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Immunology

A protective monoclonal antibody recognizes a linear epitope in the precursor to the major merozoite antigens of *Plasmodium chabaudi adami*

(malaria/hybridoma/peptide)

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ABSTRACT **The monoclonal antibody 5C10/66 was shown to afford strong protection in mice against fulminating *Plasmodium chabaudi adami* infection. This was remarkable, as immunity to this organism is regarded to be mainly T-cell mediated. This antibody identified a 250-kDa molecule in schizonts and an 83-kDa fragment in merozoites. A cDNA clone selected by 5C10/66 was the homologue of the *Plasmodium falciparum* precursor to the major merozoite surface antigen (PMMSA). Comparison with the *P. falciparum* sequence showed that the *P. chabaudi adami* clone encoded the middle portion of the gene and that it can also be divided into variable and conserved blocks. Screening of a set of all possible octamer peptides predicted by the cDNA clone revealed that the core epitope of 5C10/66 was Glu-Thr-Thr-Glu-Thr. This region resides in a variable block of PMMSA.**

In the search for a molecular vaccine against human malaria, considerable effort has been marshalled to identify and characterize plasmodial antigens that may elicit a protective immune response. There are various developmental stages in the life cycle of *Plasmodium* and this structural diversity is one of the reasons for the oganism's antigenic complexity. During the asexual blood phase, the parasite leaves the protection of its intraerythrocytic environment and emerges as free merozoites. Hence, this stage would seem a readily accessible target for immune attack. Fragments of a high molecular weight antigen have been found on the merozoite surface of human, simian, and rodent *Plasmodium* (1). This antigen, which is synthesized during schizogony, has been termed the precursor to the major merozoite surface antigens (PMMSA) and has a size range of 185–250 kDa depending on the strain and species of *Plasmodium*. Of relevance to PMMSA's candidacy as a vaccine, the various alleles of *Plasmodium falciparum* have been found to be derived from two primordial genes and so is essentially dimorphic and not polymorphic (2, 3).

Evidence of at least partial protection by PMMSA has been obtained from passive transfer of monoclonal antibodies (mAbs) *in vivo* for rodent malaria (4, 5), inhibition of reinvasion *in vitro* (6, 7), and active immunization studies in rodent and primate models (8–11). Recently, a synthetic vaccine containing a PMMSA peptide protected humans against *P. falciparum* (12). The sequence of this peptide was derived from block 1 [as designated by Tanabe *et al.* (2)].

We describe a mAb (mAb 5C10/66) that protects mice against *Plasmodium chabaudi* challenge. This antibody was used to select a cDNA clone of 1.4 kilobases (kb), which showed sequence homology with PMMSA of *P. falciparum* and *Plasmodium yoelii*.‖ The clone corresponds to the middle portion of PMMSA and the epitope of mAb 5C10/66 was mapped to a core region of five amino acids.

MATERIALS AND METHODS

Parasites. *P. Chabaudi adami* DS was originally obtained from D. Walliker (Edinburgh) and *P. Chabaudi adami* IC was a gift from Ian Clark (Australian National University, Canberra, Australia).

mAb. CBA × BALB/c mice were immunized with Triton X-114 phase preparations of *P. chabaudi adami* IC and challenged with the same parasite. Hybridomas were produced from the spleen of one mouse and screened for reactivity against *P. chabaudi adami* IC by indirect immunofluorescence. 5C10/66 is an IgG2a mAb produced by one of these hydridomas. 7.1.3 was an IgG2a mAb against the hapten azobenzenearsonate and was a gift from G. Morahan (Walter and Eliza Hall Institute, Melbourne).

Passive Protection Studies. Ascites fluid (0.5 ml) was injected intraperitoneally into 8-week-old SJL female mice on days −1 and +1 relative to infection. On day 0, all mice were infected with 10^5 *P. chabaudi adami* DS parasitized erythrocytes i.v. Percent parasitemias were determined by examination of Giemsa-stained thin tail blood smears.

Immunofluorescence. Smears of washed parasitized erythrocytes were air-dried, fixed in cold acetone, reacted with mAb, washed, and reacted with fluorescein-conjugated sheep anti-mouse immunoglobulin (Silenus, Melbourne).

Labeling. When a predominantly schizont stage of infection by *P. chabaudi adami* DS was reached, blood from a mouse was metabolically labeled for 2 hr with [^{35}S]methionine in RPMI 1640 medium without methionine or [^{3}H]myristic acid in RPMI 1640 medium or [^{3}H]ethanolamine in RPMI 1640 medium. Cells were washed and lysed for 1 hr in 0.5% Triton X-100 in phosphate-buffered saline (PBS) with leupeptin, chymostatin, pepstatin, and antipain (each at 1 μg/ml) (Sigma).

Immunoprecipitation, Western Blotting, and Isolation of Merozoites. These were done as described (13–15).

Library Construction. Blood from 80 BALB/c mice infected with *P. chabaudi adami* DS (parasitemia = 40%; 10% mature trophozoites, 26% schizonts, 4% rings) was centrifuged through Ficoll-Paque (Pharmacia) to remove lymphocytes and then lysed in 0.1% saponin. RNA was extracted by

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Abbreviations: mAb, monoclonal antibody; PMMSA, precursor to the major merozoite surface antigens.

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‖The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04568).

homogenization with 10 vol of 6 M guanidine hydrochloride and 0.1 M sodium acetate (pH 5.2), followed by centrifugation through 4.8 M CsCl. Poly(A) RNA was selected by oligo(dT)-cellulose chromatography.

About 1 μg of mRNA was copied into cDNA using the Amersham cDNA kit. This was methylated with *Eco*RI methylase, ligated to *Eco*RI linkers, and digested with *Eco*RI. Excess linkers were removed by Sephadex-G50 chromatography (Pharmacia). The *Eco*RI-linked cDNA was ligated to *Eco*RI-digested λgt11 DNA, packaged into phage, and plated onto Y1090 cells (16). About 3×10^5 recombinants were obtained.

Library Screening. The library was screened with mAb 5C10/66 from tissue culture supernatant followed by I^{125}-labeled protein A.

DNA Sequencing. DNA was made in the M13mp18 vector and sequenced by the dideoxynucleotide chain-termination procedure (17).

Epitope Analysis by Geysen Screening. A complete set of overlapping octamer peptides was synthesized on polyethylene rods using the predicted amino acid sequence (18). mAb 5C10/66 was affinity purified on protein A agarose beads from tissue culture supernatant and tested by ELISA on the series of peptides.

RESULTS

Passive Immunization by Sera. In preliminary experiments, several mAbs that reacted with schizonts by immunofluorescence were tested in passive protection studies. Only 5C10/66 affected the course of infection.

In a subsequent experiment, ascites fluid containing 1.0 mg of immunoglobulin was given to each SJL mouse on days −1 and +1 relative to challenge. Group A (seven SJL mice) received mAb 5C10/66. Group B received the isotype-matched 7.1.3 control antibody. After challenge with 10^5 *P. chabaudi adami* DS, mice in the control group developed high parasitemias, peaking at 58.0% on day 11 (Fig. 1). Moreover, there was a 75% mortality rate. In contrast, mice given mAb 5C10/66 developed low parasitemias (peak, 2.3% on day 17) and the onset of 2% parasitemia level was delayed by 8 days. None of these mice died.

Immunofluorescence. Immunofluorescence staining by 5C10/66 showed a grape-like staining pattern of mature schizonts of *P. chabaudi adami* DS and IC (data not shown).

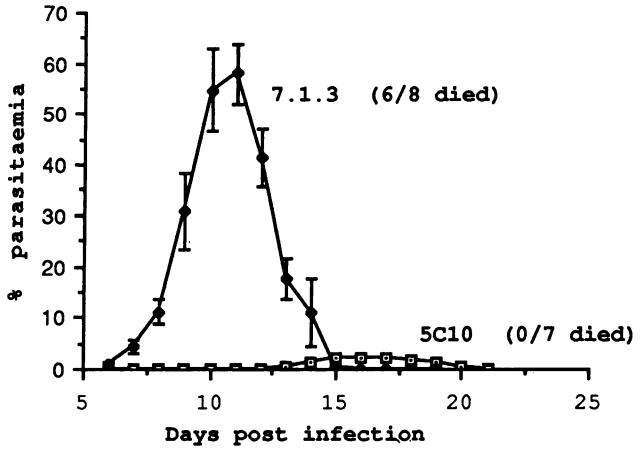


FIG. 1. Passive protection with mAb 5C10/66 against *P. chabaudi adami* DS. Ascites fluid containing 1 mg of immunoglobulin was injected intraperitoneally to each SJL mouse on days −1 and +1 relative to challenge (10^5 parasitized erythrocytes i.v.): seven mice received mAb 5C10/66 and eight mice received the isotype-matched 7.1.3 control antibody. Means ± SEM of the parasitemias of each group are shown.

A more diffuse pattern was seen in immature schizonts. The surface of free merozoites was also stained. There was no staining of rings or trophozoites.

Western Blotting and Immunoprecipitation Studies. On Western blots, mAb 5C10/66 identified a 250-kDa protein in schizonts (Fig. 2 *Left*). In accordance with the immunofluorescence findings, the only stages identified with Western blotting by mAb 5C10/66 were schizonts and merozoites. In merozoites, the antibody identified proteins of 250 and 85 kDa. Immunoprecipitation of [^{35}S]methionine-labeled Triton X-100 lysates confirmed the findings by Western blotting in that the major protein species identified was 250 kDa (data not shown). There was no change in mobility of these proteins after N-Glycanase digestion, indicating a lack of N-linked glycans (data not shown). Moreover, the 250-kDa molecule metabolically incorporated myristic acid and ethanolamine (Fig. 2 *Right*). It is probable that, like its *P. falciparum* homologue, it acquires a glycosyl phosphatidylinositol membrane anchor posttranslationally (1).

The mAb 5C10/66 Recognizes a Clone Corresponding to the Middle Portion of PMMSA. Tissue culture supernatant containing the mAb 5C10/66 was used to screen the *P. chabaudi adami* DS cDNA expression library. The antibody reacted strongly with one clone that was plaque purified and phage DNA were obtained. The 1.4-kb *Eco*RI fragment was isolated, subcloned into M13, and sequenced (Fig. 3 *Middle*). This clone encoded 478 amino acids. Its topographical relationship with the entire *P. falciparum* PMMSA gene and the *P. yoelii* PMMSA gene (20) is shown in Fig. 3 (*Top*). Comparison between the deduced amino acid sequences of *P. chabaudi adami*, *P. yoelii*, and two strains of *P. falciparum* (representing the two dimorphic forms of PMMSA) is shown in Fig. 3 (*Bottom*). In the overlapping region (positions 400–481) among these four sequences, the degree of homology indicated that the clone identified by the mAb 5C10/66 was the *P. chabaudi* PMMSA. This homology was considerably higher between the two murine malarias (*P. chabaudi adami*

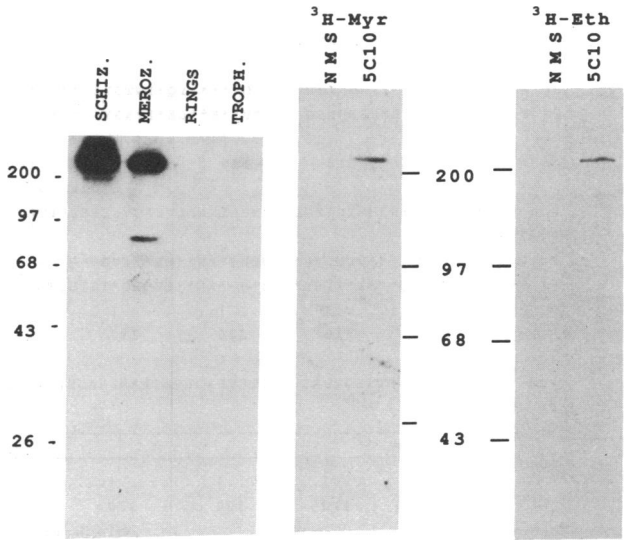
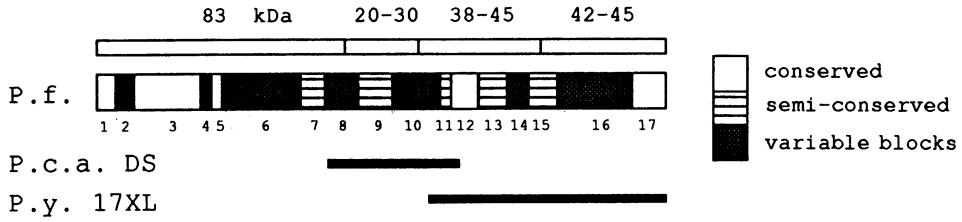


FIG. 2. (*Left*) Western blotting of *P. chabaudi adami* DS by mAb 5C10/66. Various stages of the life cycle (schiz., schizonts; troph., trophozoites) were obtained during synchronous infections and merozoites (meroz.) were isolated by concanavalin A agarose chromatography. The sizes of the prestained markers are indicated in kDa. (*Right*) Immunoprecipitation of *P. chabaudi adami* DS by mAb 5C10/66. [^{3}H]Myristic acid-labeled (^{3}H-Myr) or [^{3}H]ethanolamine-labeled (^{3}H-Eth) schizonts were lysed in Triton X-100. The proteins immunoprecipitated by either normal mouse serum (lanes NMS) or mAb 5C10/66 ascites (lanes 5C10) were electrophoresed in a $NaDodSO_4$/polyacrylamide gel. The gel was developed with scintillant, dried, and fluorographed.

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V K E T T E T T E K A T Q E T Q A A D T T T P E G T T P T E Q E A A P T A E E P  80
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Q P A T P E T P A E V P A T P A A P A A P A A P A A P A K P V M T K L Y Y L E K  120
CAACCTGCAACACCCGAAACACCAGCAGAGGTACCAGCCACACCTGCAGCTCCAGCAGCTCCAGCAGCACCAGCAGCACCTGCAAAACCAGTTATGACAAAATTATATTACCTTGAAAAA 360

L K K F F A F L I F S H K Y V L L Q [N S T] I N K D A L S K Y A L T P E E D K I R  160
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T L K R C S E L D V L L A I Q N N M P T M Y S L Y E N V V D G L Q N I Y T E L Y  200
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E K E M M Y H I Y N L K D K N P A I K A L L V K A G V I E P E P E P V A P T P A  240
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E T T T P A E P T P E T K E G A S T [N K S] E T S E G T R A P E A P S T E V P A S  320
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P P A T P A A P S A S S P A P A Q P A P A Q P V T S Q P V S G E S T N V E G S T  360
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K Y K L K L E R L Y Q K H E Q I E A A N Q K V K E I S V L K S R L L K K K K  478
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Pca DS    KMEQEPVATGES-EQVTPSSGAGTSTQTAQTTQTPPAAPAPVKETTETTEKATQETQAADTTTPEGTTPTEQEAAPTAEEPQPATPETPAEVPATPAAPA
Py 17XL
Pf Well   *VFMPK*ESLINE*KKNIK----------**GQSDNSEPST*G-*I*GQ**--*KPGQQAGSALEGDSV*AQ*QEQKQA**PV*VPVPEAK*QV--*T
Pf Fc27   *EFIPK*KDMLKK**AVL**I----------**PLV**S----****DGGHS*HTLSQSGE*EVTEE*EVTE*TVGHTTTVTITL*PKEESA*KEVKVVE
          .........|.........|.........|.........|.........|.........|.........|.........|.........|.........|
                   10        20        30        40        50        60        70        80        90        100
                             block 9
Pca DS    APAAPAAPAKP-VMTKLYYLEKLKKFFAFLIFSHKYVLLQNSTINKDALSKYALTPEEDKIRTLKRCSELDVLLAIQNNMPTMYSLYENVVDGLQNIYTE
Py 17XL
Pf Well   P**-*VNNKTEN*-S**D*****YQ*LNTSYIC***I*VSH**M*EKI*KQ*KI*K**ESK--*SS*DP**L*FN****I*V***MFDSLNNS*SQLFM*
Pf Fc27   NSIEHKSNDNSQAL**TV**K**DE*LTKSYIC***I*VS**SMDQKL*EV*N*****ENE--*KS*DP**L*FN****I*A*****DSMNID**HLFF*
          .........|.........|.........|.........|.........|.........|.........|.........|.........|.........|
                   110       120       130       140       150       160       170       180       190       200
                             block 10
Pca DS    LYEKEMMYHIYNLKDKNPAIKALLVKAGVIEPEPEPVAPTPAVPAPETAPETVPETPAQEATQEATQEATQPESAPAQEATTETTTPAEPTPETKEGAST
Py 17XL
Pf Well   I*****VCNL*K***NDK-**N**EE*KKVS----------TSVKT----------LSSSSMQPLSLTPQDKP*VSANDD*SHS----------
Pf Fc27   **Q***I*YLHK**EE*H-**K**EEQKQ*TGTSSTSS*GNTTVNTAQSATHSNSQNQ*SNASSTNTQNGVAV*SGPAVVEESHD-*LT----------
          .........|.........|.........|.........|.........|.........|.........|.........|.........|.........|
                   210       220       230       240       250       260       270       280       290       300

Pca DS    NKSETSEGTRAPEAPSTEVPASPPATPAAPSASSPAPAQPAPAQPVTSQPVSGESTNVEGSTQVKAESEDELFVVDFEVDNFYKSYLQSYLQQVDGNNTQ
Py 17XL                                                                                                    E
Pf Well   ----------------------------------------------*NLNN*LKLFENIL*LG-*NKNIYQELIGQKSSE***EKI*KDSDT-FY--*ES
Pf Fc27   ----------------------------------------------*L*ISNDLKGIVSLLNLGN*TKVPNP*TISTT*MEK**ENI*KNNDTYF---*DD
          .........|.........|.........|.........|.........|.........|.........|.........|.........|.........|
                   310       320       330       340       350       360       370       380       390       400
                             block 11                                block 12
Pca DS    FIDFIKSKKELINALTPE-KVNQLYLDIAHLKELSEHYYNRYYKYKLKLERLYQKHEQIEAANQKVKEISVLKSRLLKKKK
Py 17XL   **N*********K*****-********E***********D**ST*********N*****QLT*RQIRDL*I**A****R*Q
Pf Well   *TN*V***ADD**S*ND*S*RKK*EE**NK**KTLQLSFDL*N*********FD*KKTVGKYKMQI*KLTL**EQ*ES*LN
Pf Fc27   IKQ*V**NSKV*TG**-*TQK*A*NDE*KK**DTLQLSFDL*N******D**FN*KKELGQDKMQI*KLTL**EQ*ES*LN
          .........|.........|.........|.........|.........|.........|.........|.........|.........|.........|
                   410       420       430       440       450       460       470       480       490       500
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FIG. 3. (*Top*) The topographical relationship between the *P. chabaudi adami* (P.c.a.) DS PMMSA clone and *P. falciparum* (P.f.) PMMSA and *P. yoelii* (P.y.) PMMSA. The conserved, semiconserved, and variable blocks of *P. falciparum* PMMSA as proposed by Tanabe *et al.* (2) and their estimated relationship with the major processed fragments of PMMSA protein (1, 19) are schematically shown. (*Middle*) cDNA sequence and deduced amino acid sequence (single-letter code) of the *P. chabaudi adami* DS PMMSA clone selected by the mAb 5C10/66. Repeat sequences are underlined and potential N-linked glycosylation sites are boxed. (*Bottom*) Alignment of this clone with *P. falciparum*

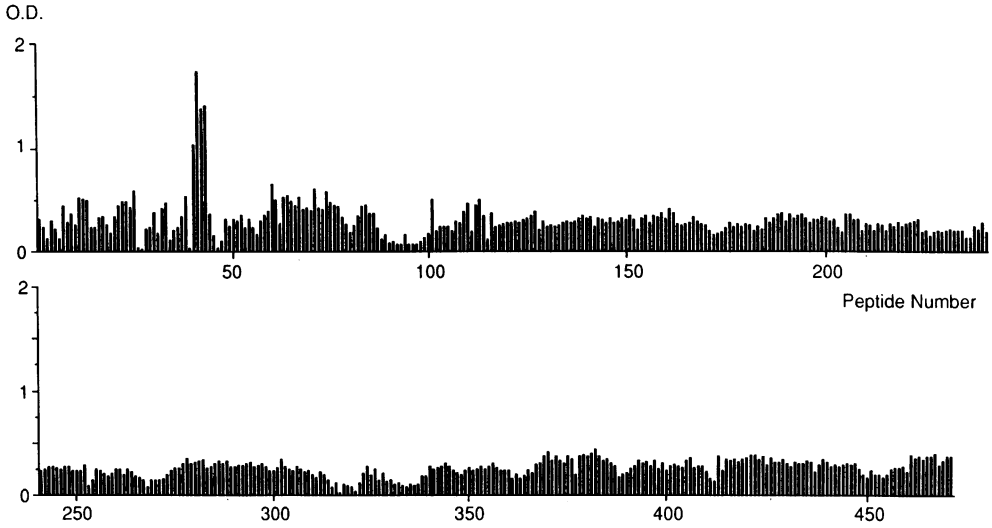

FIG. 4. Epitope analysis of mAb 5C10/66. A set of overlapping octamer peptides derived from the predicted amino acid sequence of the cDNA clone was synthesized on polyethylene rods and ELISA was performed with mAb 5C10/66. Peptides are numbered according to their amino-terminal residues. The peptides 40–43 correspond to the region Pro-Val-Lys-Glu-Thr-Thr-Glu-Thr-Thr-Glu-Lys.

and *P. yoelii* = 74%) than between either of them and *P. falciparum* (33–39%). The homology between the entire *P. chabaudi* PMMSA clone and *P. falciparum* was 23%. The blocks that are the least homologous between alleles of *P. falciparum* [the variable blocks defined by Tanabe *et al.* (2)] also show the least homology between *P. chabaudi adami* and *P. falciparum* (19–20% and 12% in blocks 8 and 10, respectively; Fig. 3 *Top* and *Bottom*). Accordingly, the more conserved blocks for *P. falciparum* have the most homology to *P. chabaudi adami* (43–45% and 37–40% in blocks 9 and 11, respectively). Block 12 is too underrepresented for *P. chabaudi adami* for valid comparison. The two variable blocks (blocks 9 and 11) also show the most variation in length compared to their counterparts in *P. falciparum; P. falciparum* PMMSA (≈195 kDa) is smaller than *P. chabaudi* PMMSA (≈250 kDa).

Typical of many *Plasmodium* molecules, the *P. chabaudi adami* PMMSA displays a series of repeats (underlined in Fig. 3 *Middle*), most of which are tandem repeats. There are seven copies of Ala-Ala-Pro; three of Glu-Thr-Thr; three of Pro-Ala-Thr-Pro; four of a degenerate repeat Pro-Glu-Thr followed by a hydrophobic residue; four of Gln-Glu-Ala-Thr; and two of Pro-Ala-Pro-Ala-Gln. All of these repeats reside in the two variable blocks 8 and 10.

Epitope Mapping. Using the Geysen peptide scanning technique, mAb 5C10/66 was shown to bind the peptides containing Glu-Thr-Thr-Glu-Thr sequence (Fig. 4). These peptides (nos. 40–43) correspond to the region Pro-Val-Lys-Glu-Thr-Thr-Glu-Thr-Thr-Glu-Lys (residues 40–50). It is interesting that no binding was observed for the peptide containing a sequence very similar to the above core sequence, Glu-Ala-Thr-Thr-Glu-Thr-Thr (residues 277–283).

DISCUSSION

A mAb 5C10/66 was generated and shown to afford strong protection against lethal challenge with *P. chabaudi adami* DS on passive immunization. The mortality rate was reduced from 75% to 0%. This antibody identified a protein that had several characteristics of PMMSA: localization to the surface of merozoites by immunofluorescence; it is labeled with myristic acid and ethanolamine and so it presumably has a glycosyl phosphatidylinositol tail and it has a high molecular mass (250 kDa). The sequence of a λgt11 cDNA clone recognized by mAb 5C10/66 identified that this clone represented the middle portion of PMMSA and, hence, that the epitope of this protective antibody resided in this region.

The degree of protection afforded by mAb 5C10/66 is remarkable because immunity to *P. chabaudi adami* is generally regarded to be mainly T-cell mediated because infections in B-cell-deficient mice occur with similar kinetics to normal mice, whereas athymic nude mice develop severe and lethal infections (23). Also, adoptive transfer of L3T4 positive cells or a cloned T-cell line was able to protect nude mice (24, 25), whereas passive protection by either hyperimmune serum or several mAbs has been undramatic (4, 23, 24, 26). It is interesting that the degree of protection afforded by mAb 5C10/66 against *P. chabaudi adami* was similar to that by mAb 302 against *P. yoelii* (5), and, like mAb 5C10/66, mAb 302 recognizes PMMSA (20).

The large size of the *P. chabaudi* PMMSA compared to the *P. falciparum* PMMSA is at least partially explained by the increased length in the two variable blocks 8 and 10 (Fig. 4 *Upper*). Interestingly, all of the repeats are found in these two blocks. Repeats are also found in blocks 8 and 10 in *P. falciparum* PMMSA, albeit less extensively than for *P. chabaudi*. It could be argued that the variable blocks are variable because of immune pressure and these repeats reside in variable blocks for a similar reason.

PMMSA is processed into various fragments at the time of merozoite maturation (1) (Fig. 3 *Top*). Although the exact cleavage sites are unknown, the sizes of these fragments can be used to estimate their topographical relationship with the sequence (1, 19). Because mAb 5C10/66 identifies the 83-kDa amino-terminal fragment in merozoites (Fig. 2 *Left*), the epitope of mAb 5C10/66 was predicted to be in the region that overlaps the region that encodes this fragment and the region corresponding to the cDNA clone. Thus, the epitope was expected to be in the C-terminal region of the 83-kDa fragment, which corresponds to the 5′ end of the cDNA clone and thus is near the start of block 8. Because this region does not overlap with the *P. yoelii* PMMSA clone of Burns *et al.* (20), the epitope of mAb 5C10/66 must reside in a different location to the *P. yoelii* mAb 302.

PMMSA (two different forms are represented by the Wellcome and FC27 strains) and *P. yoelii* PMMSA (20–22). The numbers indicated are for alignment and do not correspond to residue numbers as there are three introduced gaps in the *P. chabaudi* sequence. Asterisks indicate homology and arrows indicate start of the blocks for *P. falciparum* as proposed by Tanabe *et al.* (2).

To further define the epitope of 5C10, a complete set of overlapping octamer peptides was synthesized and examined by ELISA. mAb 5C10/66 binds to the peptides containing the Glu-Thr-Thr-Glu-Thr-Thr sequence (peptides 40–43 of the cDNA clone) (Fig. 4). This was consistent with the above prediction that the epitope resides near the start of block 8. Hence, we have defined a five-amino acid region of a malaria antigen that is an immunological target capable of preventing mice from lethal infection. The finding of such a linear epitope in *P. chabaudi adami* DS provides hope for synthetic peptide strategies in vaccination against the asexual blood stage of malaria.

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