Remnant Lipoproteins Inhibit Malaria Sporozoite Invasion of Hepatocytes

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Summary

Remnants of lipoproteins, intestinal chylomicrons, and very low density lipoproteins (VLDL), are rapidly cleared from plasma and enter hepatocytes. It has been suggested that remnant lipoproteins are initially captured in the space of Disse by heparan sulfate proteoglycans (HSPGs), and that their subsequent internalization into hepatocytes is mediated by members of the LDL-receptor gene family. Similarly to lipoprotein remnants, malaria sporozoites are removed from the blood circulation by the liver within minutes after injection by *Anopheles* mosquitoes. The sporozoite's surface is covered by the circumsporozoite protein (CS), and its region II-plus has been implicated in the binding of the parasites to glycosaminoglycan chains of hepatocyte HSPGs. Lactoferrin, a protein with antibacterial properties found in breast milk and neutrophil granules, is also rapidly cleared from the circulation by hepatocytes, and can inhibit the hepatic uptake of lipoprotein remnants. Here we provide evidence that sporozoites, lactoferrin, and remnant lipoproteins are cleared from the blood by similar mechanisms. CS, lactoferrin, and remnant lipoproteins compete in vitro and in vivo for binding sites on liver cells. The relevance of this binding event for sporozoite infectivity is highlighted by our demonstration that apoliprotein E–enriched β-VLDL and lactoferrin inhibit sporozoite invasion of HepG2 cells. In addition, malaria sporozoites are less infective in LDL-receptor knockout (LDLR−/−) mice maintained on a high fat diet, as compared with littermates maintained on a normal diet. We conclude that the clearance of lipoprotein remnants and sporozoites from the blood is mediated by the same set of highly sulfated HSPGs on the hepatocyte plasma membrane.

Chylomicron and very low density lipoprotein (VLDL)[1] remnants, generated from the metabolism of intestinal chylomicrons and hepatic VLDL, are enriched in apolipoprotein E (apoE) and rapidly cleared from the circulation by the liver (for review see references 1 and 2). Clearance and liver uptake are apoE dependent (3–8). On the basis of a large body of evidence from cell culture (9, 10) and in vivo (11) studies it has been postulated that the initial sequestration of lipoprotein particles is mediated mainly by hepatic heparan sulfate proteoglycans (HSPGs), and that this facilitates their subsequent interiorization by the low density lipoprotein receptor (LDLR), and the LDLR-related protein (LRP) (12). Lactoferrin also binds to HSPGs and LRP (12–14), and competes with remnant lipoproteins for hepatic clearance from the circulation and for internalization by hepatocytes (15, 16).

There are intriguing similarities between the clearance patterns of the major surface protein of malaria sporozoites, the circumsporozoite protein (CS) (17), and remnant lipoproteins. Within minutes after intravenous injection into mice, CS accumulates in the space of Disse on the plasma membrane of hepatocyte microvilli (18). Heparinase treatment of liver sections that have been incubated with CS, and other in vitro experiments using hepatocytes and HepG2 cells as targets, demonstrate that CS binds to HSPGs (19, 20). The proteoglycan-binding portion of CS (19, 21) is region II-plus (22), a stretch of amino acids highly conserved in all species of malaria parasites (23). The region II-plus motif is also found in thrombospondin-related adhesive protein/sporozoite surface protein 2 (TRAP/SSP2), another surface protein of malaria sporozoites (24, 25) that binds to cell surface HSPGs (26, 27). Within re-

[1] *Abbreviations used in this paper:* apoE, apoliprotein E; CS, circumsporozoite protein; EEF, exoerythrocytic forms; GAG, glycosaminoglycan; HPRT, hypoxanthine phosphorybosyl transferase; HSPG, heparan sulfate proteoglycan; LDLR, low density lipoprotein receptor; LRP, LDLR-related protein; RT, reverse transcriptase; TBS, Tris-buffered saline; TRAP/SSP2, thrombospondin-related adhesive protein/sporozoite surface protein 2; VLDL, very low density lipoprotein.

Plasmodium falciparum CS region II+

EWSPCSVTCGNG***IQVRIK***

Plasmodium berghei CS region II+

EWSQCNVTCGSG***IRVRKR***

Plasmodium yoelii CS region II+

EWSQCSVTCGSG***VRVRKR***

Apolipoprotein E (aa 142-161)

RKLRKRLLRDAED***LQKRLAV***

Lactoferrin (aa 10-28)

CAVSQPETKCFQ***R***NM***RKVR***

Figure 1. Heparin binding motifs of *P. falciparum*, *P. berghei*, and *P. yoelii* CS, apoE, and lactoferrin. Boldface italics indicate amino acid residues of CS protein required for binding to HSPGs (22). Amino acid residues forming a similar motif in the heparin-binding domains of apoE (30, 31) and lactoferrin (16, 28, 29) are also highlighted.

gion II-plus, the positively charged amino acids and interspersed hydrophobic residues in its COOH-terminal end (see Fig. 1) are critical for binding to HSPGs (22). An ionic interaction between these basic residues and the negatively charged sulfate moieties of HSPGs is most likely involved since chlorate, a metabolic inhibitor of sulfation, markedly decreases CS binding to HepG2 cells (Sinnis, P., manuscript in preparation). As shown in Fig. 1, a similar motif is found in the heparin-binding domains of apoE and lactoferrin (16, 28–31). In this paper we provide experimental evidence in support of the hypothesis that CS, malaria sporozoites, remnant lipoproteins, and lactoferrin are recognized by the same set of cell surface HSPG molecules in vitro and are cleared from the circulation by hepatocyte HSPGs in vivo.

Materials and Methods

Materials. CS protein, 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 NH_2- and COOH-terminal amino acids 1-26 and 412-424 have been deleted and five histidine residues have been added to the COOH terminus to facilitate purification (32). The recombinant protein used in these studies was generously provided by Dr. Bela Takacs (F. Hoffmann-La Roche Ltd., Basel, Switzerland). Recombinant human apoE (E3 isoform), obtained from *E. Coli* (33), was a gift from Dr. Tikva Vogel (Biotechnology General, Rehovot, Israel). mAb 2A10 is directed against an epitope contained in the $(NANP)_n$ repeat domain of *P. falciparum* CS (34) and mAb 2E6, a gift from Dr. Moriya Tsuji (New York University Medical Center), reacts with the liver stage of *Plasmodium berghei* (35). β-migrating VLDL (β-VLDL; d <1.006 g/ml) was prepared from the plasma of rabbits fed for 4 d with a 2% (wt/wt) cholesterol, 10% (vol/wt) coconut oil diet, as described (36). ApoE-enriched β-VLDL was prepared by coincubating apoE and β-VLDL at a ratio of 1:1 for 1 h at 37°C before use.

Mice. LDLR−/− mice were created by targeted gene disruption as described (37). All other mice, including apoE−/− mice, were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice on special diets were fed either normal mouse chow (Purina rodent chow #5001; Purina Mills, St. Louis, MO) or a 1.25% cholesterol, high saturated fat diet as described (38) for 5 d before each experiment. Briefly, the high fat diet consisted of three parts normal chow mixed with one part mouse chow containing cholesterol, cocoa butter, casein, and sodium cholate (TD78399 from Harlan Teklad Premier Laboratory Diets, Madison, WI). The final high fat diet contained 1.25% cholesterol, 7.5% (wt/wt) cocoa butter, 7.5% casein, and 0.5% (wt/wt) sodium cholate.

Binding of CS to HepG2 Cells. Assays were carried out as described (22). Briefly, HepG2 cells were grown in 96-well plates, fixed with 4% paraformaldehyde, and blocked with 1% BSA in Tris-buffered saline (TBS/BSA). When lactoferrin or transferrin (both from Sigma Chemical Co., St. Louis, MO) was used as an inhibitor, cells were coincubated with 2.5 μg/ml of CS and the inhibitor at the indicated concentrations for 1 h at 37°C. After washing, cells were incubated with ^{125}I-labeled mAb 2A10, washed, and counted in a gamma counter. In the experiments with apoE, β-VLDL, and apoE-enriched β-VLDL, the inhibitors were preincubated with the cells for 30 min and then CS protein was added to a final concentration of 2.5 μg/ml. CS and the inhibitor were then coincubated with the cells for 45 min at 37°C, and the assay was processed as above.

Clearance Experiments. Mice were anesthetized with intraperitoneal sodium pentobarbital injection (~80 μg/gm) and injected intravenously with 7×10^5 cpm of ^{125}I-labeled CS, prepared as described (18) and representing ~0.1 μg of protein in 200 μl TBS with 0.1% BSA. At the indicated time points, 50-μl blood samples were collected by retroorbital puncture and counted in a gamma counter. To study the effect of lactoferrin on CS clearance, radiolabeled CS was injected 5 min after the injection of 3 mg of lactoferrin in 250 μl TBS or TBS alone. CS clearance in LDLR−/− and apoE−/− mice was performed 5 d after the animals were put on a high fat diet, and plasma cholesterols were measured on the day of the clearance experiment using an assay kit obtained from Sigma Chemical Co. When organs were harvested for counting, the mice were injected with 4×10^5 cpm of CS; 2 min later, the mice were killed, exsanguinated, and their organs were removed, rinsed in TBS, and counted in a gamma counter.

Assay for Sporozoite Infectivity In Vitro. This was performed as described (39, 40) with modifications as described (21). Briefly, HepG2 cells were plated in chamber slides, grown for 2 d, and incubated for 15 min with each inhibitor before 50,000 *P. berghei* sporozoites were added to each well. 2 h after parasites were added, the cells were washed and then grown for an additional 2 d after which they were fixed with methanol and stained with mAb 2E6, followed by goat anti–mouse Ig conjugated to horseradish peroxidase and 3,3′-diaminobenzidine. The number of exoerythrocytic forms (EEF) in each well were counted microscopically using a × 20 light microscope objective.

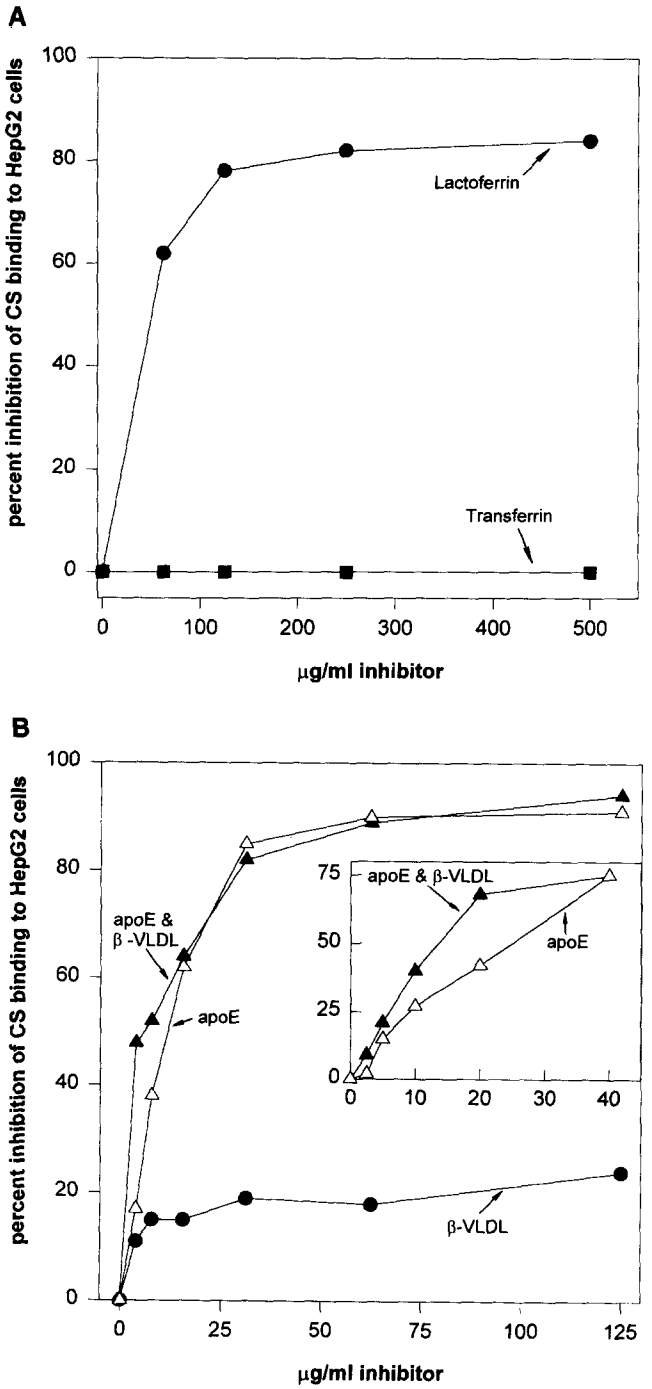

Figure 2. Inhibition of CS binding to HepG2 cells by lactoferrin, apoE, and apoE-enriched β-VLDL. (*A*) Paraformaldehyde-fixed HepG2 cells in 96-well plates were coincubated with CS and either lactoferrin (*circles*) or transferrin (*squares*) for 1 h. After washing, cells were incubated with iodinated mAb 2A10, washed and bound antibody was counted in a gamma counter. Shown is percent inhibition of binding of CS to HepG2 cells in the presence of inhibitor compared with results obtained in the absence of inhibitor. Each inhibitor concentration was assayed in triplicate and standard deviations were not greater than 5%. (*B*) The assay was carried out as above except that cells were preincubated with apoE (*open triangles*), β-VLDL (*closed circles*), or apoE-enriched β-VLDL (*closed triangles*) for 30 min. CS was then added for an additional 45 min, cells were washed, and bound CS was quantified as above. (*Inset*) Results of a similar experiment using lower concentrations of apoE (*open triangles*) and apoE-enriched β-VLDL (*closed triangles*). Each inhibitor concentration was assayed in triplicate and standard deviations were less than 5%.

Quantitative PCR Assay for Sporozoite Infectivity In Vivo. LDLR−/− mice were fed either a normal or high fat diet for 5 d and then injected intravenously with 7,500 *Plasmodium yoelii* sporozoites. Measurement of parasite rRNA was then performed using a competitive PCR assay as described (41). Briefly, 40 h after sporozoite injection, the mice were killed, their livers were harvested, frozen in liquid nitrogen, crushed, added to 8 ml of RNAzol (Biotecx Laboratories Inc., Houston, TX), and vortexed. 1 ml of liver homogenate was used for RNA purification performed according to manufacturer's instructions (Biotecx Laboratories, Inc.). Reverse transcriptase (RT) and PCR reactions were performed using a RT-PCR kit (Perkin-Elmer, Branchburg, NJ). RNA was quantified by absorbance at 260 nm, and RT reactions were performed with 1 μg of RNA and random hexamers supplied by the manufacturer. PCR of this cDNA was performed using parasite rRNA primers that recognize *P. yoelii*–specific sequences within the 18S rRNA (5′-CGGGATCCAGGATGTATTCGCTTTAT-3′ and 5′-GGGGTACCTTCTTGTCCAACCAATTC-3′) in the presence of a competitor template constructed by insertion of a 355-bp lambda DNA fragment into the cloned 393-bp rRNA parasite amplification product. The competitor molecule is 748 bp in length and the parasite target is 393 bp. Mouse hypoxanthine phosphorybosyl transferase (HPRT) primers and competitor were used as positive controls to assess the efficiency of RT reactions as described (42). Amplification products were analyzed by electrophoretic separation on 2% agarose in 0.04 M Tris-acetate, 0.001 M EDTA, stained with 0.5 μg/ml ethidium bromide and photographed under long wavelength UV light.

Results

To examine the relationship between hepatocyte-binding sites for CS and apoE/lactoferrin, we performed in vitro competition experiments using HepG2 cells as targets. We have previously shown that CS binds to these cells in a saturable fashion, and that binding is abolished when cells are treated with heparitinase (19). Fig. 2 illustrates the inhibition of CS binding to HepG2 cells by lactoferrin and apoE. Between 80 and 90% inhibition is obtained with 1.6 μM lactoferrin. Transferrin, a protein with 59% identity to lactoferrin but lacking heparin-binding domains, has no effect on CS binding even at much higher concentrations. In Fig. 2 *B*, we compare the abilities of β-VLDL (isolated from plasma of hyperlipidemic rabbits and used as an experimental source of lipoprotein remnants), apoE, and apoE-enriched β-VLDL to inhibit CS binding to HepG2 cells. CS binding is inhibited by >80% with 1 μM recombinant apoE. While β-VLDL alone is inactive, upon addition of apoE, it inhibits CS binding in a dose-dependent fashion. This finding is in agreement with previous studies that have shown that β-VLDL must be enriched in apoE in order to bind with high avidity to cell surface proteoglycans and to LRP (9, 10, 38, 43). At low concentrations, the

Table 1. *Lactoferrin and ApoE-enriched Remnant Lipoproteins Inhibit Sporozoite Invasion of HepG2 Cells*

Experiment	Inhibitor*	No. of EEF‡	Percent inhibition§
1	Medium alone*	251,273,305‡	
	Transferrin 500 μg/ml	200,281,254	11
	Lactoferrin 500 μg/ml	63,67,80	74
	Lactoferrin 250 μg/ml	77,100,114	64
	Lactoferrin 125 μg/ml	138,137,190	43
2	Medium alone	779,730,725	
	Transferrin 500 μg/ml	493,686,736	14
	Lactoferrin 500 μg/ml	274,266,270	63
	Lactoferrin 250 μg/ml	462,476,425	38
	Lactoferrin 125 μg/ml	479,515,535	31
3	Medium alone	459,488,424	
	ApoE and β-VLDL 250 μg/ml	112,138,180	68
	ApoE and β-VLDL 125 μg/ml	220,253,265	46
	ApoE and β-VLDL 62 μg/ml	323,358,357	23
4	Medium alone	928,958,1068	
	β-VLDL 250 μg/ml	1053,930,984	
	β-VLDL 125 μg/ml	1051,981,981	
	ApoE and β-VLDL 250 μg/ml	486,454,459	51
	ApoE and β-VLDL 125 μg/ml	769,938,792	15
5	Medium alone	166,114,149	
	ApoE and β-VLDL 250 μg/ml	41,59,52	64
	ApoE and β-VLDL 125 μg/ml	69,105,122	31
	ApoE and β-VLDL 62 μg/ml	107,110,111	24

*HepG2 cells in chamber slides were incubated for 15 min with each inhibitor before 50,000 *P. berghei* sporozoites were added to each well. 2 h after parasites were added, the cells were washed and grown for an additional 2 d at which time they were fixed and stained.
‡The number of EEF per 20 fields under ×20 magnification in triplicate wells.
§Calculated using the mean number of parasites in the control group, in which sporozoites were allowed to invade in the presence of medium alone, and the mean from the experimental group.

inhibitory activity of apoE-enriched β-VLDL is higher than that of apoE alone (Fig. 2 *B inset*). The increased activity of apoE-enriched β-VLDL over apoE alone is probably greater than that shown in Fig. 2 *B*, since the molar concentration of apoE after incorporation into lipoprotein particles is lower than that of free apoE. Previous studies (9, 10, 12) have shown that the binding of apoE-enriched β-VLDL to HepG2 cells is inhibited by lactoferrin, or by prior heparitinase treatment of the cells. Our findings, together with these data, indicate that CS, lactoferrin, and apoE compete for the same set of HSPGs on the surface of HepG2 cells.

Next, we asked whether lactoferrin and apoE-enriched β-VLDL could prevent the infection of HepG2 cells by *P. berghei*, a rodent malaria parasite. Table 1 shows that 3.2 μM (250 μg/ml) lactoferrin, but not an equivalent amount of transferrin, inhibits sporozoite invasion by ~50%. Whereas β-VLDL alone is inactive, after its enrichment with apoE, it inhibits sporozoite invasion of HepG2 cells by 50–68%.

Lactoferrin and remnant lipoproteins compete with CS not only for binding to hepatocytes in vitro, but also for CS clearance by the liver. In Fig. 3 *A* we show that the removal of CS from the circulation is delayed when mice are preinjected with 3 mg of lactoferrin. To verify that lactoferrin was inhibiting clearance of CS because it was competing with CS for hepatic binding sites, we performed another experiment in which mice preinjected with lactoferrin were killed after radiolabeled CS injection and their organs were harvested and counted. As shown in Fig. 4 *A*, mice preinjected with 3 mg of lactoferrin had 24% of the injected counts in the liver, whereas control mice preinjected with either 3 mg of transferrin or buffer alone had 60% of the injected counts in their livers. Most of the remaining counts were found in the blood, and ~10% of the counts

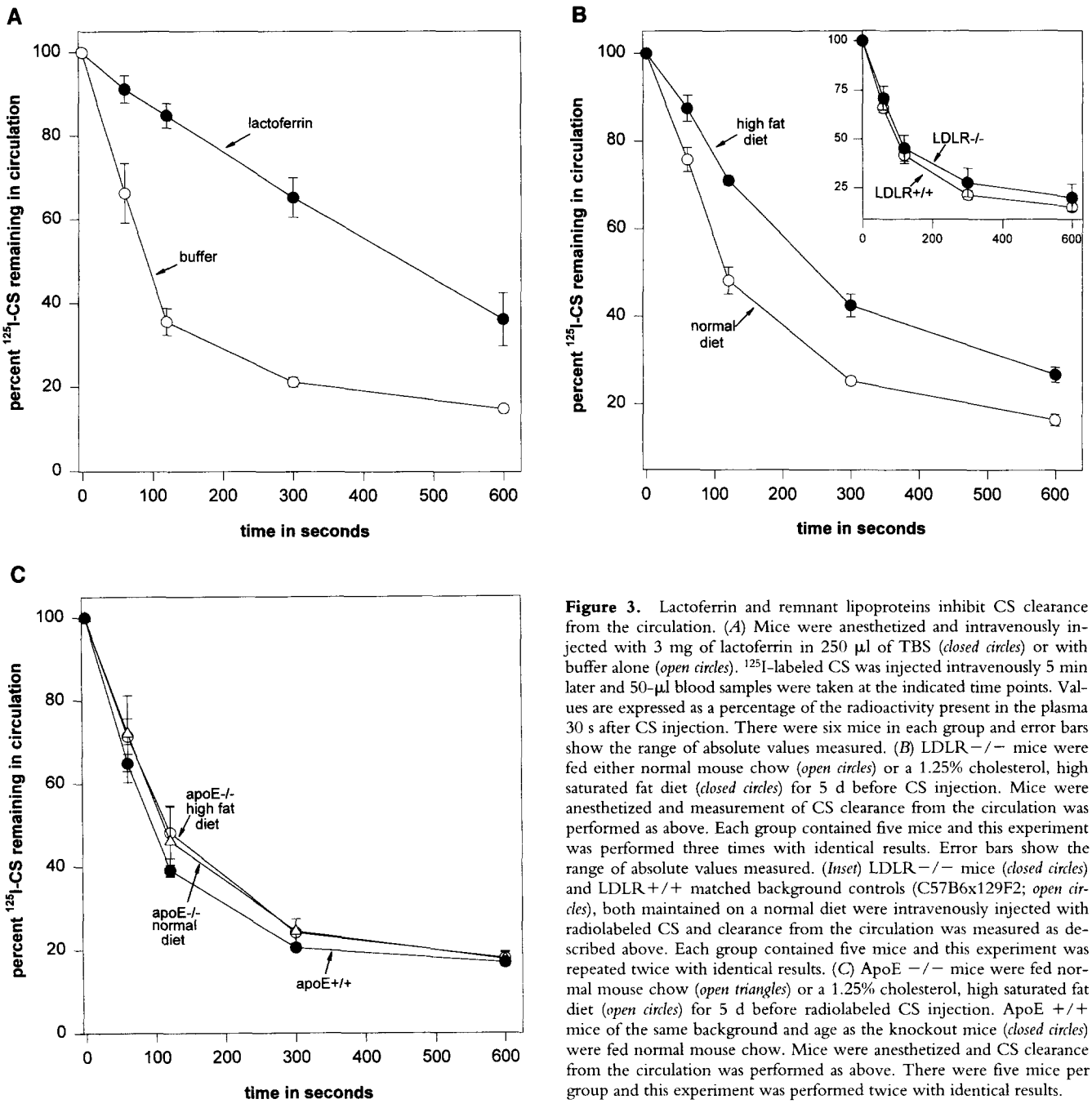

Figure 3. Lactoferrin and remnant lipoproteins inhibit CS clearance from the circulation. (*A*) Mice were anesthetized and intravenously injected with 3 mg of lactoferrin in 250 μl of TBS (*closed circles*) or with buffer alone (*open circles*). ^{125}I-labeled CS was injected intravenously 5 min later and 50-μl blood samples were taken at the indicated time points. Values are expressed as a percentage of the radioactivity present in the plasma 30 s after CS injection. There were six mice in each group and error bars show the range of absolute values measured. (*B*) LDLR−/− mice were fed either normal mouse chow (*open circles*) or a 1.25% cholesterol, high saturated fat diet (*closed circles*) for 5 d before CS injection. Mice were anesthetized and measurement of CS clearance from the circulation was performed as above. Each group contained five mice and this experiment was performed three times with identical results. Error bars show the range of absolute values measured. (*Inset*) LDLR−/− mice (*closed circles*) and LDLR+/+ matched background controls (C57B6x129F2; *open circles*), both maintained on a normal diet were intravenously injected with radiolabeled CS and clearance from the circulation was measured as described above. Each group contained five mice and this experiment was repeated twice with identical results. (*C*) ApoE −/− mice were fed normal mouse chow (*open triangles*) or a 1.25% cholesterol, high saturated fat diet (*open circles*) for 5 d before radiolabeled CS injection. ApoE +/+ mice of the same background and age as the knockout mice (*closed circles*) were fed normal mouse chow. Mice were anesthetized and CS clearance from the circulation was performed as above. There were five mice per group and this experiment was performed twice with identical results.

were evenly distributed in various organs without any focal accumulation (data not shown).

To study the effect of lipoprotein remnants on CS clearance, we used LDLR−/− mice. When fed a normal diet, these mice accumulate LDL, but not lipoprotein remnants, and their total plasma cholesterol levels are slightly elevated (≅250 mg/dl; 37, 38). In preliminary studies we found that there was no significant difference in CS clearance between LDLR−/− and LDLR+/+ mice fed normal diets (*inset*, Fig. 3 *B*). When LDLR−/− mice are fed a diet high in saturated fat, their plasma cholesterol levels rise because of the accumulation of lipoprotein remnants and LDL (37, 38). As shown in Fig. 3 *B*, CS clearance was delayed in LDLR−/− mice fed a high fat diet (plasma cholesterol 1,120 ± 79 mg/dl) when compared with LDLR−/− littermates fed a normal diet (plasma cholesterol 179 ± 31 mg/dl). To verify that the delay in CS clearance was due to an inhibition of CS binding in the liver, we performed another experiment in which the mice were killed after radiolabeled CS injection and their organs were harvested and counted. When LDLR−/− mice fed a high fat diet were killed 2 min after radiolabeled CS injection, 43% of the in-

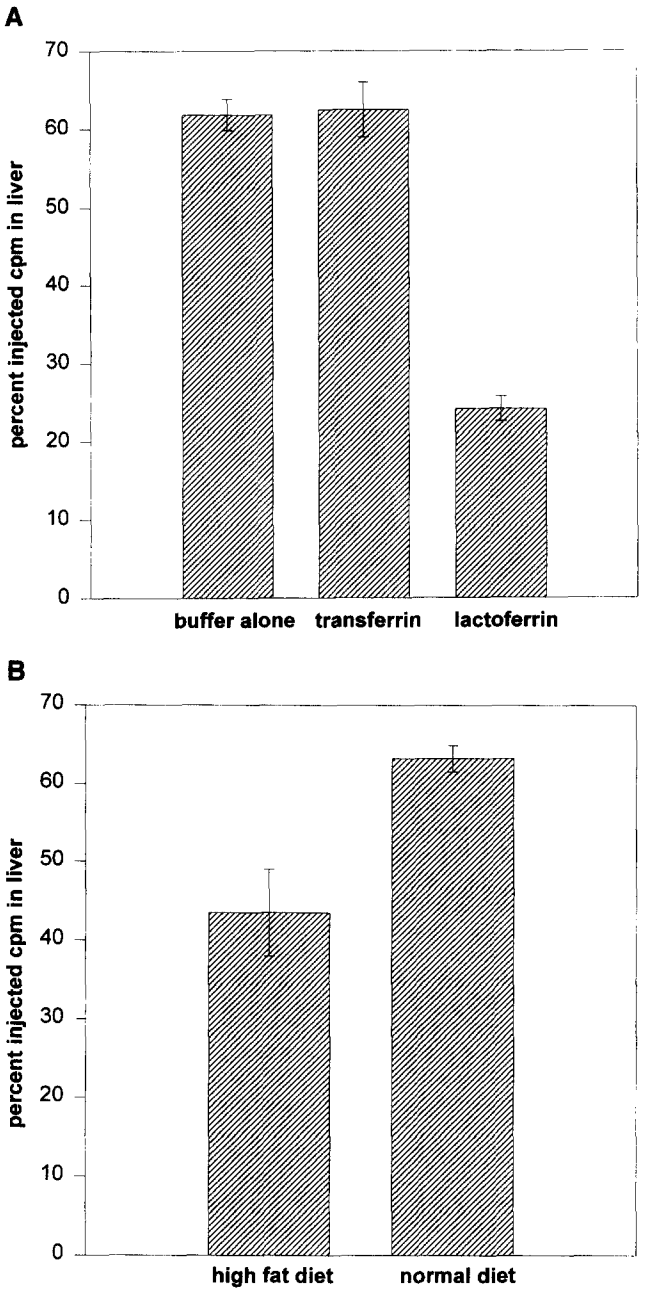

Figure 4. Lactoferrin and remnant lipoproteins inhibit CS clearance to the liver. (*A*) Mice were injected with either 3 mg of lactoferrin or transferrin in 200 μl of TBS or with buffer alone. 5 min later they were injected with ^{125}I-labeled CS and then killed 2 min later. The mice were exsanguinated, their organs harvested, and the radioactivity in the organs was determined. Values are expressed as a percentage of the radioactivity injected. There were three mice in each group and error bars show the range of absolute values measured. (*B*) LDLR−/− mice were fed either normal mouse chow or a 1.25% cholesterol, high saturated fat diet for 5 d. On day 5, the mice were injected with ^{125}I-labeled CS and 2 min later the mice were killed, exsanguinated, and their organs were harvested and counted in a gamma counter. Values are expressed as a percentage of the radioactivity injected. There were six mice in each group.

jected counts were found in the liver, whereas 63% of the injected counts were found in the livers of mice fed a normal diet (Fig. 4 *B*). Of note, the inhibition of CS clearance observed in mice preinjected with lactoferrin was greater than that observed in LDLR−/− mice fed a high fat diet (compare Fig. 3, *A* and *B*). This can be explained by differences in the plasma concentrations of the inhibitors. In the experiments with lactoferrin, plasma levels of the inhibitor were ~2 mg/ml, whereas in LDLR−/− mice maintained on a high fat diet, plasma apoE levels increase two- to fourfold, i.e., from 0.08 to 0.4 mg/ml (38, 44).

It could be argued that the delay in CS clearance in LDLR−/− mice fed a high fat diet is not due to CS competition with apoE-enriched remnant particles, but rather to secondary, nonspecific effects of high plasma cholesterol levels. To exclude this possibility, we compared CS clearance in apoE knockout mice (apoE−/−) and controls. ApoE−/− mice fed a normal diet have plasma cholesterol levels between 500 and 700 mg/dl. When fed a high fat diet, their cholesterol levels rise to over 2,000 mg/dl due to the accumulation of apoE-deficient remnant lipoproteins (7, 8, 38). As shown in Fig. 3 *C*, CS clearance is not significantly different between apoE−/− mice fed a normal diet (plasma cholesterol 726 ± 62 mg/dl) and those fed a high fat diet (plasma cholesterol 2,159 ± 496 mg/dl). In addition, CS clearance in apoE+/+ mice (plasma cholesterol <130 mg/dl) was not significantly different from clearance in apoE−/− mice. Thus, if apoE is missing, high levels of circulating remnant lipoproteins do not compete with CS for binding to liver HSPGs.

Finally, we tested whether we could inhibit malaria infection in a rodent model of the disease. Using a quantitative PCR assay, we compared the amounts of parasite rRNA in the livers of LDLR−/− mice infected with *Plasmodium* sporozoites 40 h earlier. We found that LDLR−/− mice fed a high fat diet had eightfold less parasite rRNA in their livers than LDLR−/− littermates fed a normal diet (Fig. 5).

Discussion

Here we show that CS, lactoferrin, and remnant lipoproteins compete for the same hepatic-binding sites. Although the nature of the liver molecules involved in the rapid clearance of these ligands from the circulation has not been unequivocally established, a large body of evidence derived from in vitro (for a review see reference 12) and in vivo (11) studies suggest that they are HSPGs. This idea is supported by the recent observation that the injection of heparinase into mice delays the clearance of lactoferrin and lipoprotein remnants from the circulation (45). Our data, demonstrating that CS can compete with these physiological ligands for clearance by hepatocytes, provide additional support for this hypothesis since injected CS binds almost exclusively to the microvilli on the basolateral domain of hepatocytes (18) and in vitro, this binding is completely eliminated when liver sections are treated with heparitinase (19).

Although HSPGs are widely distributed in animal tissues, CS, apoE, and lactoferrin are retained almost exclusively in

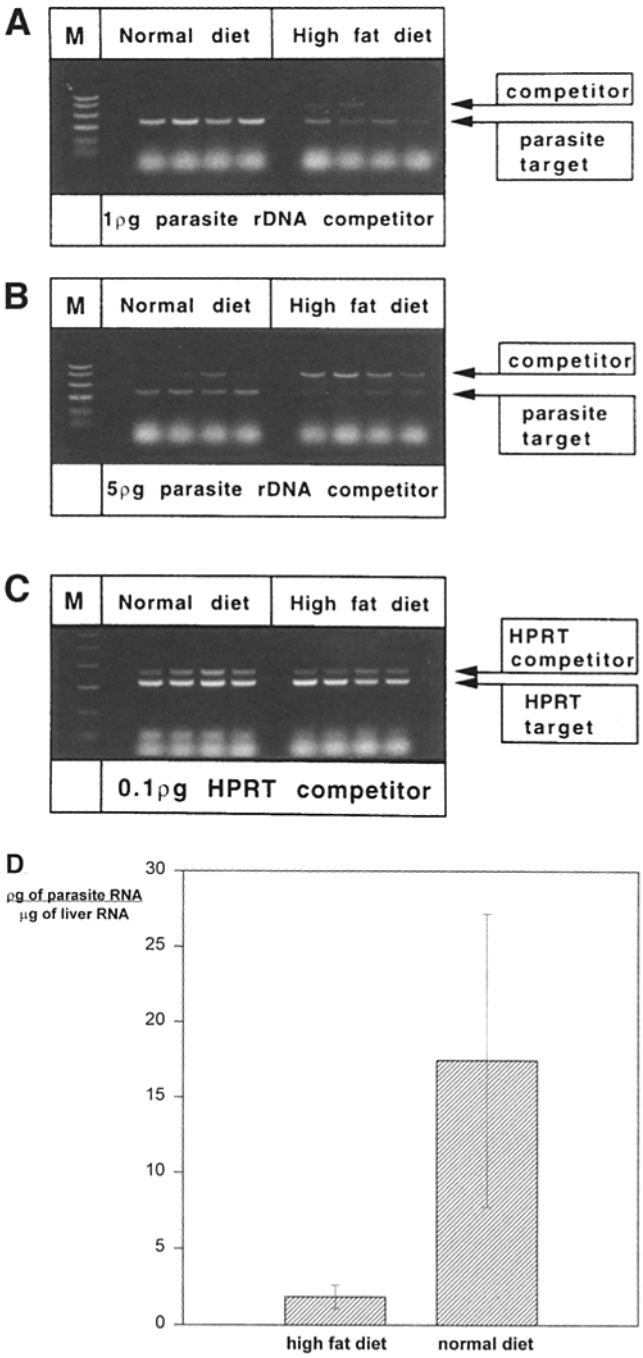

Figure 5. Remnant lipoproteins inhibit infectivity of *P. yoelii* sporozoites in mice. LDLR−/− mice were fed either a normal or high fat diet (four mice per group) for 5 d and then injected intravenously with 7,500 *P. yoelii* sporozoites. 40 h after sporozoite injection, the mice were assayed for malaria infection by measurement of parasite rRNA using quantitative RT-PCR. Shown are PCR products of reactions using parasite rRNA primers and (*A*) 1 pg or (*B*) 5 pg of parasite rRNA competitor. (*C*) primers for mouse HPRT and 0.1 pg of the HPRT competitor were used. The competitor band is not visible in PCRs using cDNA from the four mice fed a normal diet, whereas faint bands can be seen in reactions using cDNA from the four mice fed a high fat diet (*A*). When the amount of competitor in the reactions is increased (*B*), the competitor band is clearly more abundant than the parasite target in the mice fed the high fat diet, and is less than or equal to the parasite target in mice fed normal chow. In reactions using HPRT primers and competitor, an equal ratio of the intensities of the competitor and target bands for all mice, regardless of diet, indicates that the efficiency of the RT reactions was equivalent in both groups. Molecular size markers (M); bp: 1,000, 750, 500, 300, 150, and 50. (*D*) The photograph in *B* was analyzed by densitometry. For each amplification reaction, a target/competitor ratio was calculated and this ratio was used to determine the amount of parasite RNA per microgram of liver RNA. The mean for each group of mice is plotted with error bars showing the range of values calculated.

the liver. This can be explained in part by the accessibility of hepatocyte HSPGs to the circulation since the endothelial cells lining the hepatic sinusoids have open fenestrations, allowing for direct contact between hepatocytes and the blood circulation. However, endothelial cells of other organs also have contact with the circulation and bear HSPGs on their plasma membranes. Structural diversity of HSPG glycosaminoglycan (GAG) chains, generated by post-polymeric modifications such as de-N-acetylation, N-sulfation and O-sulfation, is associated with defined biological functions (for a review see reference 46) and may explain the selective binding of CS, apoE, and lactoferrin to hepatocyte HSPGs. Recent studies have demonstrated that hepatic HSPGs have levels of N- and O-sulfation that are at least 50% higher than most other HSPGs (47), whereas the levels of N- and O-sulfation of endothelial cell HSPGs are low (47, 48). Although the structure of the GAGs that bind CS and the other ligands is not known, the degree of sulfation of HSPGs of HepG2 cells is critical for CS binding and sporozoite invasion (Sinnis, P., manuscript in preparation), suggesting that these ligands can indeed discriminate between different GAG chain structures.

The present studies also demonstrate that remnant lipoproteins inhibit sporozoite infectivity in mice. This suggests, for the first time, that the in vitro demonstration that HSPGs are the hepatic-binding sites for CS, is of relevance in malaria infection. The precise step at which CS binding to hepatic HSPGs is required during sporozoite invasion of hepatocytes is not known, but the clearance data presented here and elsewhere (18) suggest that CS mediates the initial arrest of the parasites in the liver. Previous studies demonstrating that only multimers of CS bind in a stable fashion to HSPGs (21, 22), suggest that the adhesion of sporozoites to hepatocytes involves a multimeric interaction between sporozoite CS, which forms a dense coat on the parasite surface (49), and hepatocyte HSPGs, which are present on the cell surface in the range of 4×10^6 molecules per cell (50, 51). The abundance of HSPG molecules on the cell surface, together with the large number of hepatocytes in the liver (10^{11}), create a high capacity capture system that can, in part, explain the well known efficiency of sporozoite infection. TRAP/SSP2, which also contains a region II-plus motif, may contribute to sporozoite binding in the liver. In contrast to CS, however, TRAP/SSP2 is detected as clusters in restricted regions of the parasite plasma membrane (27, 52, 53) and its relative contribution to sporozoite adhesion to hepatocytes is not known.

After sequestration by HSPGs in the liver, sporozoites must enter hepatocytes in order to successfully complete their development. The mechanism by which they enter

cells is unknown although our results raise the possibility that sporozoites, like remnant lipoproteins, are interiorized by LDLR and/or LRP. Alternately, the attached sporozoites may use their own actin-based motility system to actively invade hepatoctyes, an idea supported by videomicroscopic observations (54) and recent studies of cell invasion by *Toxoplasma gondii* (55), a parasite that belongs to the same phylum as *Plasmodium*.

An unresolved issue is the anatomical localization of the GAGs that bind lipoprotein remnants and, as suggested by the present results, malaria sporozoites. It is generally assumed that lipoprotein remnants traverse the fenestrated endothelium of the liver sinusoids and enter the space of Disse where they are retained by HSPGs. The diameter of sporozoites (1 μM), however, is greater than the average diameter of the fenestrae (0.1 μM; 56, 57), making this an unlikely model for sporozoite attachment to hepatic HSPGs. Another possibility is that the interaction between the GAG chains of hepatic HSPGs with their physiologic ligands, as well as with sporozoites, takes place not in the space of Disse, but within the sinusoids. If one considers the fact that the space of Disse is a narrow, loose matrix of proteins and proteoglycans rather than a true basement membrane, it is possible that the long HSPG GAG chains of the hepatocytes protrude through the fenestrae and are in direct contact with the blood circulation. This model, strengthened by the finding that the bulk of the sulfation of the hepatcyte HSPG GAG chains is found along the distal portion of the molecules (47), would greatly increase the likelihood of productive encounters between the positvely charged regions of the ligands and HSPGs.

The utilization by sporozoite and lipoprotein remnants of a common pathway of retention by the liver is unexpected, and brings together two different areas of research. Our findings raise the intriguing possibility that the lower parasite densities and fewer episodes of clinical malaria observed in neonates (58, 59) are, at least in part, due to the high concentration of lactoferrin (60) and the high fat content of breast milk (61). The present findings thus provide new perspectives for the development of prophylactic agents against malaria, and for the understanding of malaria pathology and epidemiology.

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