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Effect of Antibodies to Recombinant and Synthetic Peptides on *P. falciparum* Sporozoites in Vitro

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Antibodies were raised in mice immunized with several recombinant and synthetic peptides of the circumsporozoite protein of *Plasmodium falciparum*. The antibodies were evaluated for protective activity in a human hepatocyte culture system. They exerted their protective effect against the parasite at three points: sporozoite attachment to the hepatocyte surface, entry, and subsequent intracellular development. Inhibition of attachment and entry were found to be related to the antibody titer against the authentic circumsporozoite protein on the sporozoite surface, especially when peptides were administered with alum or complete Freund's adjuvant. Even when invasion was not totally inhibited, the presence of abnormal trophozoites and a frequent inhibition of schizont development in long-term cultures suggested continued activity of antibodies at the intracellular level after sporozoite penetration had been completed.

DESPITE PROMISING RESULTS FROM earlier vaccination experiments against human malaria involving irradiated sporozoites (1, 2), progress on a purified sporozoite vaccine suitable for use in humans has been delayed for lack of the required immunogens. Recently, however, synthetic peptides and recombinant DNA circumsporozoite (CS) protein derivatives were produced and characterized (3, 4), and both are now available for experimentation. In the present study, we took advantage of a recently described system for cultivation of the liver stages of *Plasmodium falciparum* (5) to study the ant sporozoite activity of antibodies raised against synthetic peptides and recombinant CS protein products of this species.

Human hepatocytes obtained from liver biopsies (6) were seeded at a concentration of 10^5 cells per chamber in eight-chamber plastic Lab-Tek slides (Miles) and cultured for 24 to 48 hours before sporozoite inoculation. Sporozoites of *P. falciparum* were obtained from *Anopheles stephensi* and

Anopheles freeborni mosquitoes after feeding on gametocytes from cultures of the NF54 strain through an artificial membrane. Salivary glands were aseptically dissected and, after removal of medium from the culture chambers, sporozoites were added in 50 μ l of medium containing test or control serum. This medium was replaced 3 hours later by medium without antibody, and thereafter was changed daily.

Sera from groups of five mice immunized with recombinant or synthetic peptides were pooled for testing (3, 4). The animals had received two injections at a 4-week interval of one of the following recombinant protein constructs of the repeat region of the CS protein: R16tet32 (R16), R32tet32 (R32), or R48tet32 (R48) administered with complete Freund's adjuvant (CFA), alum, murabutide (7), or phosphate-buffered saline (PBS) or two injections of one of the following synthetic peptides administered with CFA: 8- or 16-amino acid peptide sequences of the repeat region of the CS protein or of region I or region II, which are

conserved amino acid sequences occurring outside the repeat regions in the CS proteins of both *P. falciparum* and *Plasmodium knowlesi*. Controls consisted of normal mouse serum, serum from mice injected with alum only, and monoclonal antibodies recognizing either the CS protein of *P. falciparum* or that of *Plasmodium vivax*. Monoclonal antibodies were purified from ascitic fluids and titrated at least $1:10^5$ against the homologous sporozoites. The level of antibody reactivity against the immunizing peptides was measured by the enzyme-linked immunosorbent assay (ELISA) (3, 4), while the reactivity with the authentic CS protein on the surface of the sporozoite was determined by the immunofluorescent antibody test (IFAT).

Both qualitative and quantitative experiments were performed. In the qualitative experiments, five pairs of salivary glands dissected in a 1:20 dilution of test or control sera were added to each chamber. In quantitative experiments, 25 μ l of a 1:5 dilution of the serum was added to each chamber. Salivary glands were homogenized with culture medium in a tissue homogenizer, sporozoites counted, and an equal number added to each chamber in a volume of 25 μ l. Depending on the number available, 1×10^4 to 5×10^4 sporozoites per chamber were used to inoculate each of the cultures. In any one experiment, the size of the inoculum was the same for all chambers, including controls. Unless otherwise stated, all quantitative experiments were carried out in a final serum dilution of 1:10.

Cultures were examined with a fluorescent assay (5). The number of sporozoites that invaded a liver cell and developed into a liver form was determined for each culture chamber 24 hours after the inoculum was added to the chamber. This delay was required to identify unequivocally intracellular parasites. In preliminary experiments, test cultures were trypsinized at 24 hours to remove parasites attached to the cell surface. Examination of these cultures confirmed that the remaining forms were indeed intracellular. In short-term cultures (24 to 48

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hours), the first antibody used to detect the presence of liver forms was a mouse monoclonal antibody directed against *P. falciparum* CS antigen. In long-term cultures (3 to 6 days), a human polyclonal serum was used as the detecting antibody. Preparations were then stained with fluorescein-labeled antimouse or antihuman immunoglobulin as appropriate. The number and appearance of extracellular sporozoites and intracellular parasites were studied with epifluorescence

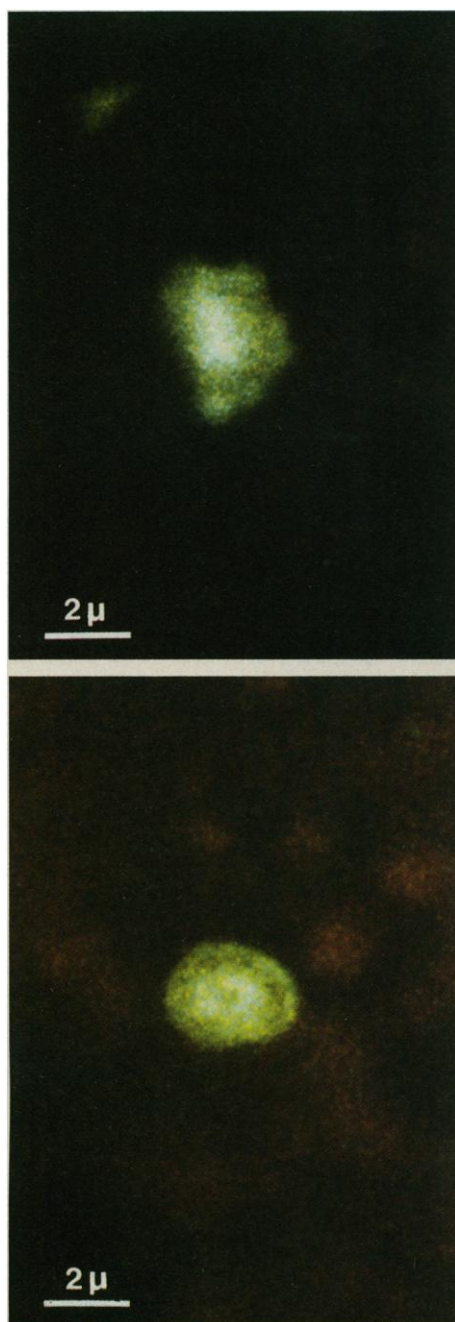


Fig. 1. Liver-stage *P. falciparum* trophozoite in 24-hour culture, stained by fluorescence after incubation of sporozoites with (bottom) normal mouse serum or (top) mouse serum containing antibodies to R32 murabutide, both at 1:10 dilution in medium (magnification $\times 1000$).

at $\times 250$ and $\times 1000$ magnification. Results are expressed as the percentage of inhibition calculated from the ratio of the number of parasites in test cultures compared with the number in control cultures for the same experiment. Cultures contaminated with fungi or bacteria, and cultures in which the number of parasites in control wells was lower than 20, were not included in the results.

In qualitative experiments, schizonts were frequently found in cultures fixed after 2 to 5 days of cultivation. These cultures had been exposed to antibodies directed against the 8- or the 16-amino acid synthetic peptide in a 1:20 dilution. Although only small numbers of parasites were detected, their appearance and size were similar to those in control cultures. Since total inhibition was not consistently obtained, all other tests were performed quantitatively to measure the percentage of sporozoite attachment, penetration, and development.

The addition of immune serum had an obvious effect at three levels: attachment of the sporozoite to the hepatocyte, entry of the sporozoite into the hepatocyte, and intracellular development of the parasite.

Reduction of sporozoite attachment. In control cultures, only 0.1 to 0.5 percent of sporozoites normally penetrated hepatocytes and developed into rounded liver forms. Many of the remaining sporozoites simply attached to the cell surface, where they remained extracellular without detectable morphological alteration. In cultures containing antibodies, the number of attached sporozoites was reduced up to 96 and 100 percent when exposed to sera produced against the R32 alum or R16 CFA preparations, respectively. Even when total attachment inhibition was not obtained, the effects of antibodies on the few sporozoites that remained attached were readily visible, with the majority showing evidence of circumsporozoite precipitation. High-titer monoclonal antibody to *P. falciparum* CS protein totally inhibited attachment.

Reduction of penetration and initial development (24 to 48 hours). The number of parasites developing in cultured hepatocytes was found to be markedly reduced by most of the antibodies tested. When sporozoites managed to escape the extracellular effects of antibodies, however, many of the resulting intracellular parasites appeared abnormal. Their size was similar to those of control cultures, but their surface was broadly scalloped (Fig. 1).

The inhibitory effect was related to the adjuvant and type of antigen used to raise the antiserum, the dilution of the antiserum, and its IFAT titer against sporozoites. Detailed results from short-term cultures are summarized in Fig. 2. The results show that

a high level of inhibition (80 to 100 percent) could only be achieved with sera from immunized mice when CFA or alum was used as the adjuvant. Differences between these two adjuvants did not appear to be significant, although the only serum that totally inhibited penetration was prepared with CFA. When no adjuvant or murabutide was used, lower levels of inhibition (0 to 78 percent) were recorded. Among the different types of antigens, R32 was apparently the more consistently effective in producing inhibitory sera. No inhibition was noted with the use of sera from mice receiving alum alone, monoclonal antibodies to unrelated antigens, such as antibody to *P. vivax* CS antigen, or sera from mice immunized with region I or region II. In contrast, monoclonal antibody to *P. falciparum* CS protein totally inhibited attachment and penetration.

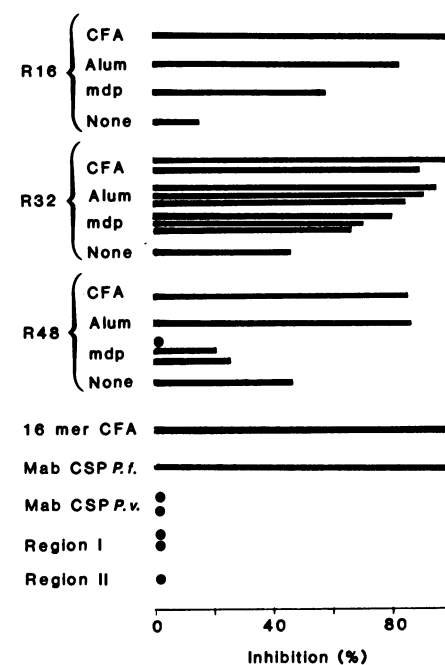


Fig. 2. Inhibition of *P. falciparum* sporozoite penetration in cultured liver cells by antibodies to a variety of antigens. Each bar represents a single experiment with antibodies raised to recombinant and synthetic peptides and control sera (1:10 dilution). Percentages of inhibition were estimated by counting the number of intracellular trophozoites stained by immunofluorescence in cultures fixed at 24 to 48 hours compared with the number in the corresponding control culture. Control sera were monoclonal antibodies (Mab) to surface antigens of either *P. falciparum* (P.f.) or *P. vivax* (P.v.). Sixteen mer is a synthetic sequence of four copies of the tetrapeptide repeat of the *P. falciparum* CS protein (CSP). Regions I and II are synthetic sequences of the conserved regions of this CS protein. The closed circles represent cultures in which no inhibition was observed. The abbreviation mdp represents murabutide (*N*-acetylmuramyl-L-alanyl-D-glutamyl- α -*n*-butyl ester).

In each experiment, the possible degradation of antibodies during incubation of mouse sera was monitored by comparing the IFAT titer of the test serum with that of the corresponding spent culture medium collected after 3 hours. In every case, the titers remained unchanged. The inhibitory effect of the sera was found to be closely related to the concentration of specific anti-sporozoite surface antibodies in the test culture. Dilution of sera consistently resulted in lowered levels of inhibition (R32 CFA 1:10, 97 percent inhibition; 1:20, 91 percent; 1:40, 80 percent), and inhibition obtained with each serum was directly related to its IFAT titer (Fig. 3). Results obtained with antibodies to the R16 construct illustrate the relation between inhibition and antibody level. Depending on the adjuvant used, inhibitory effects varied widely (15 to 100 percent), as did the levels of antibodies induced (no adjuvant, 1:500; murabutide, 1:1000; alum or CFA, 1:8000).

In contrast, ELISA titers against the recombinant molecule appeared to be independent of the IFAT results [for example, two sera with a 1:400 ELISA titer produced very different inhibition (0 and 81 percent) and IFAT titers (1:500 and 1:8000, respectively)]. Two other sera having similar IFAT titers (1:1000) and inhibition levels (46 percent) titrated 1:1600 and 1:200 by ELISA.

Delayed effect in long-term cultures. In long-term cultures the effect of immune serum was more pronounced, although total inhibition was not always achieved. For example, in a culture with sera produced against R32 alum, a normal-looking schizont measuring 35 μ m was found. However, total inhibition occurred in four of five quantitative experiments and in several qualitative experiments with a 1:20 dilution of antise-

Table 1. Percentage of inhibition of liver stages after infection with sporozoites in the presence of antisera to recombinant proteins. Numbers in parentheses indicate the ratio of the number of parasites found in a test culture to the number in its paired control. For each antibody tested and at each time interval, the samples, including controls, consisted of all the parasites in a single culture chamber. Cultures testing each antibody were started at the same time with a single hepatocyte and sporozoite source.

Immunizing preparation	Percentage of inhibition		
	24 hours	3 days	6 days
R32 with murabutide	57 (15/35)	82 (6/32)	100 (0/32)
R32 with alum	88 (9/73)	92 (2/26)	95 (1/22)
R32 without adjuvant	49 (18/35)	94 (2/32)	100 (0/32)

rum. Sera raised with R32 murabutide gave a mean inhibition of 71 percent at 24 hours and of 91 percent at 3 days. This delayed effect was not unexpected, since in our earlier experiments a morphological difference had already been noted between 24-hour and 3-day cultures. It was suspected that the abnormal trophozoites which exhibited an altered, scalloped border at 24 hours would probably not complete their development.

Experiments were conducted to confirm the preliminary observation that antisera continues to exert an effect on intracellular parasite development. Hepatocyte cultures containing a 1:10 dilution of antisera were infected with identical inocula from a single preparation of sporozoites. A test culture and its paired control were examined at 1, 3, and 6 days. The number of liver forms in the test culture, compared with the number in the paired control of the same age, showed a

progressive increase in inhibition from 24 hours to 6 days (Table 1). This long-term effect, in contrast with results from short-term cultures, was independent of antibody levels to sporozoite surface antigens. For instance, regardless of the antigen used, 100 percent inhibition at 6 days was achieved, with sera titrating only 1:1000 from mice immunized using murabutide or no adjuvant (Table 1), whereas total protection was not even achieved with antibodies against R32 titrating to 1:8000.

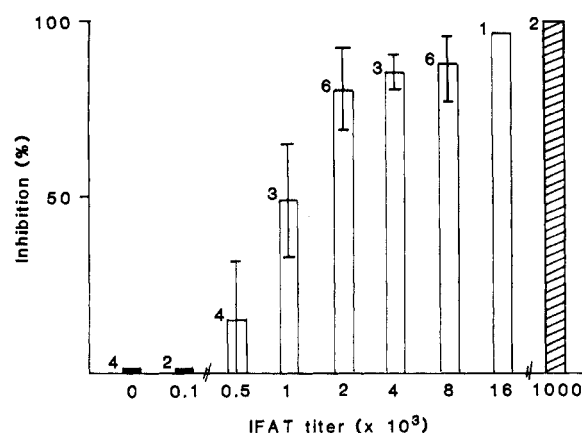
Under the conditions of these in vitro experiments, the attachment, penetration, and development of *P. falciparum* sporozoites in human hepatocytes were markedly inhibited, but not always totally blocked, by antisera raised in mice to synthetic or recombinant peptides of the repeat region of the CS protein. Results show a clear relation between the antisporezoite antibody levels and their effect on both attachment and penetration. The continuing effect of antisera after penetration of sporozoites was surprising, considering that these antisera had been removed from the cultures after 3 hours of incubation.

Inhibition of schizont development may result from an alteration by antibodies of some sporozoites in a way that does not prevent penetration. Alternatively, antibodies to the CS protein may exert an effect on trophozoites at the intracellular level, since the CS protein is detectable in cultured liver forms. The wavy appearance of the outer surface of some young liver forms could be the result of an antigen-antibody interaction similar to circumsporozoite precipitation but at the trophozoite stage. Whether or not antibodies directed against the parasite can enter hepatocytes directly is not known; however, it has been shown that immunoglobulin A antibodies can enter hepatocytes (8, 9) and, although entry of immunoglobulin G (IgG) has not been reported, hepatocytes are known to have Fc receptors (10, 11). It has been shown, however, that IgG-albumin immune complexes penetrate these cells in vivo (12), and IgG antibodies have been detected in the nuclei of hepatocytes from patients infected with hepatitis B virus (13).

Our data suggest that even when penetration is not totally blocked, antibodies to the CS protein may still exert an inhibitory effect on development at the intracellular level. On the basis of these observations, it would appear that the activity of antibodies should not be assessed solely by penetration assays.

To what extent this in vitro model is predictive is not yet established. We do not know if the humoral response in man will be as high in all individuals as that developed in

Fig. 3. Relation between inhibition of sporozoite invasion and titer of antibody to sporozoite surface antigens measured by IFAT. Open bars with standard errors represent the average inhibitory effect of mouse sera to recombinant and synthetic repeat tetrapeptides. The number at the top of each bar indicates the number of sera giving the end point titer indicated at the bottom of the bar and is independent of the immunizing preparation used to prepare each serum. The cross-hatched bar represents a mouse monoclonal antibody to *P. falciparum* CS antigen. Filled bars represent antibodies to regions I and II. Sera were titrated by testing serial dilutions in an immunofluorescence assay on glutaraldehyde-fixed (0.01 percent) *P. falciparum* sporozoites. Antibodies to mouse immunoglobulins G, A, and M (Cappel) and antihuman immunoglobulins G, A, and M (Institut Pasteur Production) were used at 1:100 dilution in 0.05 percent Evans blue.



highly responsive, genetically inbred mice. Cultured parasites may be more susceptible to inhibitory agents than parasites developing in vivo. Since a single developing schizont is sufficient to induce a blood infection, and since some sporozoites manage to develop even in the presence of antibodies, the humoral protection resulting from immunization may vary from one individual to the other and may depend in part on the size of the challenge inoculum.

In our culture assay, only diluted sera could be tested. Since a positive relation was found between the degree of invasion inhibition and the level of antibody, a correspondingly greater effect might be expected in vivo, where the antibody would be undiluted. Antibodies may also cooperate with

other components of the immune system, such as macrophages and Kupffer cells, particularly if sporozoites have to transit these cells in vivo to reach the hepatocyte (14). Sensitized T lymphocytes could also play a toxic role or interfere indirectly by releasing products such as gamma interferon.

Finally, it is encouraging that alum, which is licensed for use in man, was nearly as effective as CFA in inducing protective antibodies.

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Thyrotropin-Releasing Hormone Precursor: Characterization in Rat Brain

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To characterize the precursor of mammalian thyrotropin-releasing hormone (TRH), a rat hypothalamic λ gt11 library was screened with an antiserum directed against a synthetic peptide representing a portion of the rat TRH prohormone. The nucleotide sequence of the immunopositive complementary DNA encoded a protein with a molecular weight of 29,247. This protein contained five copies of the sequence Gln-His-Pro-Gly flanked by paired basic amino acids and could therefore generate five TRH molecules. In addition, potential cleavage sites in the TRH precursor could produce other non-TRH peptides, which may be secreted. In situ hybridization to rat brain sections demonstrated that the pre-proTRH complementary DNA detected neurons concentrated in the parvocellular division of the paraventricular nucleus, the same location as cells detected by immunohistochemistry. These findings indicate that mammalian TRH arises by posttranslational processing of a larger precursor protein. The ability of the TRH prohormone to generate multiple copies of the bioactive peptide may be an important mechanism in the amplification of hormone production.

THYROTROPIN-RELEASING HORMONE (TRH, pyroGlu-His-ProNH₂) has a central role in the regulation of the hypothalamic-pituitary-thyroid axis (1). Although TRH was the first hypophysiotropic peptide to be characterized structurally (2), the mechanism of its biosynthesis has been controversial. The initial hypothesis that TRH was synthesized by a nonribosomal mechanism (3) has not been confirmed (4). The alternative view, that TRH arose by a messenger RNA (mRNA)-directed ribosomal mechanism, was suggested by several subsequent studies (5) and is supported by the isolation of a complementary DNA (cDNA) encoding a portion of the TRH precursor from frog skin (6), a tissue that has a relative abundance of TRH (7).

To determine whether TRH in the mam-

malian hypothalamus also arises from the posttranslational cleavage of a larger precursor protein, we raised an antiserum (No. 342) against a synthetic peptide hypothesized to represent a portion of the mammalian TRH prohormone (8). Immunocytochemical studies indicated that this antiserum recognizes the rat TRH prohormone rather than the fully processed peptide (8). This provided us with the means to identify a cDNA that encodes the TRH precursor from a rat hypothalamic λ gt11 bacteriophage expression library based on methods described by Young and Davis (9). Antisera against TRH itself cannot be used to screen such libraries because of the extensive posttranslational processing undergone by this peptide. Because of the small size of the TRH sequence and the degeneracy of the

codons representing the three amino acids, the application of conventional hybridization techniques to identify a cDNA encoding mammalian hypothalamic TRH would have presented several problems. Furthermore, it seemed likely that the nucleotide sequences encoding the amphibian and mammalian TRH precursors have diverged.

The expression library was prepared by isolating polyadenylated RNA from 65 adult Sprague-Dawley rat hypothalami to generate double-stranded cDNA (10). Approximately 3×10^7 recombinant phage with an average insert size of 500 base pairs (bp) were generated from 1 μ g of insert cDNA. The library was plate-amplified to a titer of 10^{10} plaque-forming units per milliliter. Plaques (7.5×10^5) from the rat hypothalamic λ gt11 library were screened with the proTRH antiserum. Of these, eight immunopositive bacteriophage clones were identified and purified by sequential low-density plating (Fig. 1). The largest of the cDNA's (pLW 4-2, 1322 bp) was subcloned into the plasmid pUC-12 for further study.

The restriction map and sequencing strategy used to characterize pLW 4-2 are illustrated in Fig. 2A. The nucleotide and corresponding amino acid sequence are depicted in Fig. 2B. This cDNA includes an open

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