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REPEAT REGION OF *PLASMODIUM FALCIPARUM* CIRCUMSPOROZOITE PROTEIN DOES NOT RECOGNIZE OR BIND TO HUMAN HEPATOMA TARGET CELL

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Antibodies directed against the repeat region of the Plasmodium falciparum *circumsporozoite protein can completely inhibit sporozoite invasion of hepatic cells, suggesting that this protein region may have a role in hepatic cell recognition or invasion. To directly test this possibility we assayed recombinant proteins containing a large portion of the repeat region of the circumsporozoite protein of* P. falciparum *for binding affinity to a human hepatoma cell line, HepG2-A16, under conditions where invasion by sporozoites is known to take place. No specific binding of the repeat region could be detected. Nonspecific attachment was seen to occur with an earlier form of the recombinant protein, R32tet$_{32}$, but this binding was shown to be dependent on a highly charged, plasmid derived terminal sequence of amino acids. When this sequence was removed by either enzymatic treatment or recombinant manipulation, no binding was detected. No significant inhibition of* in vitro *invasion by competition with the repeat region recombinant protein was seen. These data suggest that the repeat region of the* P. falciparum *CS protein is not directly involved in attachment of the sporozoite to the hepatocyte. While immune sera raised against an unmodified repeat sequence of amino acids clearly do inhibit sporozoite invasion of liver cells, a better understanding of the recognition and invasion process and the specific role of the CS protein could greatly increase the efficacy of any potential vaccine against this stage of malaria.*

Monoclonal antibodies against the repeat region of the circumsporozoite (CS) protein have been shown to inhibit sporozoite invasion of liver cells *in vivo* by Yoshida et al. (1980) and to inhibit both attachment and invasion *in vitro* by Hollingdale et al. (1984). Whole IgG as well as monovalent Fab fragments of these monoclonal antibodies were shown by Potocnjak et al. (1980) to be equally active *in vitro* and *in vivo*. Clyde et al. (1973) demonstrated that sera from human volunteers experimentally immunized with *Plasmodium falciparum* sporozoites and resistant to sporozoite-hepatocyte interactions and may thus be a candidate anti-malarial vaccine. Young results suggest that the CS protein repeat region may elicit protective antibodies that prevent sporozoite – hepatocyte interactions and may thus be a candidate anti-malarial vaccine. Young et al. demonstrated that sera from mice immunized with molecular constructs containing only repeat region sequences of the *P. falciparum* CS protein blocked sporozoite invasion of hepatoma cells *in vitro*. Thus, sporozoite invasion of hepatic cells may be mediated by interaction of the CS protein with the hepatic cell surface. In this note we report that purified recombinant proteins containing extended repeat region sequences from *P. falciparum* CS protein do not bind to a cloned human hepatoma cell line, HepG2-A16, under conditions where sporozoite attachment and invasion are known to occur. Furthermore, recombinant protein containing the CS repeat region had no effect on sporozoite *in vitro* invasion of HepG2-A16 cells (ISI). We suggest that the lack of binding of a substantial segment (128aa) of the *P. falciparum* CS repeat region indicates that this region is not directly involved in liver cell attachment.

MATERIALS AND METHODS

The recombinant proteins R32tet$_{32}$, prepared and described by Tonney et al. (1985) and R32tet$_2$ (manuscript in preparation) were generously provided by Dr. Young (Smith, Kline and French Laboratories, Philadelphia, PA). These two constructs both contain 128 amino acids from the sequence of the *P. falciparum* CS protein repeat region with 32 and 2, respectively,

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additional amino acids on their carboxy terminal end derived from the host plasmid (Table I). Protein A, asialo-fetuin, Chymotrypsin-TLCK, and trypsin-TPCK were obtained through SIGMA (SIGMA, St. Louis, MO).

For iodination, $R32tet_2$ was first coupled to methyl-p-hydroxy-benzimidate (MHBI, Pierce Chemical Company, Rockford, IL) through the N terminal amino group as described by Tolan et al. (1980). Derivatized $R32tet_2$ and native protein A were radiolabeled using $Na^{125}I$ (Amersham Corporation, Arlington Heights, IL) and IODOGEN (Pierce) as described by Fraker & Speck (1978). Uncoupled iodide was removed by passing the radiolabeled proteins through DOWEX 1 (SIGMA).

Human hepatoma cloned cell line HepG2-A16 was grown on Thermolux coverslips (LUX, Miles Laboratories, Inc., Naperville, IL) using medium and procedures previously described. HepG2-A16 cells were grown in MEM (Earl's) medium supplemented with 10% fetal bovine serum, 50U/ml penicillin, and 50μg/ml streptomycin. For binding tests proteins were applied in 0.45ml complete medium to coverslips of confluent HepG2-A16 cells in 24 well plates (Falcon) and incubated in 5% CO_2 in air at 37°C for one hour. Coverslips were then washed with phosphate buffered saline and bound protein determined by one of two procedures. Coverslips were incubated only with unlabeled protein were reacted sequentially with a fixed amount of anti-*falciparum* repeat region monoclonal and ^{125}I-protein A, each for 30 min at 22°C followed by extensive washing. Total bound iodine was then determined by counting the coverslips directly in an LKB CliniGama counter (LKB Wallac, Turku, Finland). Coverslips incubated with iodinated proteins were washed extensively and counted directly as above.

For inhibition of sporozoite invasion (ISI), 30000 to 40000 *P. falciparum* sporozoites were added to HepG2-A16 cells in the presence of dilutions of $R32tet_2$. Cultures were incubated for 3hrs at 37°C and the numbers of invaded sporozoites determined as described by Hollingdale et al. (1983).

RESULTS AND DISCUSSION

As increasing amounts of $R32tet_{32}$ were added to cell cultures, the level of binding increased until all available sites were saturated at a concentration of about 1.0μg/ml protein (Fig. 1A). Background levels of binding were, however, very high and appeared to have the same binding saturation curve on blank plastic (Fig. 1B) or glass (data not shown) coverslips. Examination of the sequence of $R32tet_{32}$ suggested that the binding properties of this construct might be due to strong charge interactions with the plasmid derived 32 amino acid tail. Of these 32 amino acids, 12 were highly positive arginine residues, 6 were histidine residues, and none were acidic amino acids (Table I), resulting in a dramatic shift in charge distribution and pI of the construct as compared to the repeat region alone.

Because the *P. falciparum* repeat sequence does not contain positively charged amino acids, treatment of the construct with trypsin to cleave the polypeptide at arginine residues would result in separating the malaria repeat sequence from the bulk of the charged, plasmid derived tail. Ideally the resulting polypeptide would contain only malaria repeat sequence plus two amino acids, leucine and arginine. When $R32tet_{32}$ was treated with 100μg/ml trypsin-TPCK for 10 min and further trypsin activity inhibited by the addition of a five fold excess of trypsin egg white inhibitor, all binding to either cells or substrate decreased to background levels (Fig. 1A and B) over a wide range of concentrations. Incubation of $R32tet_{32}$ with trypsin-TPCK that had been previously incubated with a five fold excess of inhibitor (SHAM, Fig. 1A and B) showed no difference in binding level.

The purified construct $R32tet_2$, produced by the genetic elimination of the major part of the translated plasmid sequence, was used to confirm the trypsin analysis. $R32tet_2$ was derivitized with MBHI and labeled with ^{125}I to a specific activity of about 42μCi/μg. The radiolabeled construct was added to cell cultures in the presence of varying amounts of unlabeled $R32tet_2$ and total bound construct determined directly. The ratio of Bound $R32tet_2$ to free did not vary significantly over the concentration range of 0.03μg/ml to 140μg/ml (Fig. 2B). In contrast, when asialofetuin was used as a ligand, the bound to free ratio showed the expected inverse linear relationship to concentration of bound protein (Fig. 2A) under similar circumstances.

The effectiveness of $R32tet_2$ to compete for sporozoite invasion was assayed by ISI with the recombinant protein replacing the test immune serum. No inhibition could be detected, even with up to 100μg/ml of recombinant protein (data not shown).

The lack of binding by *P. falciparum* CS protein repeat region may be explained in three ways. Firstly, the amino acid sequence may have been altered during the isolation procedure, for

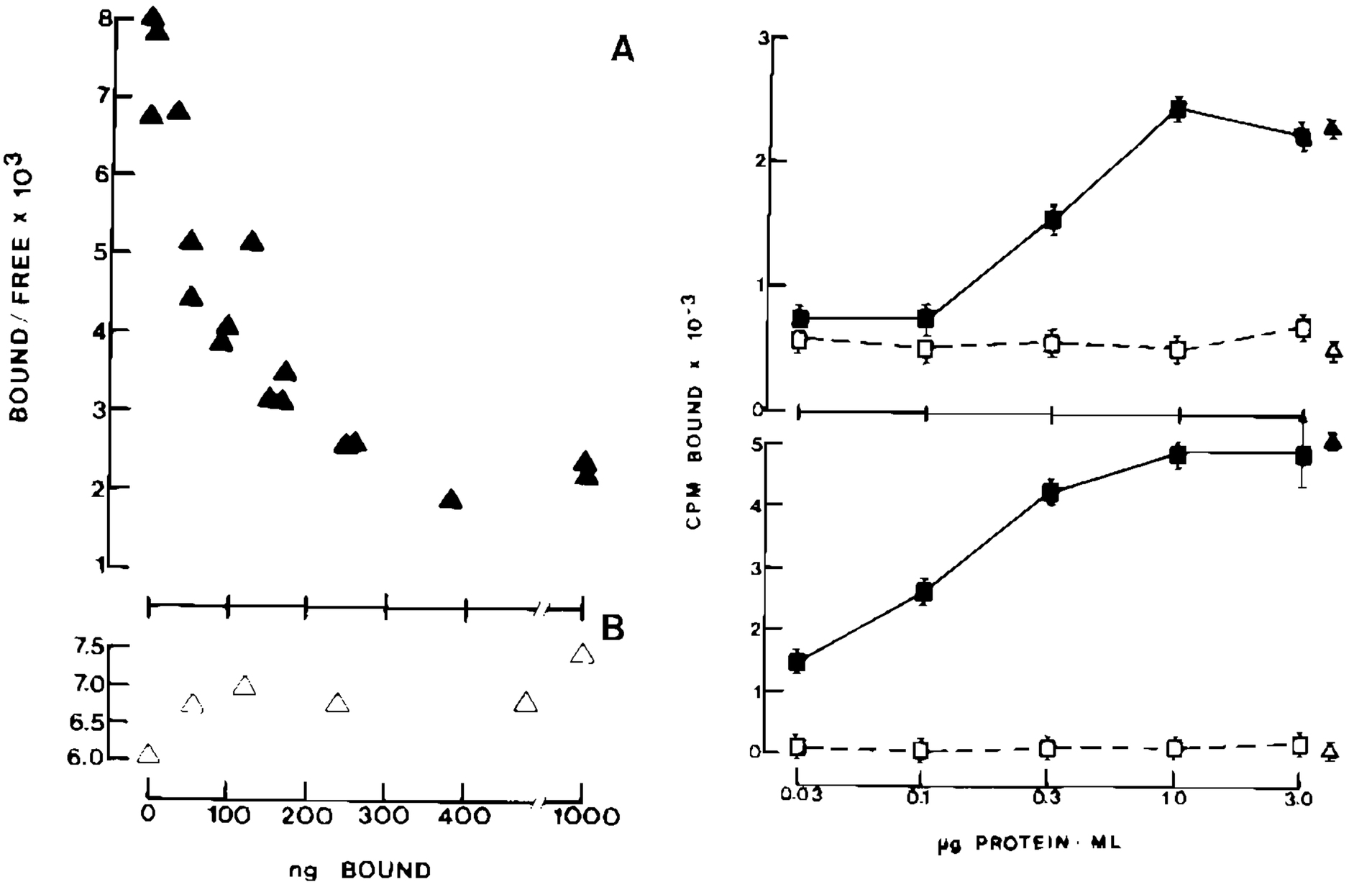

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Fig. 1: Binding of $R32tet_{32}$ to (A). HepG2 Cells. (B). Thermolux coverslips. Bound protein detected by rabbit sera against the *P. falciparum* CS repeat region. (—■—) binding of whole $R32tet_{32}$. (– –□– –) binding of trypsin treated $R32tet_{32}$. (▲) Sham trypsinized sample (trypsin plus inhibitor added to $R32tet_{32}$) and run at 3 µg/ml of initial recombinant protein. (△) background labeling by rabbit antibody in the absence of any form of $R32tet_{32}$. Each point plotted is the mean ± standard deviation of two determinations.

Fig. 2: Scatchard type analysis of (A). Asialo-fetuin and (B). $R32tet_2$ to cell monolayer, plotted as (µg bound protein)/(µg/ml of protein) versus µg bound protein. (▲) Asialo-fetuin, demonstrating combination of specific binding via asialo-glycoprotein receptor (at low protein concentration) plus a non-specific, low affinity component seen at high ligand concentration (high protein concentration). (△) $R32tet_2$, binding in only a non-specific (background or very low affinity) fashion to the monolayer.

TABLE I

Complete amino acid sequence of recombinant proteins $R32tet_{32}$ and $R32tet_2$

Common amino terminal sequence consisting of *P. falciparum* repeat and variant:

Met – Asp – Pro – (Asn Ala Asn - Pro)$_{15}$ – Asn – Val – Asp – Pro – (Asn – Ala – Asn – Pro)$_{15}$ – Asn – Val – Asp – Pro –

Carboxy terminal sequence of $R32tet_{32}$:

– Leu – Arg – Arg – Thr – His – Arg – Gly – Arg – His – His – Arg – Arg – His – Arg – Cys – Gly – Cys – Trp – Arg – Leu – Trp – Arg – Arg – His – His – Arg – Trp – Gly – Arg – Ser – Gly – Ser – COOH

Carboxy terminal sequence of $R32tet_{32}$:

– Leu – Arg – COOH

example by deamination of some of the many asparagine residues during the initial boiling of protein preparation as described by Aheim & Klibanov (1985). Experiments in this laboratory have, however, demonstrated that even chemically synthesized repeat region analogues do not demonstrate affinity for hepatic cells. Secondly, the repeat region of the native CS protein could contain essential post-translational modifications, such as glycosylation or fatty acid acylation, although no such modifications have been presented in the literature. Thirdly, the primary site of sporozoite recognition may reside outside of the repeat region of the CS protein. We are cur-

rently investigating amino acid sequences from other (non-repeat) regions of the CS protein for possible ligand activity.

These data suggest that the repeat region of the *P. falciparum* CS protein is not directly involved in attachment of the sporozoite to the hepatocyte, and that any indirect role would require either substantial post-translational modification or interaction with other regions of the CS protein or other sporozoite components. While immune sera raised agains an unmodified repeat sequence of amino acids clearly do inhibit sporozoite invasion of liver cells, a better understanding of the recognition and invasion process and the specific role of the CS protein could greatly increase the efficacy of any potential vaccine against this stage of malaria.

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