Rapid Clearance of Malaria Circumsporozoite Protein (CS) by Hepatocytes

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Summary

The circumsporozoite protein (CS) covers uniformly the plasma membrane of malaria sporozoites. In vitro, CS multimers bind specifically to regions of the hepatocyte plasma membrane that are exposed to circulating blood in the Disse space. The ligand is in the region II-plus of CS, an evolutionarily conserved stretch of the protein that has amino acid sequence homology to a cell adhesive motif of thrombospondin. We have now found that intravenously injected CS constructs bind rapidly to the basolateral surface of hepatocytes, provided that the recombinant proteins contain region II-plus, and that they are aggregated. Significant amounts of CS were not retained in any other organ. The striking parallelism between these in vitro and in vivo findings with the target specificity of malaria sporozoites, reinforces the hypothesis that the attachment of the parasites to hepatocytes is via region II-plus of CS.

The binding of malaria sporozoites to liver cells in vitro appears to involve an interaction between region II-plus of the circumsporozoite protein (CS)[1] with glycosaminoglycan (GAG) chains of heparan sulfate proteoglycans (HSPG) on the surface of hepatocytes (1). The CS is the major surface protein of malaria sporozoites. A second region II-plus-containing protein, TRAP (2–4) or SSP-2 (5–7), has been identified on the surface of sporozoites from some species of malaria sporozoites, but it is scarcely represented on their plasma membranes. Although the binding of region II-plus to GAG chains may also represent the initial recognition event in vivo, some investigators have suggested that before hepatocyte invasion, sporozoites are retained by specific receptors located either on the fenestrated endothelium that lines the liver sinusoids, or on Kupffer cells (8). The attached sporozoites would then traverse the endothelium, gain access to the Disse space, and enter hepatocytes. Nevertheless, the putative non-hepatocyte receptors that mediate the attachment of sporozoites to liver cells have not been identified.

Here we have approached this question experimentally, by following the fate of recombinant CS injected intravenously into mice. Based on our previous in vitro results, we expected that CS would home specifically to hepatocytes in vivo, in a region II-dependent manner. In addition, we hoped that these experiments might reveal nonhepatocyte receptors for CS.

[1] *Abbreviations used in this paper:* CS, circumsporozoite; GAG, glycosaminoglycan; HSPG, heparin sulfate proteoglycans.

Materials and Methods

Recombinant Proteins. A schematic representation of the CS constructs is shown in Fig. 1. The *Escherichia coli*–derived recombinant CS27IVC (27-123[NANPNVDP]$_3$[NANP]$_{21}$300-411) represents the complete *Plasmodium falciparum* CS sequence from the T4 isolate, except that the hydrophobic clusters of NH_2- and COOH-terminal amino acids 1–26 and 412–424 have been deleted (9, 10). The yeast-derived Falc-1 *P. falciparum* T4 recombinant protein (amino acids 43–348) was obtained from Chiron Corporation (Emeryville, CA). Falc-1 terminates in the proline residue that precedes the first pair of cysteines in region II-plus (11).

Antibodies. The mAb 2A10 (12) is directed against an epitope contained in the $(NANP)_n$ repeat domain of the *P. falciparum* CS.

Mice. BALB/c males, from Taconic Farms, Inc. (Germantown, NY), weighing between 15 and 20 g were used.

FITC Labeling of mAb 2A10. The antibody labeling was performed as described by Harlow and Lane (13).

^{51}Cr Labeling of RBC. Mouse blood (200 μl) was collected from the retroorbital sinus, and washed with PBS, pH 7.4, containing 1% BSA (BSA/PBS). The RBC pellet was incubated with 10 μCi of $Na^{51}CrO_4$ for 30 min, and then washed with BSA/PBS.

^{125}I Labeling. The recombinant proteins were labeled using iodogen to a specific activity of ~2.5 × 10^5 cpm/μg. To reduce the amount of oxidative damage to the proteins, 1 mCi of ^{125}I in 10 μl of 0.1 M sodium phosphate buffer was oxidized in a glass tube precoated with iodogen for 5 min. 10 μl were then transferred to another tube, and incubated for 5 min on ice with 10 μl (10 μg) of CS27IVC or Falc-1. The free iodide was removed by filtration in Sephadex G-25 (Isolab Inc., Akron, OH), and dialyzed against 50 mM Tris-HCl, 75 mM NaCl, pH 7.4 (buffer A).

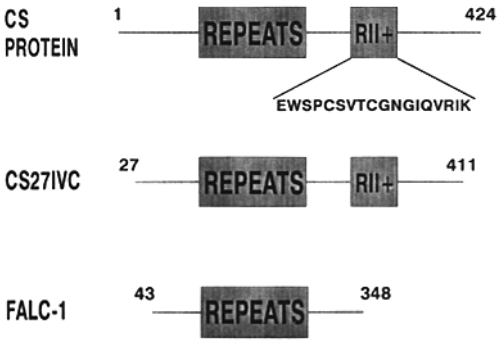


Figure 1. Schematic representation of the recombinant CS.

Isolation of CS27IVC Multimers. Radiolabeled CS27IVC was applied to a 1-ml heparin–Sepharose column (Sigma Chemical Co., St. Louis, MO) that had been preequilibrated with 50 mM Tris-HCl, 75 mM NaCl, 1% BSA, 0.05% Tween-20 (Bio-Rad Laboratories, Hercules, CA), pH 7.4 (buffer B), and washed with five-column volumes of the same buffer. The bound material was eluted with 1 ml 50 mM Tris-HCl, 1.5 M NaCl, pH 7.4, and dialyzed against buffer A.

Clearance Studies. Mice were anaesthetized with ether, and injected with 10^5 cpm of ^{125}I-labeled CS27IVC or Falc-1 via the periorbital sinus. At 2, 5, and 15 min after injection, the mice were exsanguinated, the organs removed, rinsed in Tris-buffered saline blotted dry on filter paper, and counted for radioactivity. To estimate the amounts of blood contaminating the various organs, we repeated the same procedure in mice injected with 100 μl (1.5×10^5 cpm) of ^{51}Cr-labeled RBC. The mean volumes of blood in the liver, spleen, and kidney of the exsanguinated animals were 75, 24, and 35 μl, respectively. In all other organs the amount of blood was negligible, i.e., <15 μl. The percent of injected dose of CS that was retained in the various organs was calculated as described in the legend to Fig. 4, after subtracting the CS counts from the contaminating blood.

In other experiments, we used light and electron microscopy to localize CS in the various organs. The mice were injected with 100 μg (i.v.) of unlabeled CS27IVC or Falc-1 via the retroorbital plexus. They were exsanguinated 5 min after injection, the organs were removed, rinsed in TBS, blotted dry, and then snap frozen in liquid nitrogen for light microscopy. For electron microscopy, the organs were cut into small pieces and fixed in a mixture of 0.1% glutaraldehyde and 4% paraformaldehyde.

Light Microscopy. The frozen tissue was embedded in Tissue Tek ornithine carbonyl transferase (Miles Inc., Naperville, IL) and cut into 5-μm sections. Sections were dried for 30 min, fixed for 10 min in 4% paraformaldehyde, and either used immediately or stored at 4°C in PBS containing 1% BSA, 0.5% Tween-20. After blocking with the same buffer, the sections were incubated with mAb 2A10 conjugated to FITC, and examined in a fluorescence microscope.

Electron Microscopy. The fixed liver specimens were dehydrated in ethanol and embedded in Lowicryl K4M (Ted Pella, Redding, CA) at −20°C. After UV polymerization, sections were cut with a RMC MT-7 ultramicrotome (Research and Manufacturing Co., Tucson, AZ) and stained by sequential incubation with 15 μg/ml mAb 2A10 and protein A gold 10 nm (PAG10) (1:30, Amersham Corp., Arlington, IL) (14). Control sections were stained only with the gold conjugate. Photographs were taken with an electron microscope (EM 910; Carl Zeiss, Inc., Thornwood, NY).

Results

Isolation of CS27IVC Multimers by Affinity Chromatography on Heparin–Sepharose. We have previously found that only the multimers of CS27IVC bind in vitro to the basolateral domain of hepatocytes, and to HepG2 cells (14). In those studies we used molecular sieving chromatography to separate multimers from monomers of CS27IVC. However, the separation of multimers from monomers by this procedure is sometimes incomplete. Since the CS multimers bind to the GAG chains of HSPG (1), in the present studies we isolated the CS multimers by affinity chromatography on heparin–sepharose.

As shown in Fig. 2 *a*, ~30% of the radiolabeled CS27IVC bound to the heparin–sepharose column, and the bound molecules were eluted with a buffer containing a high salt concentration. SDS-PAGE analysis under nonreducing conditions showed that the breakthrough peak contained only CS monomers, while the heparin-binding material contained various CS multimers as well as monomers (Fig. 2 *b*). Under reducing conditions, a single band corresponding to a CS monomer was detected in proteins under both peaks, indicating that the CS multimers consist of mixed aggregates of disulfide-linked dimers, trimers, etc., and noncovalently bound monomeric CS. The CS27IVC fractions obtained by filtration in sizing columns (14), and by affinity chromatog-

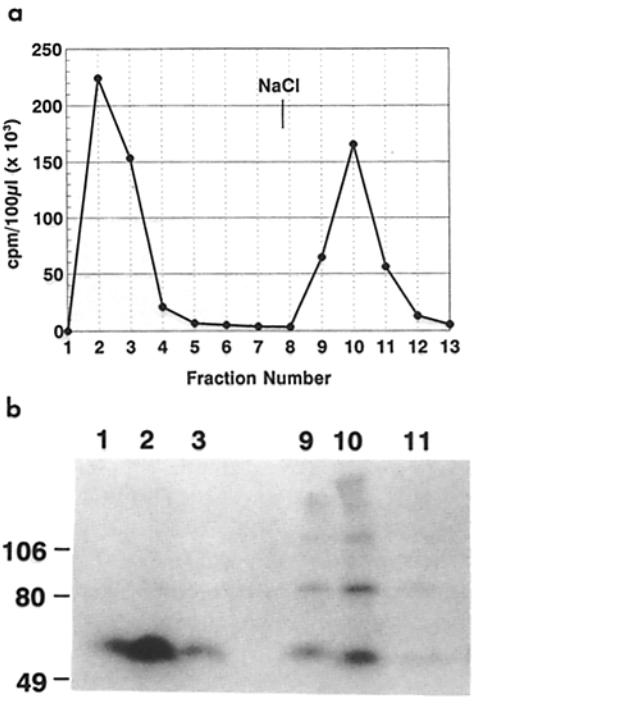


Figure 2. Isolation of CS27IVC aggregates by affinity chromatography in a heparin–sepharose column. (*a*) ^{125}I-labeled CS27IVC was applied to a heparin–sepharose column preequilibrated in buffer A. The column was washed with 5 ml of the same buffer, and the bound CS27IVC was eluted with 50 mM Tris-Cl, 1.5 M NaCl, pH 7.4. The ordinates represent the cpm in 10 μl of each fraction. (*b*) 10 μl of selected fractions were run on 10% SDS-PAGE under nonreducing conditions, the gel was dried, and subjected to radioautography. As shown, only the aggregated CS27IVC bound to the heparin–sepharose column.

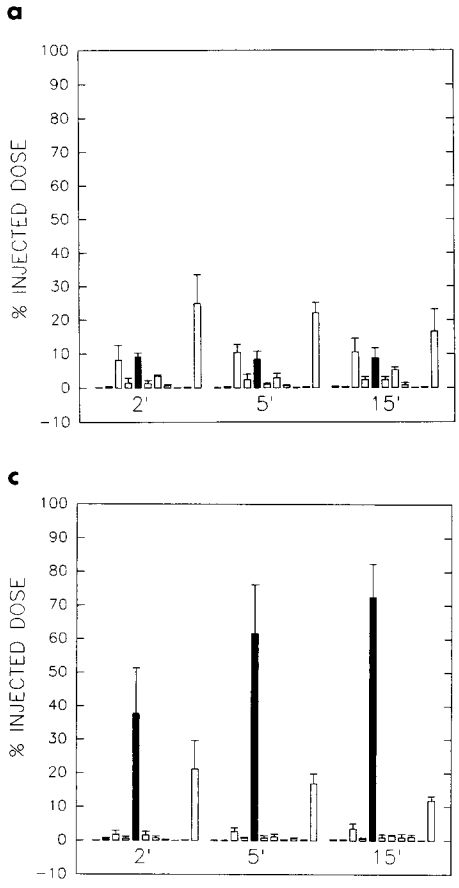
raphy on heparin–sepharose have similar properties; i.e., only the CS multimers bind to HepG2 cells (data not shown).

Clearance of CS27IVC and Falc-1. Radiolabeled Falc-1, and the aggregated and monomeric fractions of radiolabeled CS27IVC proteins were injected intravenously into mice. The animals were killed 2, 5, and 15 min later, and the amounts of CS in the blood, and in the various organs were measured. There were striking differences in the pattern of clearance of multimers of CS27IVC, as compared with that of Falc-1, or of the monomers of CS27IVC. Only ~40% of the injected CS monomers, or of Falc-1 counts were recovered in the blood and in various organs between 2 and 15 min after injection, and 4% or less were associated with the liver at any time (Fig. 3, *a* and *b*). In sharp contrast, 80% or more of the injected multimer CS counts were recovered, and most were in the liver, i.e., 45% at 2 min, 55% at 5 min, and 70% at 15 min. There was practically no accumulation of CS multimers in the bladder, heart, kidney, large intestine, lung, small intestine, stomach, spleen, or thyroid (Fig. 3 *c*).

The CS27IVC Aggregates Accumulate on the Microvilli of Hepatocytes. To define the cellular localization of the CS, we performed immunofluorescence staining of frozen sections of various organs of mice injected intravenously 15 min earlier with larger amounts (100 μg) of unlabeled CS27IVC or Falc-1. No staining with FITC-labeled mAb 2A10 was seen in the livers (Fig. 4 *a*) or kidneys of mice injected with Falc-1. In animals injected with CS27IVC, the recombinant was readily detected in the liver (Fig. 4 *b*), but not in the kidney (Fig. 4 *c*) or other organs. In the liver, the bound CS closely followed the contours of the sinusoids, suggesting accumulation in the Disse spaces. By electron microscopy, the gold label was detected in the Disse spaces of the liver (Fig. 5 *a*), where single or doublets of gold particles were found in close association with the microvilli of hepatocytes (Fig. 5 *b*). No gold label was found on endothelial or Kupffer cell surfaces.

Discussion

We show here that intravenously injected CS rapidly enters the Disse space and binds to the basolateral surface of hepatocytes, but not to endothelial or Kupffer cells. Only aggregated CS-containing region II-plus bound to hepatocytes. As shown elsewhere (14), these were precisely the requirements for the binding of CS to HepG2 cells, and to frozen sections of liver. Similar mechanisms appear therefore to be involved in the recognition of the CS by hepatocytes in vitro and in vivo. The in vivo liver staining pattern shown in Fig. 4 is almost identical to that obtained by direct incubation of CS27IVC with frozen sections of that organ (14). The only difference between the two patterns resides in the central veins, which are negative by in vivo labeling, and positive by in vitro labeling. The most likely explanation for this apparent discrepancy is that there are no fenestrae in the centrolobular vein endothelium (15). Therefore, the underlying



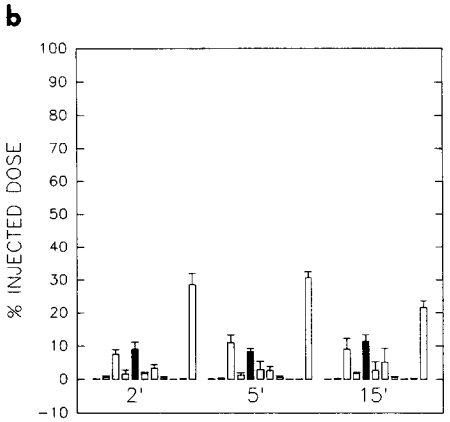


Figure 3. Clearance of aggregated CS27IVC by the liver. Radiolabeled monomeric CS27IVC (*a*), Falc-1 (*b*), and multimeric CS27IVC (*c*) were injected into the retroorbital sinuses of mice. The animals were killed 2, 5, and 15 min after injection. To estimate the amounts of blood contaminating the various organs, we repeated the same procedure in mice injected with 100 μl (1.5×10^5 cpm) of ^{51}Cr-labeled RBC. The mean volumes of blood in the liver, spleen, and kidney of the exsanguinated animals were 75, 24, and 35 μl, respectively. In all other organs the amount of blood was negligible, i.e., less than 15 μl. The percent of the injected CS counts retained in the organs of each mouse (percent retained cpm) was calculated after subtraction of the CS counts from the contaminating blood. Percent retained cpm = corrected cpm per organ/total injected dose × 100. The total blood volume was calculated as in (16). From left to right, the bars represent cpm associated with: bladder, heart, two kidneys, large intestine, liver, two lungs, small intestine, stomach, spleen, thyroid, and blood. Each bar represents mean values ± SD of cpm in organs from three mice.

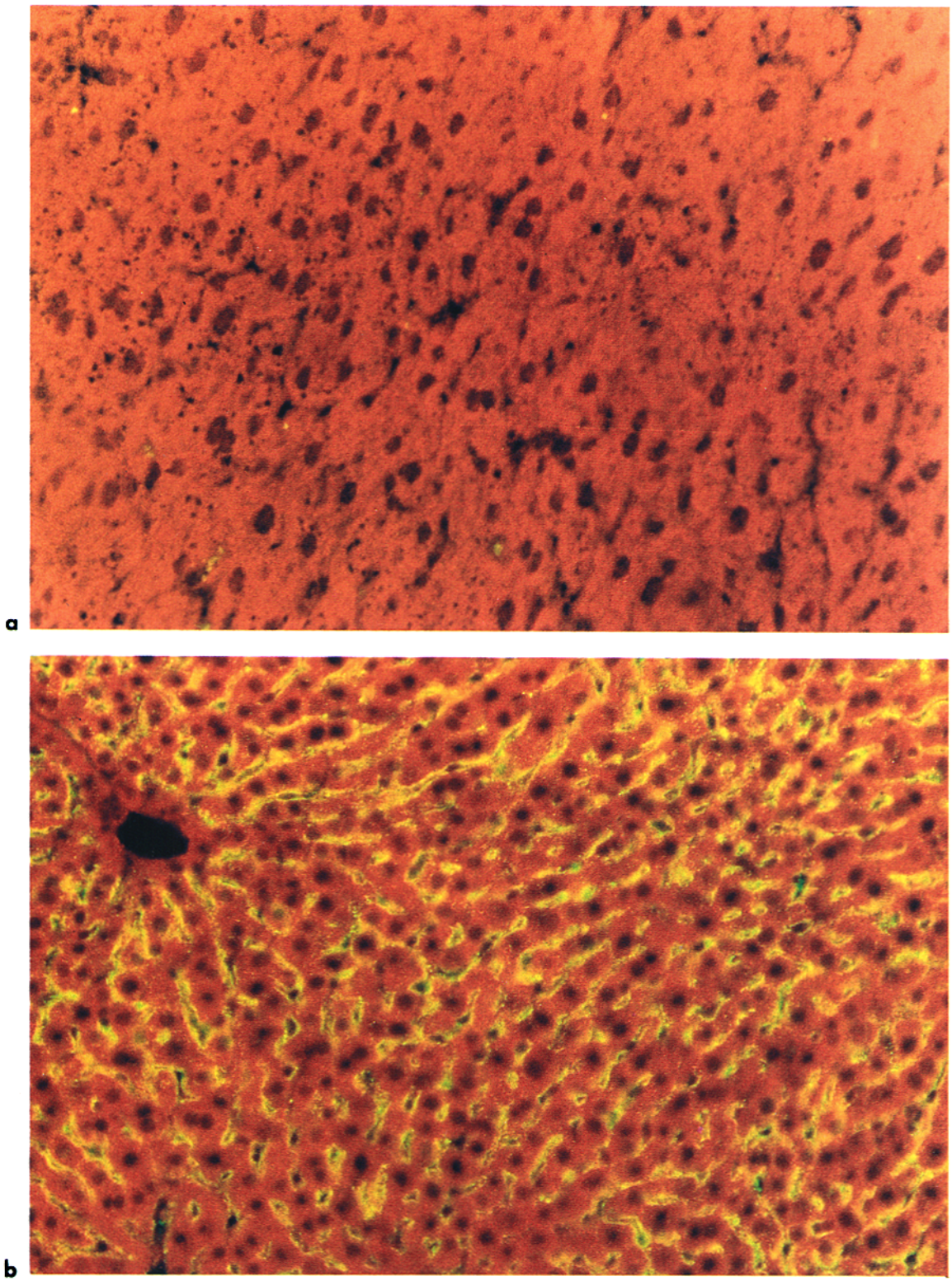
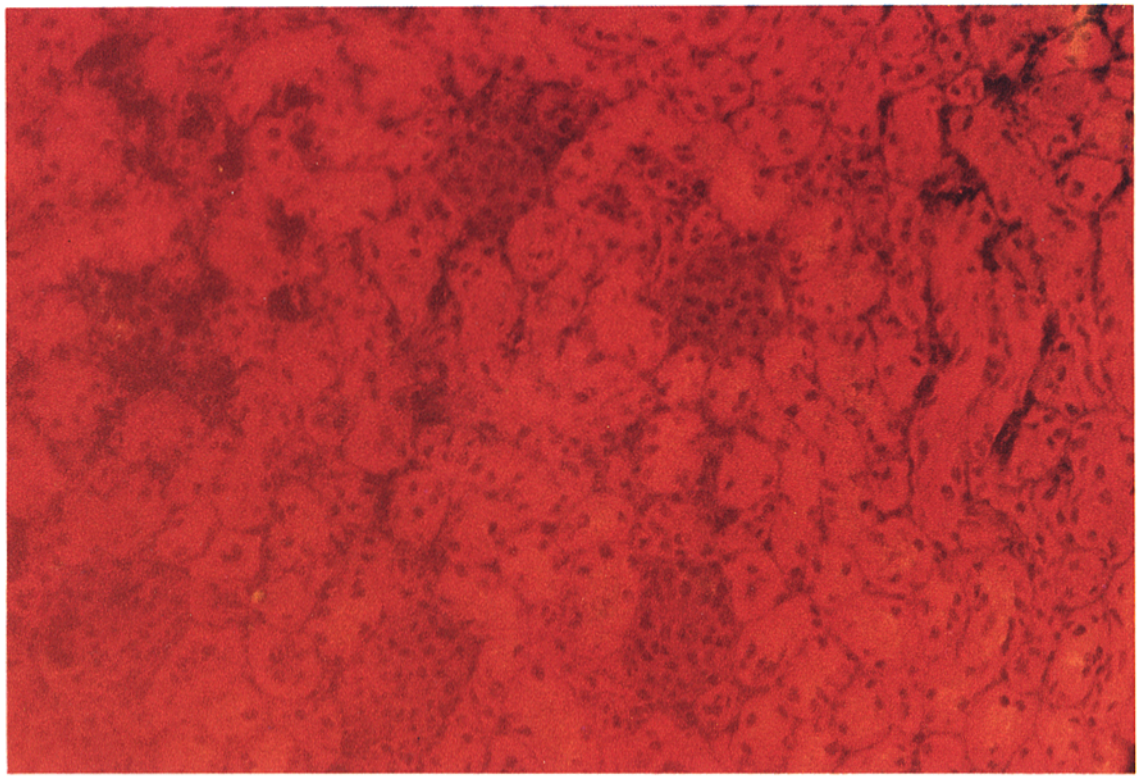


Figure 4. CS27IVC localizes to the sinusoids of the liver. Mice were injected with 100 μg CS27IVC or Falc-1, and 5 min later they were killed. The livers and kidneys were removed, washed in TBS, blotted dry, and snap frozen in liquid nitrogen. Frozen tissues were stained with mAb 2A10 conjugated to FITC. (*a*) Liver of a mouse injected with Falc-1. (*b*) Liver of a mouse injected with CS27IVC. (*c*) Kidney of a mouse injected with CS27IVC.



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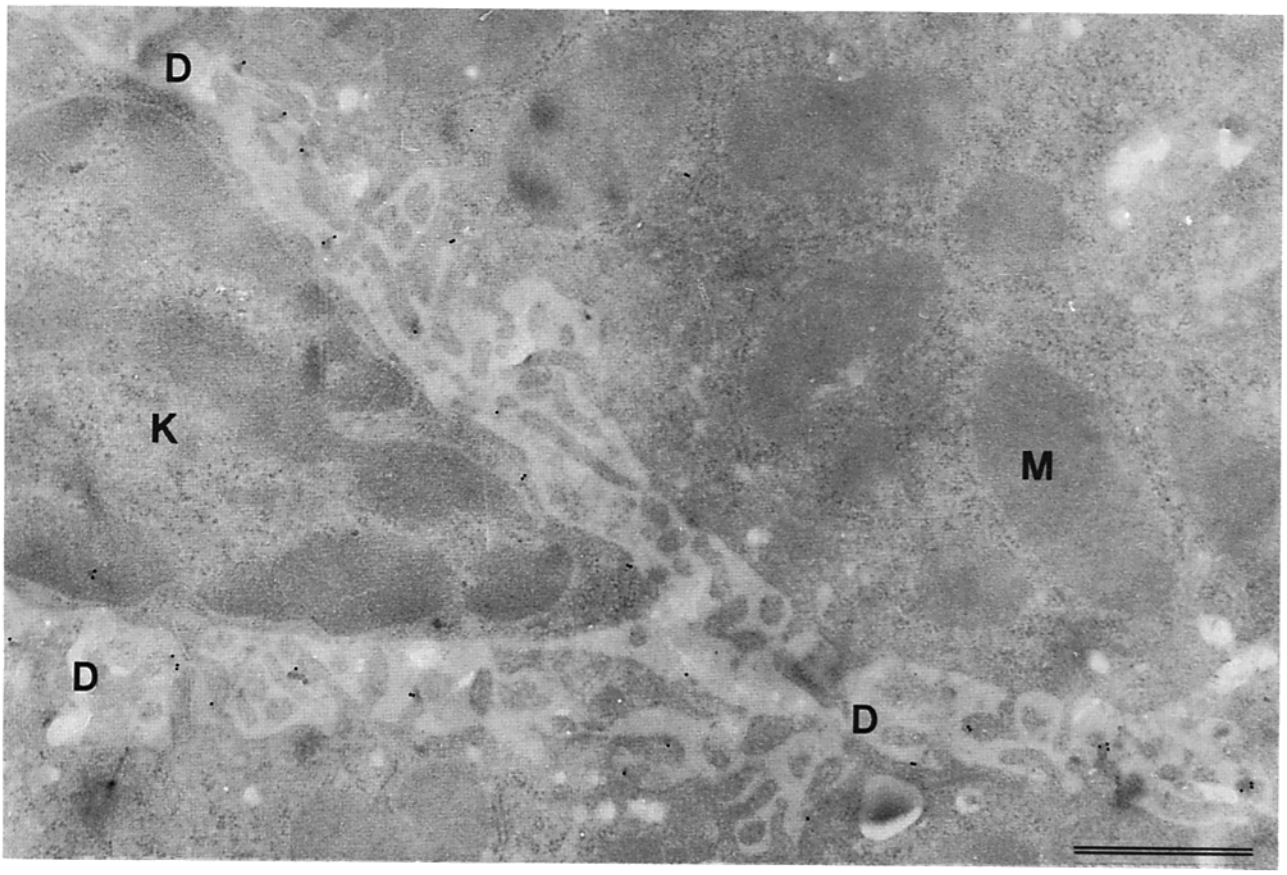

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Figure 5. *(continued)*

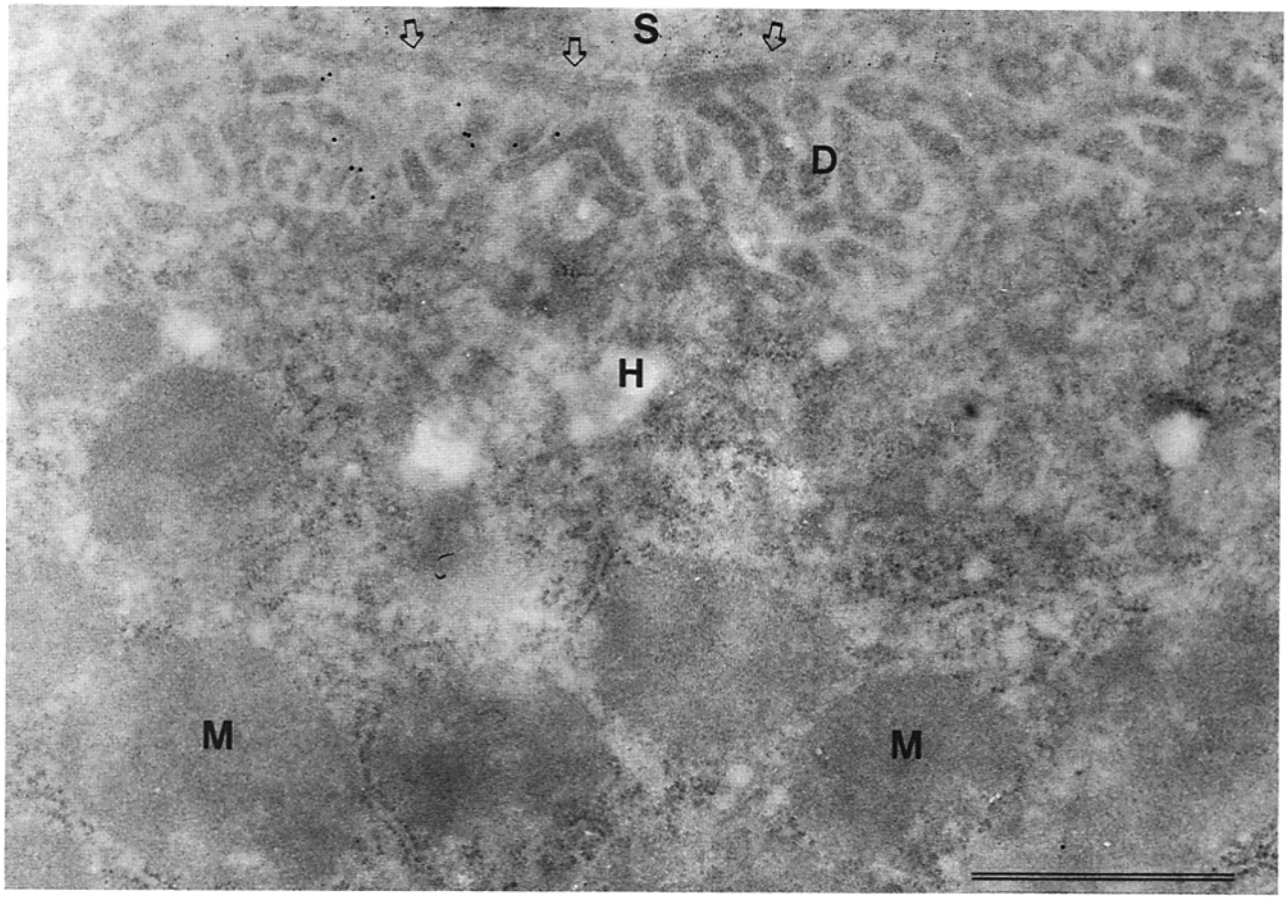

Figure 5. Ultrastructural localization of CS27IVC in the liver. Mouse liver was fixed 15 min after intravenous injection of 100 μg CS27IVC. The tissue was embedded in Lowicryl K4M, and sections were stained with mAb 2A10 and protein A–coated gold particles PAG10. (*a*) The gold label is associated with hepatocyte microvilli in the Disse space (*D*). There is no label on a Kupffer cell (*K*) in the sinusoidal lumen. (*b*) CS is found between the hepatocyte (*H*) microvilli within the Disse space (*D*), whereas the endothelial cell surface facing the sinusoidal lumen (*S*) is unlabeled (*arrows*). M, mitochondrium. Bars, 1 μm.

hepatocytes are not accessible to the recombinant CS27IVC circulating in the sinusoids. In an analogous fashion, the basolateral domain of the kidney proximal epithelia are stained by CS27IVC in frozen sections (1), but there is no accumulation of CS27IVC in vivo in the kidney (Fig. 4 *c*). The renal tubular epithelia are separated from the blood circulation by fenestrated epithelia, but the fenestrae in this case are closed by a diaphragm.

Although the Disse space is accessible to the blood circulation and to the purified CS, a confounding remaining question is how sporozoites cross the fenestrated endothelium of the liver sinusoids, since the diameter of the parasites (1 μm) is greater than the diameter of the fenestrae (average, 0.1 μm). However, the diameter of the fenestrae may vary in vivo with changes in blood pressure, or sporozoites may be flexible and squeeze through a small aperture. Furthermore, hepatocyte microvilli (or the GAG chains of the HSPGs from the surface of microvilli), may in fact traverse the fenestrae and protrude in the lumen of the sinusoids, thus entering in direct contact with the circulating blood.

The present findings do not prove that during infection by malaria sporozoites the initial recognition event is the binding of region II–plus of CS, and perhaps of TRAP/SSP2, to hepatocyte microvilli. Nevertheless, they provide a plausible explanation for the target cell specificity of malaria sporozoites, and, more importantly, suggest new experimental strategies to prevent sporozoites from reaching their target. Outside the malaria field, our findings raise the intriguing possibility that the in vivo specificity of region II–plus for hepatocytes can be utilized for the targeting of drugs, or of genetic materials to hepatocytes in vivo. For example, the incorporation of the region II–plus amino acid sequence into surface proteins of a recombinant virus may deliver it to hepatocytes.

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