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P/00/008
Section 29(1)
Regulation 3.1(2)

AUSTRALIA

Patents Act 1990

NOTICE OF ENTITLEMENT

NEW YORK UNIVERSITY, the applicant in respect of Application No. 15936/88 state the following:-

The Nominated Person is entitled to the grant of the patent because the Nominated Person derives title to the invention from the inventor Victor NUSSENZWEIG by assignment.

The Nominated Person is entitled to claim priority from the application listed in the declaration under Article 8 of the PCT because the Nominated Person made the application or has entitlement from the applicant in respect of the application listed in the declaration under Article 8 of the PCT, and because that application was the first application made in a Convention country in respect of the invention.

DATED this 16th day of September, 1991.

.....
a member of the firm of
ARTHUR S. CAVE & CO. for
and on behalf of the
applicant(s).

3075M/VMJ

5986/23992-AU

224146/GHG

Regulation 12(2)

PATENT DECLARATION FORM (CONVENTION)

COMMONWEALTH OF AUSTRALIA

Patents Act 1952

DECLARATION IN SUPPORT OF A CONVENTION APPLICATION FOR A PATENT

In support of the Convention application made for a patent for an invention entitled: IMMUNOGENIC POLYPEPTIDE AND METHOD FOR PURIFYING IT

I/We ..Isaac T. Kohlberg..... (full name of declarant) of ....70 Washington Square South, New York, New York 10012.... (full address) do solemnly and sincerely declare as follows:-

1. I am/~~we are~~ authorised by NEW YORK UNIVERSITY the applicant for the patent to make this declaration on its behalf.

2. The basic Application(s) as defined by Section 141 of the Act was/were made in the following country or countries on the following date namely:-
in United States of America on 30 March 1987 ~~by New York University~~

3. Victor Nussenzweig of 110 Bleeker Street, New York, New York 10012, United States of America is the actual inventor of the invention and the facts upon which the applicant(s) are entitled to make the application are as follows:

The Applicant is the Assignee of the said invention from the actual inventor.

4. The basic application(s) referred to in paragraph 2 of this Declaration was the first application(s) made in a Convention country in respect of the invention the subject of the application.

Declared at New York this 29th day of Oct. , 19 90

To:
The Commissioner of Patents

ARTHUR S. CAVE & CO.
PATENT AND TRADE MARK ATTORNEYS
SYDNEY

Signature of Declarant(s)
Isaac T. Kohlberg
Vice President - Industrial Liaison

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(56) Prior Art Documents
AU 17914/88 C07K
WO 87/00533

(57) This invention relates to a synthetic polypeptide (preferably produced by recombinant DNA techniques) comprising an amino acid sequence incorporating
(a) a portion of the P.vivax circumsporozoite (CS) protein including the region of the repeat immunodominant epitope of said protein and
(b) another portion of such protein which is conserved among the different malarial species;
and to a method for purifying such a polypeptide.

CLAIM

1. A method for purifying a polypeptide consisting essentially of an amino acid sequence consisting essentially of at least two tandemly repeating sequences idential to those of the immunodominant epitope region of the circumsporozoite protein of P.vivax, from an impure preparation of said polypeptide said impure preparation also containing one or more other protein species as impurities, said method comprising the steps of:

(a) subjecting said preparation to a temperature of 100'C for a period of time sufficient to cause denaturation and precipitation of the bulk of said protein impurities, while leaving said polypeptide in solution;

.../2

(b) removing and discarding said precipitate and retaining the supernatant;

(c) separating the solid constituents of said supernatant;

(d) redissolving said solid constituents in an aqueous ionic buffer solution;

(e) subjecting said solution to ion-exchange chromatography on a first chromatography column, thereby causing said polypeptide to be retained on said column, while a portion of the remaining impurities of said polypeptide are excluded in the effluent, and eluting said polypeptide using an ionic gradient;

(f) decreasing the ionic strength of said eluant containing said polypeptide;

(g) subjecting said eluant to a molecular-sieve chromatography on a second chromatography column, and eluting said polypeptide from said colum.

6. A synthetic polypeptide comprising at least one epitope of the circumsporozoite protein of P.vivax other than the immunodominant epitope which consists of a repeating sequence of amino acids, said polypeptide being expressed by a recombinant organism and having an amino acid sequence containing:

(a) a repetitive amino acid sequence of said circumsporozoite protein which represents a repeating immunodominant epitope region of said protein; and

(b) a portion of the amino acid sequence of the circumsporozoite protein of Plasmodium vivax which immediately precedes the repeating epitope region of said protein proximal to the N-terminal of said protein and which is conserved in the CS proteins of different malarial species.

16. A synthetic polypeptide having an amino acid sequence comprising:

(a) an immunogenic repetitive sequence corresponding to the repetitive immunodominant epitope region of P.vivax circumsporozoite protein; and

.../3

(b) at the N-terminal of said repetitive sequence a non-repetitive amino acid sequence which in the native circumsporozoite P.vivax protein (1) immediately precedes and is located at the 5'-end of the repetitive immunodominant epitope region of said protein, and (2) is conserved among the different malarial species.

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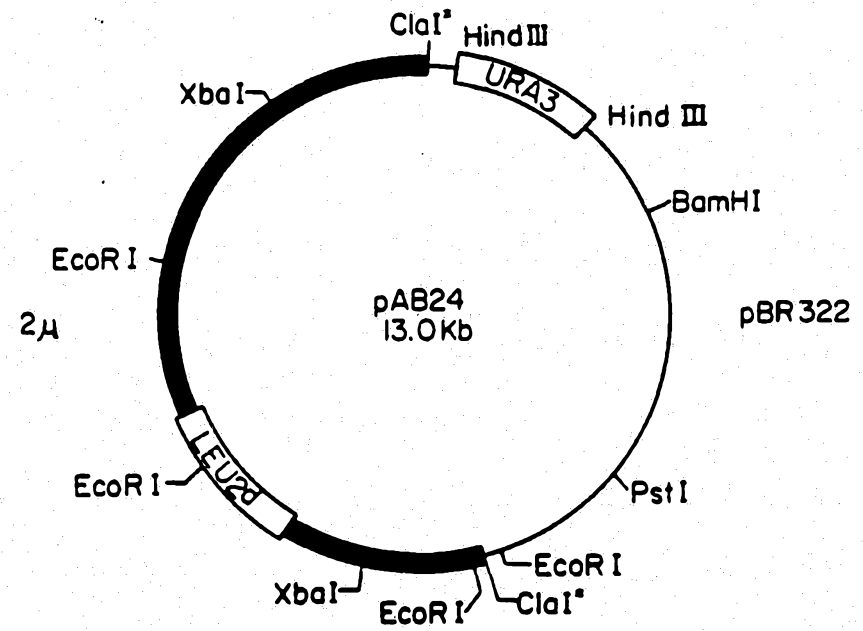

YEAST EXPRESSION PLASMID pAB24

(57) Abstract

This invention relates to a synthetic polypeptide (preferably produced by recombinant DNA techniques) comprising an amino acid sequence incorporating (a) a portion of the *P.vivax* circumsporozoite (CS) protein including the region of the repeat immunodominant epitope of said protein and (b) another portion of such protein which is conserved among the different malarial species; and to a method for purifying such a polypeptide.

IMMUNOGENIC POLYPEPTIDE AND METHOD FOR PURIFYING IT

Field of the Invention

This invention relates to an immunogenic polypeptide expressed by a recombinant yeast and comprising an amino acid sequence incorporating a portion of the P.vivax circumsporozoite (CS) protein including the region of the repeat immunodominant epitope of said protein; and to a method for purifying this polypeptide.

The immunogenic properties of the P.vivax CS protein and, in particular, those of a subsequence of this protein have been previously described, in the above-identified Arnot et al application and in Arnot, D.E. et al. Science 230:815-818, 1985 (also incorporated by reference).

In fact, the entire P.vivax CS gene has been identified and its sequence described in the above-mentioned documents. As described therein, the P.vivax CS protein comprises a central region of 19 tandem repeats of the sequence:

Asp-Arg-Ala-Asp/Ala-Gly-Gln-Pro-Ala-Gly

or, by another system of notation:

D R A D/A G Q P A G

This region contains the repeat immunodominant epitope of the P.vivax CS protein.

(The correspondence between the two notation systems is as follows: A = alanine, C = cysteine, D = aspartic acid, E = glutamic acid, F = phenylalanine, G = glycine, H = histidine, I = isoleucine, K = lysine, L = leucine, M = methionine, N = asparagine, P = proline, Q = glutamine, R = arginine, S = serine, T = threonine, V = valine, W = tryptophan, and Y = tyrosine.)

Synthetic peptides consisting essentially of 18 amino acid residues (i.e. two repeats of the above repeating sequence and cyclic permutations thereof -- such as Asp-Gly-Gln-Pro-Ala-Gly-Asp-Arg-Ala) are recognized by antibodies to the native P.vivax CS protein and in turn generate antibodies (when injected in mammals) which recognize, and bind to, the native CS protein. Hence, such peptides have utility in a vaccine against malaria.

One possible approach to making a vaccine against P.vivax malaria would be to synthesize immunogenic peptides including a sequence corresponding to that of the immunodominant epitope of the P.vivax CS protein (i.e., containing one and preferably two or more repeats). In the case of P.vivax, however, because the sequence of the repeating unit of the CS protein is rather long (in contrast to that of P.falciparum) classical peptide synthesis cannot be reliably used. Use of classical synthetic techniques to make longer peptides is especially inconvenient when practiced on a large scale, as would be necessary for manufacture of a malaria vaccine that would be distributed to millions of people worldwide.

Moreover, coupling of the synthesized peptide to a larger molecule that would play the role of a carrier or adjuvant would be necessary. In addition, in most instances, only a minor proportion of antibodies to a synthetic peptide recognize the same sequence in the native protein. That is, even if the synthetic peptide coupled to a carrier protein is shown to be immunogenic, most of the antibodies produced may

not mediate protective immunity against the pathogen. In this respect, the synthetic peptide $(NANP)_3$ which is a candidate for preparing a vaccine against P.falciparum is exceptional, since at least 70% of the antipeptide antibodies recognize the malaria sporozoites. The explanation for this unusual finding is probably that the $(NANP)_3$ peptide contains many prolines (P) and asparagines (N), amino acids which are frequently found in reverse turns of the protein molecule. Perhaps in this instance, a preferred configuration of $(NANP)_3$ in solution, mimics that of the same sequence in the native CS protein. However, from the examination of the amino acid sequence of the P.vivax repeats, it does not seem likely that a P.vivax peptide will behave similarly to $(NANP)_3$.

For these reasons, an alternative technique was sought for manufacturing an immunogenic peptide that could be used in a vaccine against P.vivax malaria. The present inventors looked to recombinant DNA and genetic engineering techniques to express immunogenic polypeptides that would be used to confer immunity to mammals against P.vivax malaria.

Although the technology was readily available for constructing recombinant bacteria that would express a portion or all of the repeating amino acid sequence of the P.vivax protein, the present inventors searched for an alternative expression system for the following reasons:

First, the expression system should be reliable and able to produce the polypeptide of interest consistently and with a high yield. Bacteria can be difficult to handle when produced in mass culture and overexpressing a foreign protein product.

Second, and more important, expression products of bacteria are often difficult to purify from pyrogenic impurities and other inflammatory and toxic agents that either are co-expressed by the bacteria, or are necessary additives in a bacterial growth medium.

Third, expression products of bacteria are most often fusion proteins, that is, they contain additional non-relevant sequences originating from the genes associated with the bac-

teria.

The present inventors looked to yeast expression systems, which have been substantially improved by recent advances in the field of recombinant DNA technology. Yeasts are hardier organisms than bacteria and much easier to grow in mass culture. Moreover, recent advances in yeast genetics and cloning have increased the yields of yeast expression systems.

Purification of expression products of recombinant yeast systems is not a priori more complicated than purification of products of bacterial recombinant systems and depends mostly on the characteristics of the particular protein sought to be purified. Nevertheless, such purification is generally more complicated than that of a peptide or protein produced by classical peptide synthetic techniques.

The present inventors chose a general yeast expression system that is the subject matter of United States Patent 4880734 filed on May 29, 1986 in the name of R.L. Burke et al and assigned to Chiron Corporation This is incorporated by reference herein. A portion of the P.vivax gene was chosen (for incorporation into the yeast organisms) which included the entire tandemly repeated sequence plus another segment preceding the repeat region (when the sequence is read from the N-terminal to the c-terminal). This segment incorporates a sequence that is highly conserved in all malarial species and has been previously found to be immunogenic in its own right. (See Australian Patent Application 48037/85 incorporated by reference herein.)

Objects of the Invention

Accordingly, it is an object of the present invention to provide an immunogenic yeast-engineered polypeptide immuno-chemically reactive with monoclonal antibodies against P.vivax circumsporozoite (CS) protein, and useful in a vaccine preparation against malaria.

It is another object of the present invention to provide a method for making the foregoing immunogenic polypeptide

which could be practiced on a large scale and with a high yield.

Another object is to provide an immunogenic polypeptide suitable for incorporation in an anti-malaria vaccine preparation.

Another object is to provide an immunogenic polypeptide suitable for use in an anti-malaria vaccine preparation without being coupled to a carrier.

Another object is to provide a method for purifying the foregoing yeast-engineered polypeptide from an impure preparation thereof comprising lysed yeast cell material and yeast culture media.

These and other objects of the invention will be apparent to those skilled in the art in view of the present specification, accompanying claims and appended drawings.

Brief Description of the Drawings

Figure 1 is a diagram of the construction of the yeast expression vector used in the present invention.

Figure 2 is a map of yeast plasmid pAB24.

Figure 3 is a polyacrylamide gel obtained using lysates from P. vivax/pAB24-transformed yeast and control yeast.

Figure 4 is a Western analysis of lysates from yeast transformed with the P. vivax/pAB24 vector.

Figure 5 is a plot of (a) the optical density profile of the material purified according to the invention (solid line); (b) the conductivity of the eluting buffer (solid line-black points); and (c) the percent activity of eluted fractions in inhibiting the binding of antibody to native CS protein.

Figure 6 is a radioautograph of a SDS-PAGE gel demonstrating the purity of the engineered polypeptide when made and purified in accordance with the present invention.

Figure 7 is a radioautograph of an isoelectric focusing gel showing the engineered polypeptide of the present invention.

Figure 8 is a standard curve for a competitive radioimmunoassay and shows the inhibition of binding of labeled, en-

sion vector,

(e) transforming yeast with said expression vector,

(f) culturing said yeast under conditions which allow expression of the protein encoded by said vector, and

(g) harvesting the medium containing said expressed protein.

Detailed Description of the Invention

The P.vivax CS gene is set forth below (together with the amino acid sequence for which it codes)

```
[N-terminus] ATGAAGAACTTCATTCTCTTGGCTGTTTCTTCCATCCTGTTGGTGGACTTG
                M  K  N  F  I  L  L  A  V  S  S  I  L  L  V  D  L
TTCCCCACGCACTGCGGGCACAATGTAGATCTGTCCAAGGCCATAAACTTAAATGGAGTAAAC
 F  P  T  H  C  G  H  N  V  D  L  S  K  A  I  N  L  N  G  V  N
TTCAATAATGTAGACGCCAGTTCACTTGGCGCGGCACACGTAGGACAAAGTGCTAGCCGAGGCAG
 F  N  N  V  D  A  S  S  L  G  A  A  H  V  G  Q  S  A  S  R  G  R
AGGCCTTGGCGAGAACCCAGATGACGAGGAAGGAGATGCTAAAAAAAAAAAAGGATGGAAAGAAA
  G  L  G  E  N  P  D  D  E  E  G  D  A  K  K  K  K  D  G  K  K
GCAGAACCAAAAAATCCACGTGAAAATAAGCTGAAGCAACCAGGAGACAGAGCAGATGGACAGC
 A  E  P  K  N  P  R  E  N  K  L  K  Q  P  G  D  R  A  D  G  Q
CAGCAGGAGACAGAGCAGATGGACAGCCAGCAGGTGATAGAGCAGATGGACAACCAGCAGGAGA
P  A  G  D  R  A  D  G  Q  P  A  G  D  R  A  D  G  Q  P  A  G  D
TAGAGCAGCTGGACAACCAGCAGGAGATAGAGCAGATGGACAGCCAGCAGGAGACAGAGCAGAT
  R  A  A  G  Q  P  A  G  D  R  A  D  G  Q  P  A  G  D  R  A  D
GGACAGCCAGCAGGAGACAGAGCAGATGGACAACCAGCAGGAGACAGAGCAGATGGACAACCAG
 G  Q  P  A  G  D  R  A  D  G  Q  P  A  G  D  R  A  D  G  Q  P
CAGGTGATAGAGCAGCTGGACAACCAGCAGGTGATAGAGCAGCTGGACAACCAGCAGGAGATAG
A  G  D  R  A  A  G  Q  P  A  G  D  R  A  A  G  Q  P  A  G  D  R
AGCAGATGGACAGCCAGCAGGAGATAGAGCAGCTGGACAGCCAGCAGGAGATAGAGCAGATGGA
  A  D  G  Q  P  A  G  D  R  A  A  G  Q  P  A  G  D  R  A  D  G
CAGCCAGCAGGAGATAGAGCAGCTGGACAGCCAGCAGGAGATAGAGCAGATGGACAGCCAGCAG
 Q  P  A  G  D  R  A  A  G  Q  P  A  G  D  R  A  D  G  Q  P  A
GAGATAGAGCAGCTGGACAGCCAGCAGGAGATAGAGCAGCTGGACAGCCAGCAGGAGATAGAGC
G  D  R  A  A  G  Q  P  A  G  D  R  A  A  G  Q  P  A  G  D  R  A
AGCTGGACAGCCAGCAGGAGATAGAGCAGCTGGACAGCCAGCAGGAAATGGTGCAGGTGGACAG
  A  G  Q  P  A  G  D  R  A  A  G  Q  P  A  G  N  G  A  G  G  Q
GCAGCAGGAGGAAACGCAGGAGGACAGGGACAAAATAATGAAGGTGCGAATGCCCCAAATG
 A  A  G  G  N  A  G  G  Q  G  Q  N  N  E  G  A  N  A  P  N
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GAAAGTCTGTGAAAGAATACCTAGATAAAGTTAGAGCTACCGTTGGCACCGAATGGACTCCATG
E K S V K E Y L D K V R A T V G T E W T P C
CAGTGTAACCTGTGGAGTGGGTGTAAGAGTCAGAAGCAGAGTTAATGCAGCTAACAAAAAACCA
S V T C G V G V R V R S R V N A A N K K P
GAGGATCTTACTTTGAATGACCTTGAGACTGATGTTTGTACAATGGATAAGTGTGCTGGCATAT
E D L T L N D L E T D V C T M D K C A G I
TTAACGTTGTGAGTAATTCATTAGGGCTAGTCATATTGTTAGTCCTAGCATTATTCAATTAA
F N V V S N S L G L V I L L V L A L F N
[C-terminus]

The DNA fragment chosen for insertion in the yeast host was:

GCAGAACCAAAAAATCCACGTGAAAATAAGCTGAAGCAACCAGGAGACAGAGCAGATGGACAGC
A E P K N P R E N K L K Q P G D R A D G Q
CAGCAGGAGACAGAGCAGATGGACAGCCAGCAGGTGATAGAGCAGATGGACAACCAGCAGGAGA
P A G D R A D G Q P A G D R A D G Q P A G D
TAGAGCAGCTGGACAACCAGCAGGAGATAGAGCAGATGGACAGCCAGCAGGAGACAGAGCAGAT
R A A G Q P A G D R A D G Q P A G D R A D
GGACAGCCAGCAGGAGACAGAGCAGATGGACAACCAGCAGGAGACAGAGCAGATGGACAACCAG
G Q P A G D R A D G Q P A G D R A D G Q P
CAGGTGATAGAGCAGCTGGACAACCAGCAGGTGATAGAGCAGCTGGACAACCAGCAGGAGATAG
A G D R A A G Q P A G D R A A G Q P A G D R
AGCAGATGGACAGCCAGCAGGAGATAGAGCAGCTGGACAGCCAGCAGGAGATAGAGCAGATGGA
A D G Q P A G D R A A G Q P A G D R A D G
CAGCCAGCAGGAGATAGAGCAGCTGGACAGCCAGCAGGAGATAGAGCAGATGGACAGCCAGCAG
Q P A G D R A A G Q P A G D R A D G Q P A
GAGATAGAGCAGCTGGACAGCCAGCAGGAGATAGAGCAGCTGGACAGCCAGCAGGAGATAGAGC
G D R A A G Q P A G D R A A G Q P A G D R A
AGCTGGACAGCCAGCAGGAGATAGAGCAGCTGGACAGCCAGCAGGAAATGGTGCAGGTGGACAG
A G Q P A G D R A A G Q P A G N G A G G Q
GCAGCAGGAGGAAACGCAGGAGGACAGGGACAAAATAATGAAGGTGCGAATGCCCCAAATG
A A G G N A G G Q G Q N N E G A N A P N
AAAAGTCTGTGAAAGAATACCTAGATAAAGTTAGAGCTACCGTTGGCACCGAATGGACTCCA
E K S V K E Y L D K V R A T V G T E W T P

This fragment includes the entire tandem repeat sequence plus a region that is substantially parallel to Region I of Dame, et al Science 225:628 (1984), precedes the repeat region, and codes for the amino acid sequence A E P K N P R E N K

L K Q P G.

This sequence includes the subsequence K L K Q P which is conserved in all malarial species that have been investigated to date.

The foregoing DNA fragment was obtained from the entire gene by subcloning a 15kb BglII fragment isolated as described by Arnot et. al (U.S. Patent Application Serial No. 754,645, and Science 230:815-818, November 15, 1985) both incorporated by reference. It was then inserted in the DNA of modified yeast plasmid pAB24 as described in Example 1 below.

For expression of the P. vivax CS antigen in yeast, a hybrid promoter comprising the strong yeast glyceraldehyde-3-phosphate dehydrogenase and the glucose regulatable alcohol dehydrogenase-2 (ADH-2) promoter was used. Fusion of this promoter to heterologous genes allows the growth of yeast cultures to high density using glucose as a carbon source. Depletion of glucose in the media during fermentative growth leads to concomitant induction of expression of the heterologous protein. Incorporation of the plasmid into high copy number, autonomously replicating yeast plasmids, and transformation of yeast cells generated strains capable of expressing high levels of CS proteins on induction.

The yeast was grown in culture in YEP medium (1% w/v yeast extract, 2% peptone) with 1% glucose as described in Example 1 below. Two hundred liters of yeast material were thus obtained and stored at - 80 °C.

The thus obtained yeast material contained a complex mixture of different yeast proteins as well as culture medium additives. Gel electrophoresis of this material on 7.5% sodium dodecyl sulfate polyacrylamide gel gave an indication of its heterogeneity (see Figure 6, lane 1). The expression procedure and plasmid construction are outlined in Figure 1 and described in Example 1, below.

It had been observed that all antibodies to the P. knowlesi CS protein recognized the immunodominant epitope region of this protein even after the CS protein had been heated at 100°C for 30 minutes or subjected to complete

denaturation by treatment with 6M guanidine and 1% beta-mercaptoethanol (Gysin, J., et al J. Exp. Med. 160:935, 1984; Godson, G.N., et al Nature 305:29, 1983).

This observation concerned the entire P. knowlesi CS protein and by no means established either that the P.vivax CS protein would show similar behavior upon heating or that fragments thereof, consisting essentially of the polypeptide employed herein and encompassing the epitope region, would continue to be immunochemically reactive with anti-P.vivax CS protein antibodies, after being subjected to heating conditions that normally result in denaturation of proteins. Another imponderable was that, in the present invention, the fragment of the CS protein was mixed with very large amounts of non-relevant materials. Heating of the mixture might lead to the formulation of aggregates with other polypeptides and masking of the epitopes and/or coprecipitation. It was also not known whether such fragments would continue to be immunogenic after being subjected to the above heating treatment.

Nevertheless, the present inventors used a heating step as the initial step in the purification of the yeast-engineered P.vivax CS polypeptide. (As used in this patent application, "polypeptide" will refer to a relatively long protein fragment, "protein" will refer to the entire protein, and "peptide" will refer to a relatively short peptide, e.g., one containing 30 amino acid residues or less. Regarding the use of the word "sequence", it will be understood that polypeptides and peptides in accordance with the present invention will be functionally equivalent if they have the same sequence whether the sequence is set forth from the N to the C terminus or from the C to the N terminus).

The mixture was then subjected to heating at 100°C, which resulted in massive precipitation of lysed yeast cell material. The supernatant was separated, dried and lyophilized. The lyophilized supernatant residue showed a substantial improvement in purity over the yeast extract (see Fig. 6, lane 4).

A solution of the lyophilized material was then further

purified sequentially by (a) anion-exchange chromatography using an electrolyte gradient to elute the engineered CS polypeptide; and (b) molecular sieve chromatography.

The fractions containing CS polypeptide activity were identified with a radioimmunoassay. A small amount of the highly purified yeast material was radiolabelled to a high specific activity with ^{125}I. Each fraction was assayed by a classical radioimmunoassay for its capacity to inhibit the binding of the labelled material to immobilized anti-P.vivax monoclonal antibody, directed against the repetitive epitope of the P.vivax CS protein.

A major advantage of the purification process of the present invention is its simplicity and its ready adaptability to scale-up. The thus purified engineered P.vivax CS polypeptide is homogeneous by SDS-PAGE and isoelectric focusing and is thus expected to be substantially free of pyrogenic, inflammatory and toxic impurities that may have been associated with the lysed yeast cell material and yeast culture media.

The yield of the combination of the yeast expression procedure and the purification process of the present invention proved to be 13 mg of pure CS polypeptide per liter of yeast culture. Given that 200 liters of this culture were produced in three days using pilot scale equipment (250 liter fermenter), it is apparent that large amounts of the engineered CS polypeptide can be made available in a short period of time.

A 200-liter stock of yeast extract contains sufficient engineered CS polypeptide to immunize about 25,000 humans against P.vivax, using 100 micrograms of polypeptide per person.

Major advantages of the CS polypeptide produced in accordance with the present invention include that the engineered peptide:

(a) can be produced in large scale free of impurities and of non-relevant antigens;

(b) represents a large fragment of the native CS molecule;

(c) has been shown to be highly immunogenic in rodents

using aluminum hydroxide as an adjuvant;

(d) the antibodies which are produced react with both the "repeats" and a conserved region of the CS molecule.

Antibodies to the "repeats" have been shown to neutralize parasite injectivity very effectively. Antibodies to a peptide containing this conserved region also neutralized parasite injectivity, but the inhibitory activity could not be accurately quantitated (Vergara, et al, J. Immunol. 134: 3445-3448, 1985). Moreover, the fact that the sequence KLKQP, which is part of the peptide, is present in all CS proteins, suggests that this region has an important function.

The present invention is described in detail below by specific examples which are intended to illustrate the invention without limiting its scope.

EXAMPLE 1: Restriction of the P.vivax CS gene, Ligation into a Vector, Transformation of the Host Yeast Cells and Expression

In the following description all restriction endonucleases and enzymes are commercially available from Pharmacia Fine Chemical Co., (Piscataway, N.J.), New England Biolabs (Beverley, MA), Boehringer Mannheim (Indianapolis, IN) or Bethesda Research Laboratories, Inc. (Gaithersburg, MD) and are used according to the manufacturer's instruction.

A pUC9 vector (Pharmacia Fine Chemical Co., Piscataway, NJ) containing the P. vivax CS protein gene, was derived by subcloning a 15-kb BglII fragment inserted into the BamHI sites of EMBL3, a bacteriophage lambda vector (as disclosed in Arnot et. al., U.S. Patent application serial number 754,645 incorporated by reference). Clone pUC9Ci was digested with BglII and XBaI and gel-purified to obtain a 4.1kb fragment encoding all the repeat sequences (Arnot et. al., Science 230:815 - 818, 1985, incorporated by reference.). This gel-purified fragment was then digested with FokI and BanI and ligated to the following 5'-phosphorylated synthetic linkers synthesized by phosphoramidite method using Applied Biosystems' 380A DNA synthesizers.

I CCTTGGAACCATGG
GGAACCTTGGTACCGTCT
NcoI Complementary to FokI overhang

II GCACCGAATGGACTCCATAG
____GCTTACCTGAGGTATCCAGCT
BanI SalI

Linker I provides for an NcoI site, while Linker II provides a SalI overhang.

The linker-containing fragment was digested with NcoI and SalI, isolated by gel electrophoresis and cloned onto NcoI/SalI digested pBS100 (construction described below). The resulting plasmid is designated pAG/P.vivax1. pBS100 is a pBR322-derived plasmid containing the ADH-2 regulated GAPDH (glyceraldehyde-3-phosphate dehydrogenase) promoter (1200bp) and GAPDH terminator (900bp) (See Figure 1) and its construction is described below.

The ADH-2 portion of the promoter was constructed by cutting a plasmid containing the wild type ADH-2 gene (plasmid pADR2, see Beier and Young, Nature, 300:724 - 728 (1982) incorporated by reference with the restriction enzyme EcoR5, which cuts at a position +66 relative to the ATG start codon, as well as in two other sites in pADR2, outside of the ADH-2 region. The resulting mixture of a vector fragment and two smaller fragments was digested with Bal31 exonuclease to remove about 300bp. Synthetic XhoI linkers having the sequence

C C T C G A G G
G G A G C T C C

were chemically synthesized and ligated onto the Bal31 treated DNA. The resulting DNA linker vector fragment (about 5kb) was separated from the linkers by column chromatography (on Sepharose CL4B from Pharmacia), cut with the restriction enzyme XhoI, religated and used to transform E. coli to ampicillin resistance. The positions of the XhoI linker additions were determined by DNA sequencing using standard techniques well known in the art. One plasmid which contained an XhoI linker located within the 5' non-transcribed region of the ADH-2 gene (position -232 from ATG) was cut with the restriction enzyme

XhoI, treated with single-strand specific nuclease S1, and subsequently treated with the restriction enzyme EcoRI to create a linear vector molecule having one blunt end at the site of the XhoI linker and an EcoRI end.

The GAP portion of the promoter was constructed by cutting plasmid pPGAP (as disclosed in European Patent Application No. 164,556 of Chiron Corporation filed on May 3, 1985, incorporated by reference and accorded the filing date of a corresponding U.S. application Serial No. 609,540 filed on May 11, 1984) with the enzymes BamHI and EcoRI, followed by the isolation of the 0.4Kbp DNA fragment. Plasmid pPGAP is a yeast plasmid containing a yeast GAPDH promoter and terminator sequences with flanking NcoI and SalI restriction endonuclease sites. The purified fragment was partially digested with the enzyme AluI to create a blunt end near the BamHI site and used to construct plasmid pJS104.

Plasmid pJS104 was constructed by the ligation of the AluI-EcoRI GAP promoter fragment to the ADH-2 fragment present on the linear vector described above.

The BamHI-NcoI ADH-GAP promoter fragment was obtained from plasmid pJS103, which is the same as pJS104 (supra) except that the GAP fragment of the ADH-GAP promoter is about 200 bp in pJS103 and 400 bp in pJS104. Construction of pJS103 was the same as that for pJS104 except that the 0.4 kb BamHI-EcoRI fragment was completely digested with AluI (instead of partially digested for pJS104) and a 200 bp fragment was isolated.

The entire above region containing the promoter, P. vivax segment and terminator, (hereinafter termed the "expression cassette") was excised by digestion with BamHI, purified by gel electophoresis and cloned into BamHI-digested pAB24. A restriction map of this plasmid is shown in Figure 2.

pAB24 is a yeast expression vector (Fig. 2) which contains the complete 2 mu sequences necessary for an autonomous replication in yeast (Broach, in: Molecular Biology of the Yeast Saccharomyces, 1:445, Cold Spring Harbor Press, 1981) and pBR322 sequences. It also contains the yeast URA3 gene derived from plasmid YEp24 (Botstein, et al., Gene (1979) 8:17 incor-

porated by reference) and the yeast $LEU2^d$ gene derived from plasmid pC1/1 (see European Patent Application Serial No. 116,201 filed on August 22, 1984 in the name of Chiron Corporation, incorporated by reference). Insertion of the expression cassette was in the BamHI site of pBR322, thus interrupting the gene for bacterial Resistance to tetracyclin.

Expression of P. vivax CS proteins

Saccharomyces cerevisiae strain AB110 isolated by Chiron Corporation (Mat, leu2-04, or both leu2-3 and leu2-112, pep4-3, his4-580, cir°) was transformed with pAB24/P. vivax1-5 according to Hinnen, et al., Proc. Natl. Acad. Sci. USA 75:1929 - 1933 (1978). Single-transformant colonies harboring GAP-regulated vectors were grown in 2ml of leu⁻ (leucine-depleted) selective media to late log or stationary phase. Only yeast harboring the plasmid can grow in this medium. Cultures were subsequently diluted 1:20 (v/v) in YEP (1% w/v yeast extract, 2% w/v peptone) with 1% glucose, and grown to saturation (about 36h) in this medium. Cells were lysed in the presence of sodium dodecyl sulfate (SDS) and dithiothreitol (DTT) and the lysates were clarified by centrifugation. Cleared lysates were subjected to polyacrylamide gel electrophoresis (Laemmli, U.K., Nature, 277:680, 1970). Following staining with Coomassie blue, a heavy band of about 38kD was observed in extracts from transformants containing the P. vivax plasmid (Figure 3). This band was detected in those cells transformed with the expression vector, while being absent from extracts of cells harboring control (pC1/1) plasmids. The fusion protein accounts for over 10% of the total cell protein. The reason for abnormal cell migration (38kD versus 23kD, predicted from DNA construction) may be attributable to anomalous SDS binding as previously reported for P. Knowlesi CS proteins (Ozaki, et al., Cell 34:185, 1983), probably due to the low proportion of hydrophobic residues.

To confirm the identity of the 38kD band, proteins synthesized by yeast were also submitted to Western analysis. Cleared yeast lysates prepared as described above were electrophoresed on polyacrylamide gels (Laemmli, supra) and proteins

were subsequently electroblotted onto nitrocellulose filters (Towbin, et. al., Proc. Natl. Acad. Sci. USA 76:3450, 1979). The filter was preincubated for 1h with 1% (BSA) in PBS and subsequently treated with a monoclonal antibody to P. vivax CS protein for 12h at 4°C. The filters were washed with 1% BSA/PBS and a second goat anti-mouse antibody conjugated with horseradish peroxidase (BioRad Laboratories, Richmond, California) added. Finally, the filters were incubated with horseradish peroxidase color development reagent (Bio-Rad, Richmond, CA) and washed. The Western analysis showed that the fusion protein reacted with the monoclonal antibodies. (Fig. 4)

EXAMPLE 2: Purification of the circumsporozoite polypeptide of the Plasmodium vivax

The yeast cultures expressing a part of the circumsporozoite polypeptide were prepared as in Example 1. The expressed polypeptide consisted of 234 amino acids including all of the repeat domain and a conserved region, namely Region I of Dame, J.B., et al, Science 225:628 (1984). The N-terminal amino acid of the expressed polypeptide is alanine in the nucleotide positions No. 385-7 (GCA) and the C-terminal amino acid is proline at nucleotide positions No. 1084-1086 (CCA); Arnot, et al, Science 230:815-818, 1985. The first step of purification of the peptide fragment expressed by yeast consisted of subjecting the extracts to temperatures of 100°C. The purification was performed as follows:

Extracts from pelleted yeast from 20 liters of yeast culture were prepared in a bead beater, diluting the yeast in an equal volume of 0.1 M sodium phosphate buffer, pH 7.3, 0.1% Triton-X, 1 mM EDTA, 1 mM PMSF (phenylmethylsulfonylfluoride) and 1 microgram per ml of pepstatin. The extract (370 ml) was added to 200 ml of boiling water containing 1 mM of PMSF and 1 microgram/ml of leupeptin. The mixture was brought to a temperature of 100°C and kept for ten minutes with constant stirring at this temperature. The mixture was cooled to 0°C and centrifuged at 18,000 rotations per minute in a Beckman Ultracentrifuge Rotor TI-19, (Beckman Instruments, Palo Alto, CA) for fifteen minutes. The supernatant was removed and then

lyophilized.

The dry material was dissolved in 120 ml of water, centrifuged to remove a small residual amount of insoluble material, dialyzed extensively against distilled water for 48 hours and lyophilized again. The powder was dissolved in 3 mM sodium potassium phosphate buffer, pH 7.5, and the conductivity adjusted with water to 0.58 mS. The solution was centrifuged to remove insoluble materials and subjected to anion exchange chromatography in a DEAE-Sephacel (Pharmacia Fine Chem Co., Piscataway, N.J.) column (5cm x 24cm) equilibrated in the same buffer. The flow rate was adjusted to 100ml per hour and 21 ml per tube were collected. The column was then washed with 500 ml of the same buffer, i.e., with about one column volume. The elution continued with a buffer formed in a linear gradient in which 1,500 ml of the initial buffer were gradually mixed with 1,500 ml of the same buffer also containing 0.75 M NaCl. The presence of the circumsporozoite polypeptide in the various fractions eluting from the column was detected using a competitive radioimmunoassay described below.

The positive fractions eluted between fraction Nos. 65-90 in a symmetrical peak with conductivities between 2 and 10 mS (Fig. 5). The full fractions 65-90 were lyophilized, redissolved in 60 ml of 0.3 M NaCl and dialyzed against 0.3 M NaCl at room temperature for several hours. The dialyzate was centrifuged to remove a small amount of insoluble material and one third, i.e. 20 ml, was subjected to molecular sieve chromatography on Sephadex G-200 (Pharmacia) equilibrated with 0.3 M sodium chloride. The Sephadex was superfine and the column was 5 cm in diameter and 100 cm long. Samples of 21 ml per tube were collected from the column. The CS polypeptide eluted in a sharp symmetrical peak between tubes 51-57. However, materials with higher and lower molecular weight in smaller amounts were distributed in tubes preceding and following this peak. The contents of tubes 51-57 were pooled, dialyzed extensively against distilled water, and lyophilized. A total amount of 89 mg of pure circumsporozoite polypeptides were recovered. On this basis, we calculated that the yield

from 20 liters of yeast is 89 x 3, i.e., 267 mg of pure protein or about 13 mg per liter of yeast culture. The purity of the recovered circumsporozoite polypeptide was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and by isoelectric focusing under denaturing conditions. At concentrations of 2 mg per ml, a single band with an isoelectric point of 4.3 was detected by isoelectric focusing (Fig. 7). By SDS-PAGE, a doublet was detected with a molecular weight between 43,000 and 45,000 (Fig. 6). The extinction coefficient at 280 nanometers of solutions of the CS polypeptide containing 1 mg of protein per ml was 0.2. A sample of the polypeptide was subjected to partial N-terminal sequence analysis and the major sequence was alanine-glutamic acid-proline-lysine-asparagine-proline, as expected. Another sample was radiolabelled with ^{125}I and immunoprecipitated with monoclonal antibodies specific to the CS protein of Plasmodium vivax. Between 75 and 85% of the counts were specifically recognized by the antibody.

A solid state competitive radioimmunoassay was used to detect the engineered CS polypeptide during the purification procedure. The standard curve relating the dose of antigen with the signal obtained in the radioimmunoassay was prepared as follows. The purified, engineered CS polypeptide was radiolabelled with ^{125}I to a specific activity of about 2×10^6 counts per minute per microgram protein using the well-known iodogen method. Mixtures containing a constant amount of radiolabelled engineered CS polypeptide and variable amounts of purified cold (i.e. unlabeled) engineered CS polypeptide (in a total volume of 100 microliters) were prepared. Thirty microliters of the mixtures were then delivered to the bottom of wells of microtiter plates pre-coated with monoclonal antibodies to the circumsporozoite protein of Plasmodium vivax (2F2). This monoclonal antibody reacts with the repetitive epitope of the circumsporozoite protein. (Nardin E.H. et al, J. Exp. Med., 156:20, 1982). The inhibitory effect of the cold peptide on the binding of the labeled peptide to the bottom of the wells was proportional to the concentration of

cold peptide. This assay detected concentrations of cold peptide as low as five nanograms per ml (Fig. 8). The assay of the column fractions and of the extracts was performed exactly as described above and the degree of inhibition obtained was referred to the standard curve (Fig. 8) to calculate the concentration of the CS protein peptide. All dilutions and washing in this assay were performed in a phosphate buffer saline (PBS) containing 1.0% bovine serum albumin (BSA) and 0.1% sodium azide.

On the basis of the results of these assays, we calculated that the yeast cultures contain about 60 mg of circumsporozoite protein per liter and that the recovery of the purified material was about 20% of the total. There were no losses in the initial step of purification, i.e., following the boiling of the extracts this step removed more than 90% of the non-relevant protein bands observed in the SDS-PAGE of the original extracts.

EXAMPLE 3: Immunization of mice with the yeast-engineered P.vivax polypeptide

The purified engineered CS polypeptide was then used as an antigen to immunize mice. Ten outbred female Swiss-Webster, 8-12 week old mice were injected with 50 micrograms of the purified peptide adsorbed on aluminum hydroxide. Three and six weeks afterwards, the mice were boosted with the same dose of antigen again using aluminum hydroxide as adjuvant. Ten days later, the mice were bled and the sera were subjected to analysis by an immunoradiometric assay to detect antibodies to the CS polypeptide. In this assay, the wells of microtiter plates are coated with purified engineered CS polypeptide (10 micrograms/ml in PBS) and then incubated the wells with 30 microliters of various dilutions of the mouse serum in PBS-BSA. The wells were then washed and re-incubated with 50,000 cpm of radiolabelled affinity-purified goat anti-mouse immunoglobulin (10^7 cpm/microgram of protein) diluted in PBS-BSA. The wells were then washed again and counted.

All sera reacted with the engineered CS polypeptide at dilutions of 1:10,000 or greater. The results of a titration

of the pooled sera are shown in Fig. 9. That these sera contained large amounts of antibodies to the "repeats" of the P. vivax CS protein was shown in two experiments described below.

1) A synthetic 18-amino acid peptide (18-mer) comprising the sequence (Asp-Gly-Gln-Pro-Ala-Gly-Asp-Arg-Ala)$_2$ and representing two tandem repeats of the P. vivax CS Protein was synthesized as described in (a) copending U.S. patent application Serial No. 754,645 filed on July 9, 1985 and incorporated by reference in the present application; and (b) Arnot, et al. Science, 230:815, 1985. This peptide was used to coat wells of microtiter plates and the antibody contents of the sera detected by the immunoradiometric assay described above. All sera reacted with titers at 1:4,000 or above. The results of titrations of pooled sera obtained after the second antigen booster injection are shown in Fig. 10.

2) The synthetic 18-amino acid peptide was also used to inhibit the binding of antibodies, present in the pooled antiserum, to the wells of plastic plates coated with the engineered CS polypeptide. In these experiments, we first incubated samples of a 1:3,000 dilution of the pooled serum from the mice (obtained after the 2nd booster rejection) with increasing concentrations of the 18-amino acid peptide described above. As a control, samples of serum were incubated with the same concentration of another 18-mer peptide with a different and unrelated sequence of amino acids. Following an incubation of one hour at room temperature, 30 microliters of the mixtures were added to wells of plates precoated with the engineered CS polypeptide. The wells were then washed, incubated with radiolabelled goat anti-mouse immunoglobulin, as described above, washed again and counted. The results shown in Fig. 11 demonstrated that the "repeat" peptide (but not the control peptide) inhibited about 50% of the reaction between the antibodies and the CS protein. All dilutions and washings in these experiments were performed with PBS-BSA.

The same sera were also analyzed for the possible presence of antibodies to Region 1 of Dame, et al., supra. For

this purpose, we used as an immobilized antigen a small synthetic peptide NH_2-Cys-Tyr-Asn-Glu-Lys-Ile-Glu-Arg-Asn-Asn-Lys-Leu-Lys-Gln-Pro-COOH. This peptide includes a sequence of five amino acids which are common to all CS proteins from all malaria parasites examined to date, Lys-Leu-Lys-Gln-Pro (Dame et al., Science, 225:593, 1984, and Enea, V., personal communication). The first two amino acids (Cys and Tyr) are extraneous to the CS protein and were added for purposes of coupling the peptide to a carrier protein and radiolabelling with ^{125}I. This peptide was used to coat wells of microtiter plates and the antibody contents in the sera were detected as described above. After the second booster, all sera recognized this small peptide at dilutions of 1:2,000 or greater. The results of titrations of a pool of these sera is shown in Fig. 12.

The sera were also tested by indirect immunofluorescence using the sporozoite of P. vivax as the antigen. All sera were positive at dilutions of 1:1,000 or greater.

Finally, another experiment demonstrated that relatively low levels of antibodies to the engineered CS protein neutralize the infectivity of P. vivax sporozoites. The assay was performed as described in J. Immunol. 132:909, 1984 (incorporated by reference) using the human hepatoma cell line Hep 62 as the target of parasite invasion. The results, set forth in Table I, below, show that a significant degree of inhibition was obtained at serum dilutions of 1:5,000. The percentages of inhibitions were calculated as 100-[(mean experimental values/mean of controls)x100]. Controls consisted of sporozoites incubated with equivalent concentrations of normal (pre-immune) mouse serum.

TABLE I

INHIBITION OF P. VIVAX SPOROZOITES INTO HEPATOCYTES IN VITRO BY ANTIBODIES TO THE YEAST-ENGINEERED CS PROTEIN

$(\text{Serum Dilution})^{-1}$	%Inhibition
50	91
250	91
1,000	77
5,000	42

WHAT I CLAIM IS:

1. A method for purifying a polypeptide consisting essentially of an amino acid sequence consisting essentially of at least two tandemly repeating sequences idential to those of the immunodominant epitope region of the circumsporozoite protein of P.vivax, from an impure preparation of said polypeptide said impure preparation also containing one or more other protein species as impurities, said method comprising the steps of:

(a) subjecting said preparation to a temperature of 100'C for a period of time sufficient to cause denaturation and precipitation of the bulk of said protein impurities, while leaving said polypeptide in solution;

(b) removing and discarding said precipitate and retaining the supernatant;

(c) separating the solid constituents of said supernatant;

(d) redissolving said solid constituents in an aqueous ionic buffer solution;

(e) subjecting said solution to ion-exchange chromatography on a first chromatography column, thereby causing said polypeptide to be retained on said column, while a portion of the remaining impurities of said polypeptide are excluded in the effluent, and eluting said polypeptide using an ionic gradient;

(f) decreasing the ionic strength of said eluant containing said polypeptide;

(g) subjecting said eluant to a molecular-sieve chromatography on a second chromatography column, and eluting said polypeptide from said colum.

2. The method of claim 1 wherein said polypeptide is a yeast-engineered polypeptide having an amino acid sequence consisting essentially of the immunodominant epitope region of the circumsporozoite protein of Plasmodium vivax flanked by amino acid sequences contiguous to said region.

3. The method of claim 2 further comprising separating said supernatant from said precipitate by centrifugation followed by at least one lyophilization of said supernatant and subsequent redissolution of the lyophilized material, prior to said first chromatography step.

4. The method of claim 3 further comprising eluting said polypeptide from said first column using a linear NaCl gradient of increasing ionic strength.

5. The method of claim 3 further comprising decreasing the ionic strength of said eluant by dialysing said eluant against a buffer of lesser ionic strength than said eluant.

6. A synthetic polypeptide comprising at least one epitope of the circumsporozoite protein of P.vivax other than the immunodominant epitope which consists of a repeating sequence of amino acids, said polypeptide being expressed by a recombinant organism and having an amino acid sequence containing:

(a) a repetitive amino acid sequence of said circumsporozoite protein which represents a repeating immunodominant epitope region of said protein; and

(b) a portion of the amino acid sequence of the circumsporozoite protein of Plasmodium vivax which immediately precedes the repeating epitope region of said protein proximal to the N-terminal of said protein and which is conserved in the CS proteins of different malarial species.

7. The polypeptide of claim 6 having the property of conferring protective immunity against Plasmodium vivax sporozoites to a mammalian host susceptible to infection with P.vivax sporozoites.

8. The polypeptide of claim 6 wherein said sequence

in (b) precedes the repeating sequence and contains the amino acid sequence KLKQP.

9. The polypeptide of claim 6 wherein said repetitive sequence is $(DRAXGQPAG)_r$ wherein X is D or A and r is an integer between 2 and 19 inclusive.

10. The polypeptide of claim 9 wherein said repetitive sequence is of the same length as the entire immunodominant epitope region of the native P.vivax circumsporozoite protein.

11. The polypeptide of claim 6 also comprising a segment of the amino acid sequence of the circumsporozoite protein of Plasmodium vivax following the repeating epitope region of said protein towards the C-terminal end of said protein which segment is conserved in the CS proteins of different malarial species and located at the C-terminal of said polypeptide.

12. The polypeptide of claim 6 also comprising the entire segment of the amino acid sequence of the circumsporozoite protein following the repetitive immunodominant epitope region and extending to the C-terminal of said protein.

13. A synthetic peptide comprising the amino acids KLKQP in sequence, said amino acid sequence being conserved in the CS protein of Plasmodium knowlesi, Plasmodium falciparum and Plasmodium vivax.

14. A synthetic polypeptide having an amino acid sequence consisting essentially of

(a) an immunogenic repetitive sequence corresponding to all or part of the repetitive immunodominant epitope region of P.vivax circumsporozoite protein; and

(b) located in at least one of the N- and C-terminal of said repetitive sequence, a non-repetitive

amino acid sequence which in the native circumsporozoite protein (1) immediately precedes or follows the repetitive immunodominant epitope region of said protein, and (2) is conserved among different malarial species.

15. The polypeptide of claim 14 wherein said sequence in (b) is located at the N-terminal of said polypeptide and is the non-repetitive conserved sequence immediately preceding the repetitive epitope region of the native circumsporozoite protein and located proximal to the N-terminal of said protein.

16. A synthetic polypeptide having an amino acid sequence comprising:

(a) an immunogenic repetitive sequence corresponding to the repetitive immunodominant epitope region of P.vivax circumsporozoite protein; and

(b) at the N-terminal of said repetitive sequence a non-repetitive amino acid sequence which in the native circumsporozoite P.vivax protein (1) immediately precedes and is located at the 5'-end of the repetitive immunodominant epitope region of said protein, and (2) is conserved among the different malarial species.

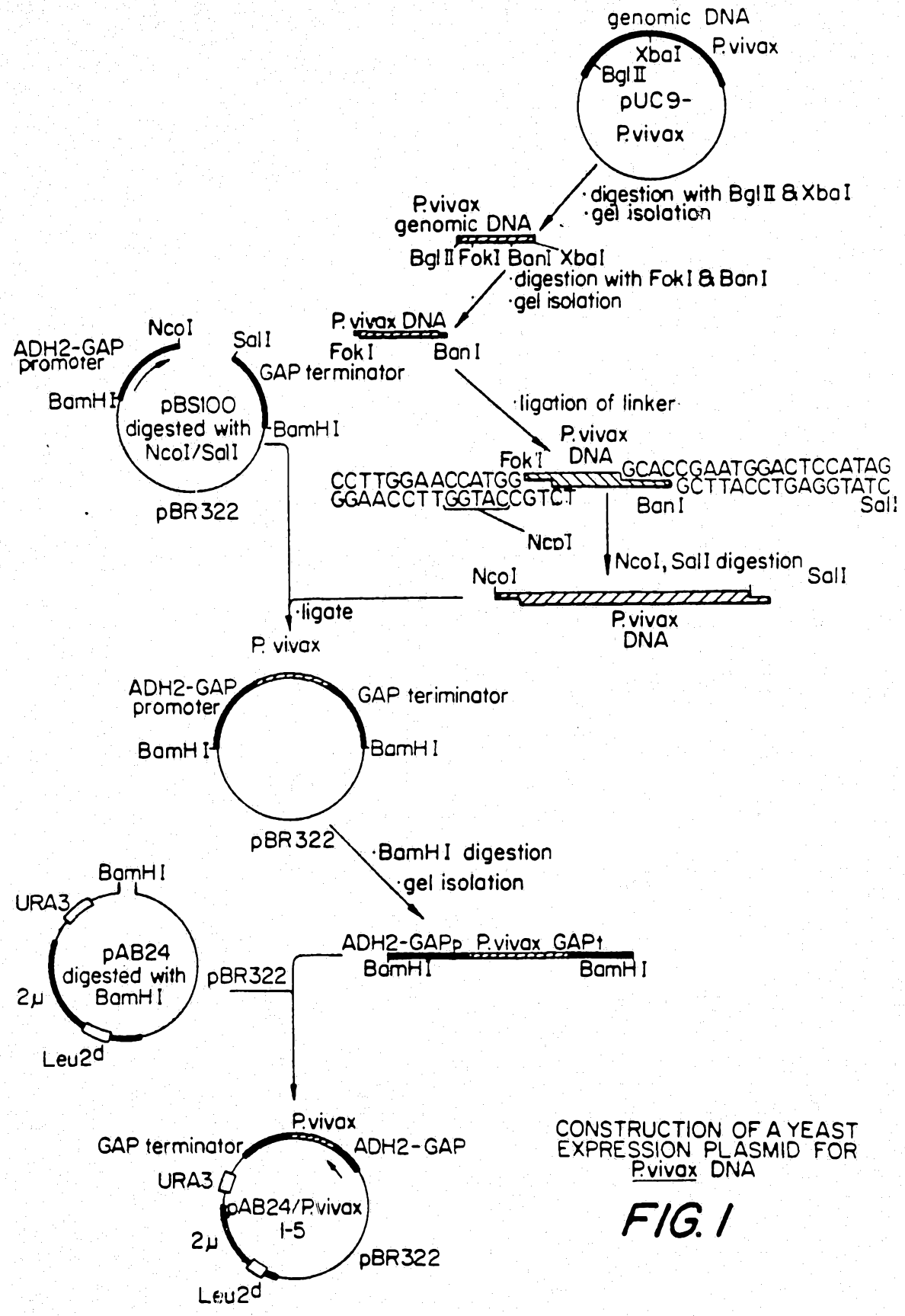
17. The polypeptide of claim 6 wherein said sequence (b) is located at the N-terminal of said polypeptide.

18. A polypeptide in accordance with claim 16, substantially as herein described with reference to the Examples.

19. A method of purifying a polypeptide substantially as herein described with refence to the Examples.

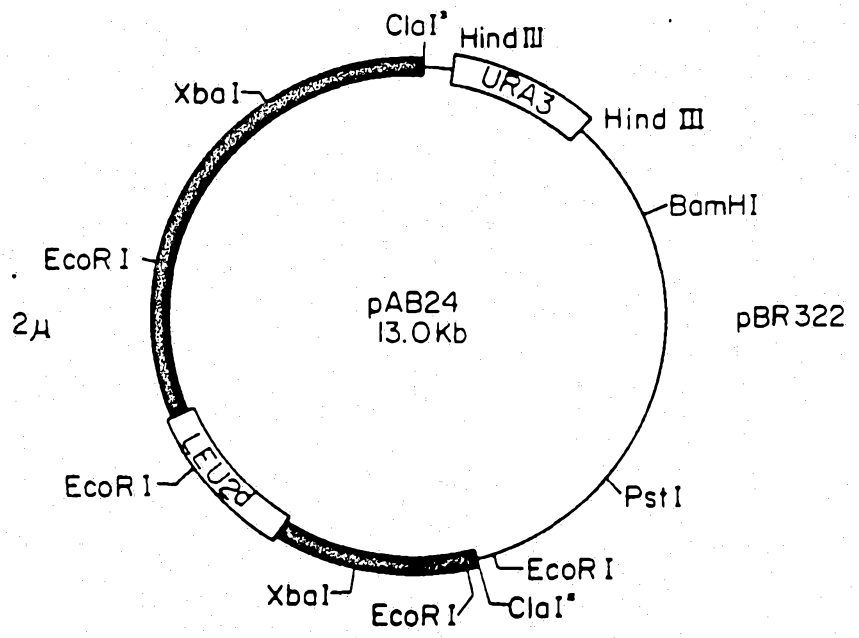
DATED this 28th day of June, 1991.

NEW YORK UNIVERSITY
By Their Patent Attorneys
ARTHUR S. CAVE & CO.



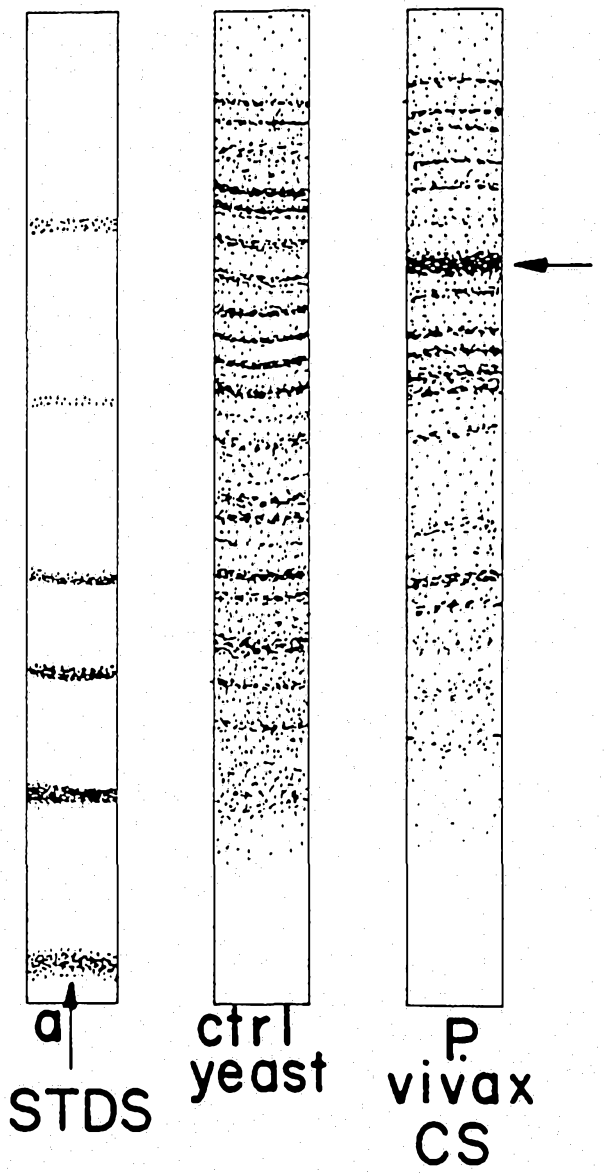
CONSTRUCTION OF A YEAST EXPRESSION PLASMID FOR P.vivax DNA

FIG. 1



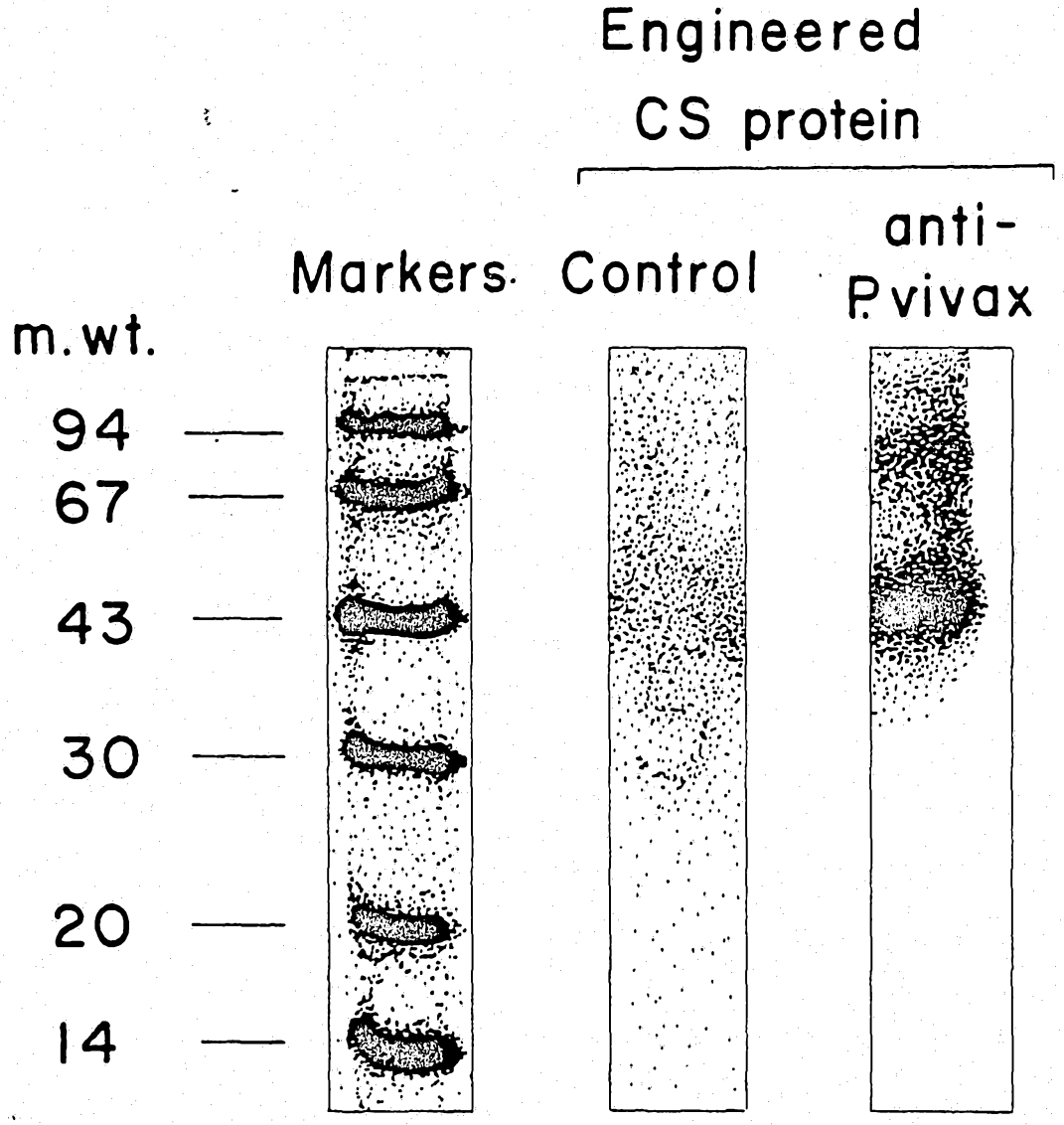
YEAST EXPRESSION PLASMID pAB24

FIG. 2



Polyacrylamide Gel

FIG. 3



Western Blot

FIG. 4

FIG. 5

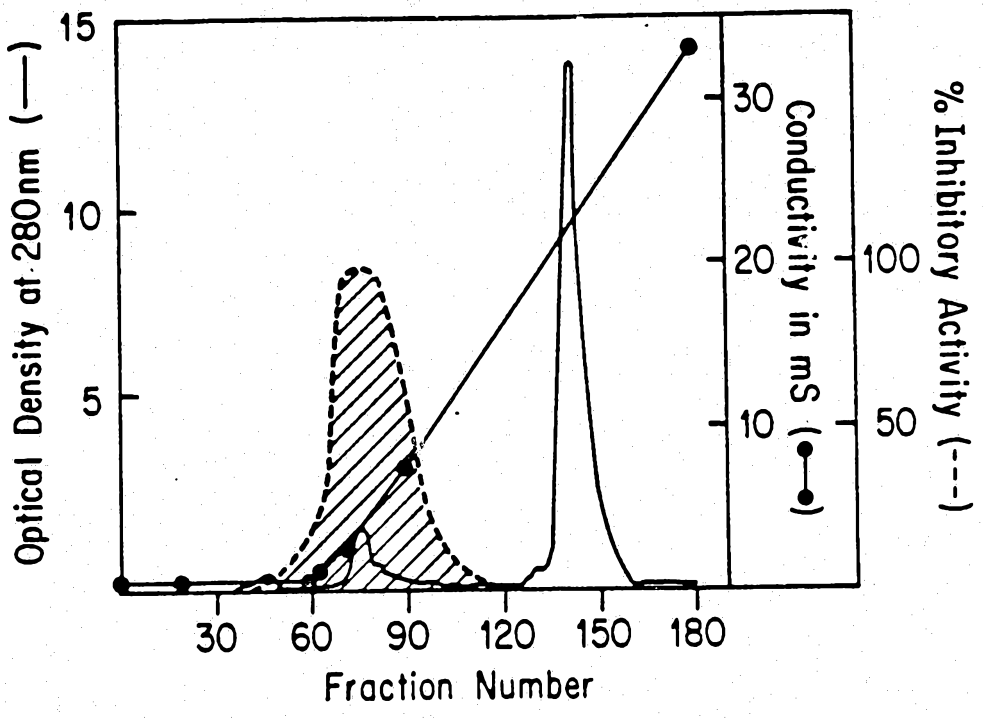
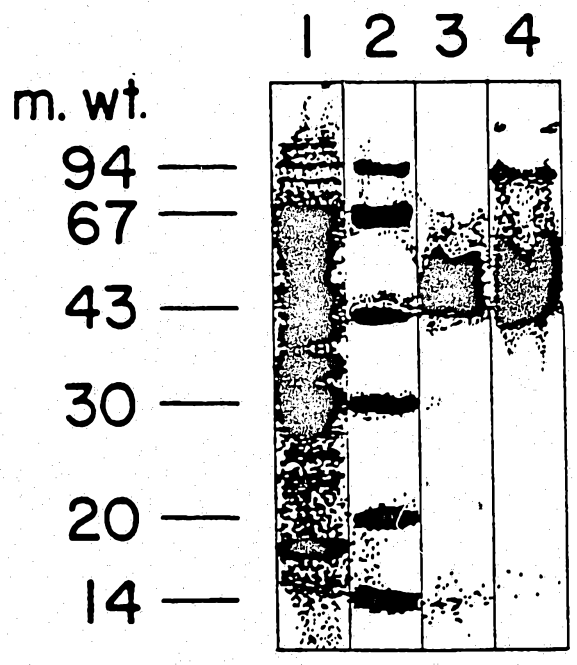


FIG. 6



1. Total extract
2. Markers
3. Purified CS
4. Extract after boiling

FIG. 7

IEF OF YEAST-ENGINEERED CS PROTEIN OF *P. vivax*

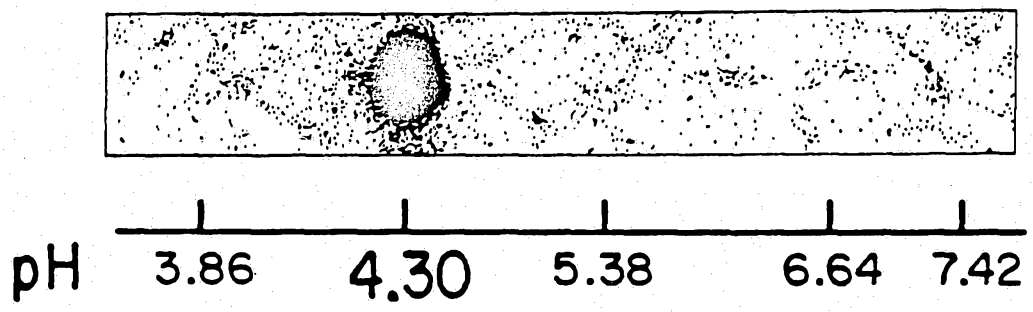


FIG. 8

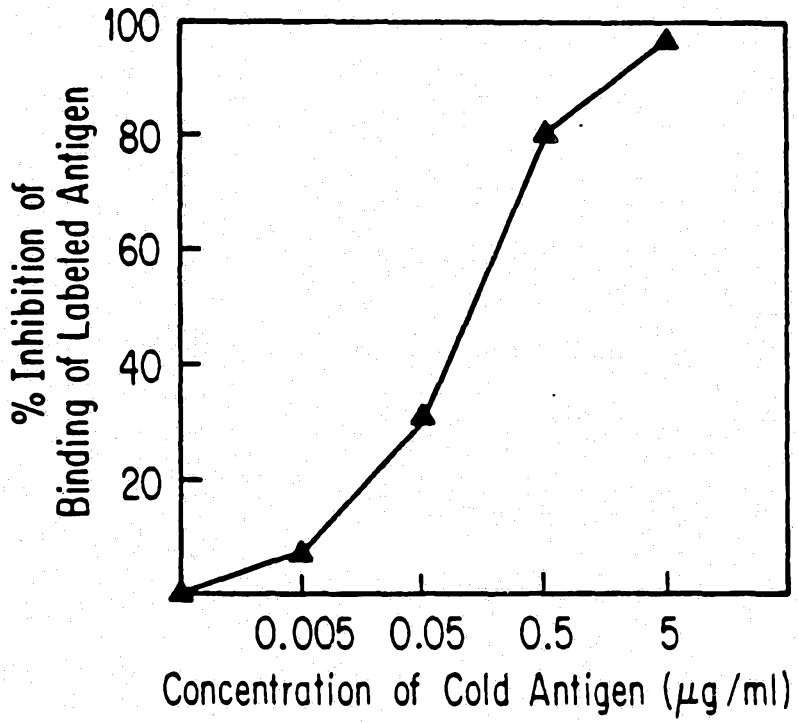


FIG. 9

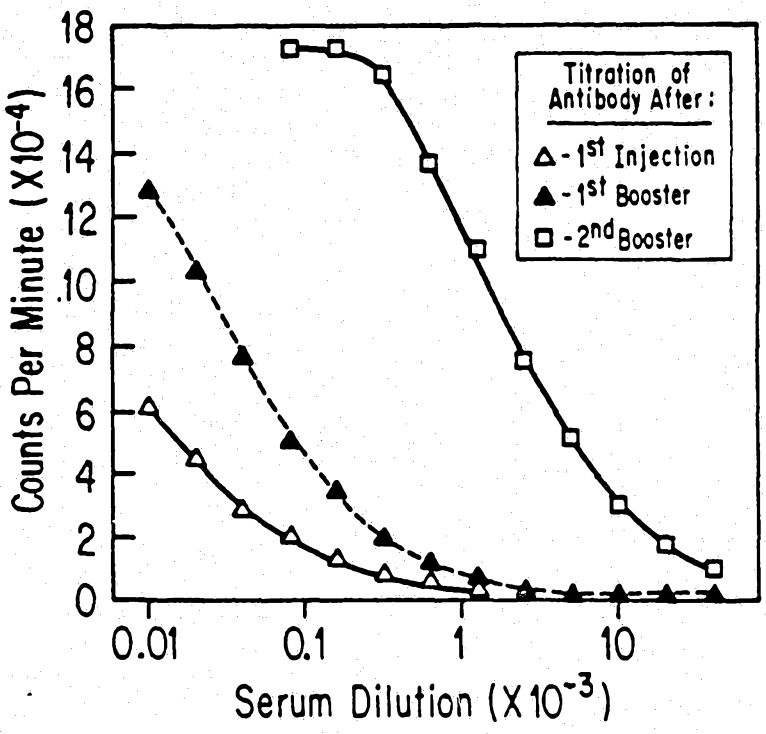

Counts Per Minute (X10⁻⁴)
18
16
14
12
10
8
6
4
2
0
0.01
0.1
1
10
Serum Dilution (X10⁻³)
Titration of Antibody After:
△ - 1st Injection
▲ - 1st Booster
□ - 2nd Booster

FIG. 10

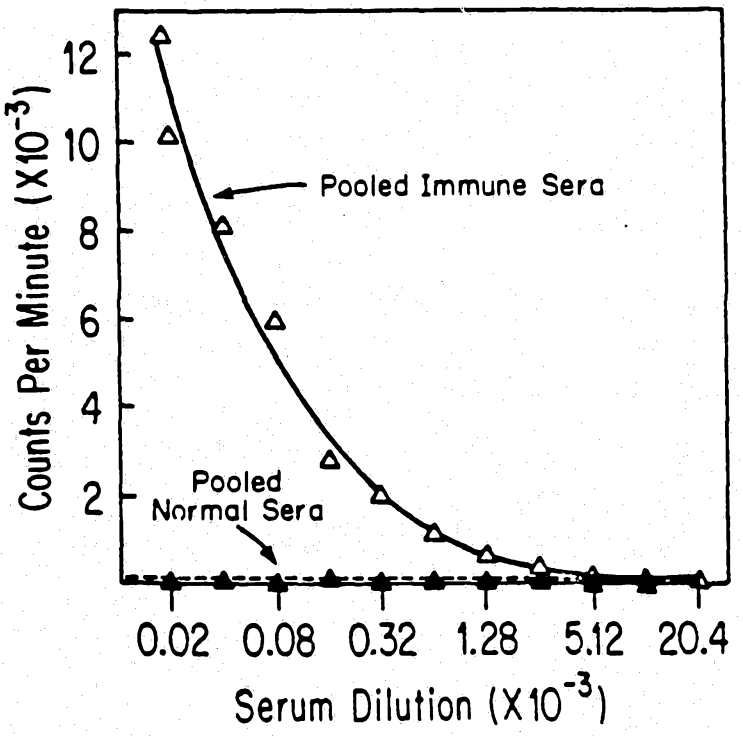

Counts Per Minute (X10⁻³)
12
10
8
6
4
2
Pooled Immune Sera
Pooled Normal Sera
0.02
0.08
0.32
1.28
5.12
20.4
Serum Dilution (X10⁻³)

FIG. 11

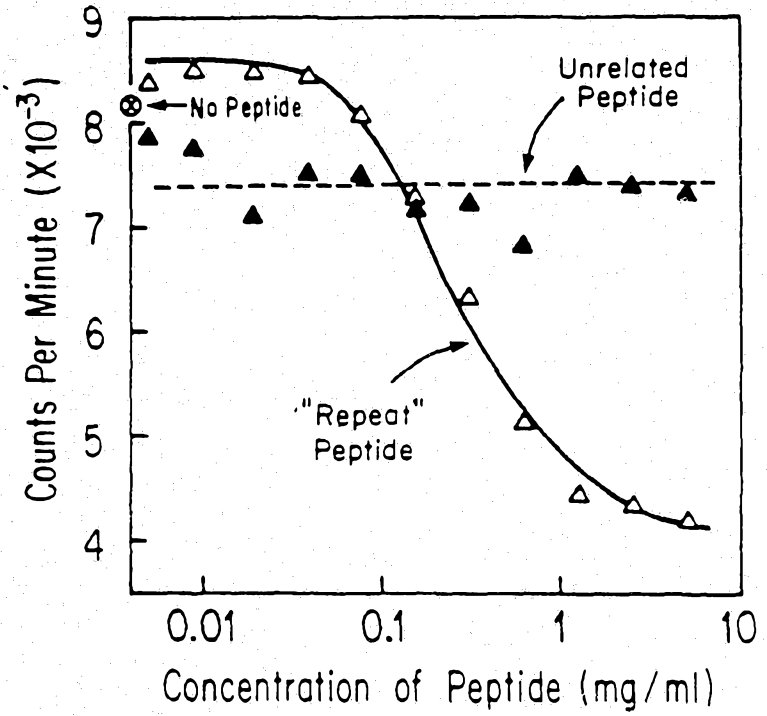
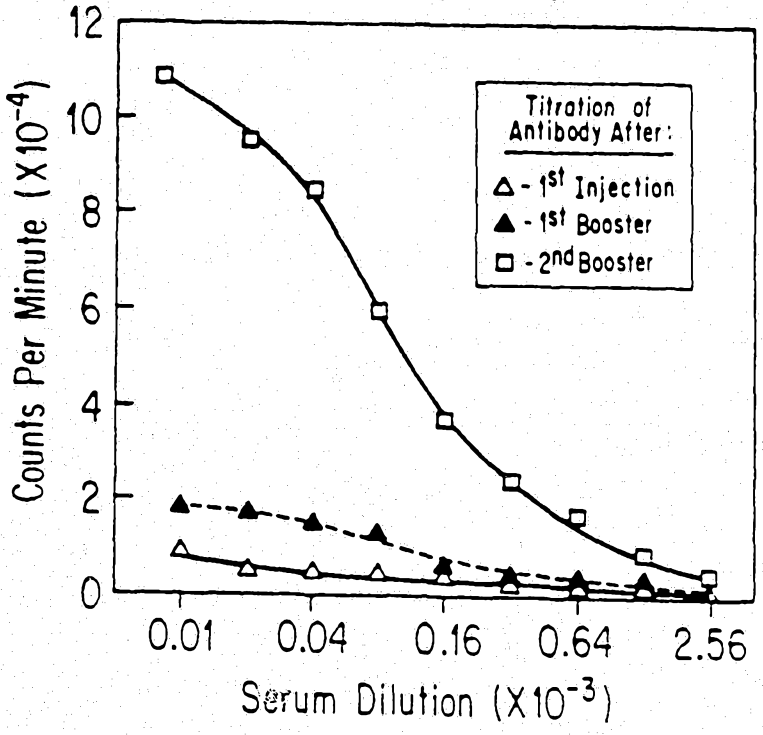

Counts Per Minute ($X10^{-3}$)
9
8
7
6
5
4
No Peptide
Unrelated Peptide
"Repeat" Peptide
0.01
0.1
1
10
Concentration of Peptide (mg/ml)

FIG. 12


Counts Per Minute ($X10^{-4}$)
12
10
8
6
4
2
0
Titration of Antibody After:
△ - 1st Injection
▲ - 1st Booster
□ - 2nd Booster
0.01
0.04
0.16
0.64
2.56
Serum Dilution ($X10^{-3}$)

INTERNATIONAL SEARCH REPORT

International Application No PCT/US88/01150

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C07K 3/22,3/28,7/06,7/08,13/00; C12P 21/00,21/02; C12N 15/00 U.S. 530/327,328,344,350; 435/68,70,172.3

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System [5]	Classification Symbols
U.S.	530/327,328,344,350,416,417,806 435/68,70,172.3,255,320 935/12,28,69

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [6]

Chemical Abstracts Data Base (CAS) 1967-1988; Biological Abstracts Data Base (BIOSIS) 1967-1988. Keywords: Plasmid, cloning, Plasmodium vivax, circumsporozoite.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category * [15]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US, A, 4,466,917 (NUSSENZWEIG ET AL) 21 AUGUST 1984, See entire document. particularly Example 5.	1-17
Y,P	US, A, 4,693,994 (MCCUTCHAN ET AL) 15 September 1987 See entire document particularly, figures 1 and 2.	1-17
Y,P	US, A, 4,707,357 (DAME ET AL) 17 November 1987 See entire document, particularly figure 2.	1-17
Y	WO, A, 8700533 (ARNOT ET AL) 29 January 1987 See entire document, particularly figure 3.	1-17

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [9]
23 JUNE 1988	30 JUN 1988
International Searching Authority [1]	Signature of Authorized Officer [10]
ISA/US	THOMAS D. MAYS

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No **
Y,P	Chemical Abstracts, Volume 107, No. 1, issued 1 July 1987 (Columbus, Ohio, USA) DE LA CRUZ ET AL) "Evolution of the Immunodominant domain of the circumsporozoite protein gene from Plasmodium vivax. Implications for vaccines". See page 181, column 2, the abstract no. 1902K, J. Biol. Chem. 1987, 262 (14), 6464-7 (Eng.).	1-17
Y,P	Chemical Abstracts, Volume 107, No. 25, issued 21 December 1987 (Columbus, Ohio USA) (ROMERO ET AL) "Antigenic analysis of the repeat domain circumsporozoite protein of Plasmodium vivax" see page 590, column 2, the abstract no. 234362z, J. Immunol. 1987, 139(5), 1679-82 (Eng.).	1-17
Y	JOURNAL OF IMMUNOLOGY (Baltimore, Maryland, USA) Volume 135, issued October 1985 (ZAVALA ET AL) "Ubiquity of the repetitive epitope of the CS protein in different isolates of human malaria parasites", See pages 2790-93.	1-17
Y	SCIENCE (Washington, D.C., USA) Volume 230, issued 15 November 1985 (ARNOT ET AL) "Circumsporozoite protein of Plasmodium vivax: Gene cloning and characterization of the immunodominant epitope". See pages 815-18.	1-17
Y	SCIENCE (Washington, D.C., USA) Volume 230, issued 20 December 1985 (MCCUTCHAN ET AL) "Sequence of the immunodominant epitope for the surface protein on sporozoites of Plasmodium vivax", See pages 1381-83.	1-17

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y,P	Chemical Abstracts, Volume 106, No. 23, issued 8 June 1987 (Columbus, Ohio, USA) (BARR ET AL.) "Expression in yeast of a Plasmodium vivax antigen of potential use in a human malaria vaccine. See page 203, column 1, the abstract no. 190316k, J. Exp. Med. 1987 165(4), 1160-71 (Eng.).	1-17

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers .... because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No [13]
A	JOURNAL OF EXPERIMENTAL MEDICINE (New York, USA) Volume 156, issued July 1982 (NARDIN ET AL) "Circumsporozoite proteins of human malaria parasites Plasmodium falciparum and Plasmodium vivax", See pages 20-30.	1-17
A	JOURNAL OF EXPERIMENTAL MEDICINE (New York, USA) Volume 157, issued June 1983 (ZAVALA ET AL) "Circumsporozoite proteins of malaria parasites contain a single immunodominant region with two or more identical epitopes". See pages 1947-57.	1-17