) the laboratory procedures for infecting mosquitoes are less complicated and require fewer facilities.

As the development of a model system requires the isolation and characterization of a mouse malaria CSP gene, we have isolated and determined the nucleotide sequence of the gene from P. yoelii. The P. yoelii gene is structurally similar to other CSP genes. Since the goal of this work is to develop a parallel rodent system to the human vaccine trials, the structural similarity of the human and rodent CSP genes is encouraging. Putative leader and anchor sequences are found at the NH₂ and COOH termini of the protein. A central immunodominant repeating region is surrounded by areas of nucleotide sequence that are common to all CSP genes. The immunodominant repeat Gln-Gly-Pro-Gly-Ala-Pro × 15 is specific to P. yoelii and yet is composed from the same restricted set of amino acids that are found in other CS protein repeats (i.e., glycine, alanine, asparagine, aspartic acid, arginine, proline, glutamine, and valine). This set of repeats is strongly reactive with monoclonal antibodies that are directed to the surface of sporozoites. A second set of amino acid repeats, Gln-Gln-Pro-Pro × 6, is also found in the central immunodominant area of the molecule. It is not known whether or not this repeat is immunogenic on the surface of sporozoites. A second set of repeats has not been found in any of the primate malaria parasites and is the only significant structural difference between the human and rodent malarial genes.

The other surprising feature of this gene is that the final 20 amino acids are considerably more similar to the COOH-terminal amino acids of P. vivax and P. knowlesi than to the COOH-terminal amino acids of P. falciparum. It has been suggested that the rodent and avian malaria are evolutionarily more similar to P. falciparum than to other primate malaria parasites (21). This observation seems inconsistent with that view of evolutionary relationships.

The effect of cell-mediated immunity on the protective response to sporozoite infection has been virtually unexplored. Investigation of this area may well have a critical effect on vaccine design and on the interpretation of the results of human vaccine trials. The work reported here should allow continued progress in the study, development, and testing of vaccine constructions.

Acknowledgement—We wish to thank Dr. Richard Beaudoin for his support with the sporozoite work.

**REFERENCES**

1. Nussenzweig, R. S., and Nussenzweig, V. (1984) Philos. Trans. R. Soc. Lond. B Biol. Sci. 307, 117–128
2. Nardin, E. H., Nussenzweig, V., Nussenzweig, R. S., Collins, W. E., Harinasuta, K., Tapchaisri, P., and Chomcharn, Y. (1982) J. Exp. Med. 156, 20–30
3. Zavala, F., Cochrane, A. H., Nardin, E. H., Nussenzweig, R. S., and Nussenzweig, V. (1983) J. Exp. Med. 157, 1947–1957
4. Dame, J. B., Williams, J. L., McCutchan, T. F., Weber, J. L.,
Circumsporozoite Gene from Plasmodium yoelii

Wirtz, R. A., Hockmeyer, W. T., Maloy, W. L., Haynes, J. D., Schneider, I., Roberts, D., Sanders, G. S., Reddy, E. P., Diggs, C. L., and Miller, L. H. (1984) Science 225, 593–599

5. Enea, V., Ellis, J., Zavaia, F., Arnot, D. E., Asavanich, A., Masuda, A., Quakyi, I., and Nussenzweig, R. S. (1984) Science 225, 628–630

6. Arnot, D. E., Barnwell, J. W., Tam, J. P., Nussenzweig, V., Nussenzweig, R. S., and Enea, V. (1985) Science 230, 815–818

7. McCutchan, T. F., Lal, A. A., de la Cruz, V. F., Miller, L. H., Maloy, W. L., Charoenvit, Y., Beaudoin, R. L., Guerry, P., Wistar, R., Jr., Hoffman, S. L., Hockmeyer, W. T., Collins, W. E., and Wirth, D. (1985) Science 230, 1381–1383

8. Young, J. F., Hockmeyer, W. T., Gross, M., Ballou, W. R., Wirtz, R. A., Trasper, J. H., Beaudoin, R. L., Hollingdale, M. R., Miller, L. H., Diggs, C. L., and Rosenberg, M. (1985) Science 228, 968–962

9. Ballou, W. R., Rothbard, J., Wirtz, R. A., Gordon, D. M., Williams, J. S., Gore, R. W., Schneider, I., Hollingdale, M. R., Beaudoin, R. L., Maloy, W. L., Miller, L. H., and Hockmeyer, W. T. (1985) Science 228, 996–999

10. Zavaia, F., Tam, J. P., Hollingdale, M. R., Cochrane, A. H., Quakyi, I., Nussenzweig, R. S., and Nussenzweig, V. (1985) Science 228, 1436–1440

11. Good, M. P., Berzofsky, J. A., Maloy, W. L., Hayashi, Y., Fujii, N., Hockmeyer, W. T., and Miller, L. H. (1986) J. Exp. Med. 164, 655–660

12. Dame, J. B., and McCutchan, T. F. (1983) Mol. Biochem. Parasitol. 8, 263–279

13. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467

14. Merrifield, R. B., and Marglin, A. (1970) Annu. Rev. Biochem. 39, 841–866

15. Nakane, P. K., and Kawaoi, A. (1974) J. Histochem. Cytochem. 22, 1084–1091

16. Ozaki, I. S., Svec, P., Nussenzweig, R. S., Nussenzweig, V., and Godson, G. N. (1983) Cell 34, 815–822

17. de la Cruz, V. F., and McCutchan, T. F. (1986) Nucleic Acids Res. 14, 4695

18. Sharma, S., Svec, P., Mitchell, G. H., and Godson, G. N. (1985) Science 229, 779–782

19. Carter, R., and Diggs, C. L. (1977) in Parasitic Protozoa (Kreier, J., ed) Vol. III, Academic Press, New York

20. Beale, G. H., Carter, R., and Walliker, D. (1977) in Rodent Malaria (Killick-Kendrick, R., and Peters, W., eds) Academic Press, New York

21. McCutchan, T. F., Dame, J. B., Miller, L. H., and Barnwell, J. (1984) Science 225, 808–811