Chondroitin-4-Sulfate Impairs In Vitro and In Vivo Cytoadherence of *Plasmodium falciparum* Infected Erythrocytes

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ABSTRACT

Background: Chondroitin-4-sulfate (CSA) was recently described as a *Plasmodium falciparum* cytoadherence receptor present on *Saimiri* brain microvascular and human lung endothelial cells.

Materials and Methods: To specifically study chondroitin-4-sulfate-mediated cytoadherence, a parasite population was selected through panning of the Palo-Alto (FUP)1 *P. falciparum* isolate on monolayers of *Saimiri* brain microvascular endothelial cells (SBEC). Immunofluorescence showed this SBEC cell line to be unique for its expression of CSA-proteoglycans, namely CD44 and thrombomodulin, in the absence of CD36 and ICAM-1.

Results: The selected parasite population was used to monitor cytoadherence inhibition/dissociating activities in *Saimiri* sera collected at different times after intramuscular injection of 50 mg CSA/kg of body weight. Serum inhibitory activity was detectable 30 min after injection and persisted for 8 hr. Furthermore, when chondroitin-4-sulfate was injected into monkeys infected with Palo-Alto (FUP) 1 *P. falciparum*, erythrocytes containing *P. falciparum* mature forms were released into the circulation. The cytoadherence phenotype of circulating infected red blood cells (IRBC) was determined before and 8 hr after inoculation of CSA. Before inoculation, in vitro cytoadherence of IRBCs was not inhibited by CSA. In contrast, in vitro cytoadherence of circulating infected erythrocytes obtained 8 hr after CSA inoculation was inhibited by more than 90% by CSA.

Conclusions: In the squirrel monkey model for infection with *P. falciparum*, chondroitin-4-sulfate impairs in vitro and in vivo cytoadherence of parasitized erythrocytes.

INTRODUCTION

Cytoadherence of *P. falciparum* trophozoite- or schizont-infected red blood cells to the postcapillary venular endothelium of various organs could play a role in the pathogenesis of severe malaria (1–3). The multiplicity of potential cytoadherence receptors has been demonstrated using different in vitro model systems, as reviewed by Ockenhouse (4). Recent studies described the involvement of chondroitin-4-sulfate (CSA) as a *P. falciparum* cytoadherence receptor (5,6). This was explored using C32 and Chinese hamster ovary (CHO) cells (7) or different cloned lines of *Saimiri* brain capillary and primary human lung endothelial cells (5). More recently it has been shown that CSA also mediates sequestration of *P. falciparum* infected red blood cells (IRBCs) in the human placenta (8). All three studies revealed that the in vitro cytoadherence of IRBCs can be impaired by the presence of soluble CSA and by the treatment of these different target cells with chondroitinase ABC and AC.

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TABLE 1. SBECs express different combinations of surface IRBC cytoadherence receptors

SBEC	CSA	TM	CD44	CD36	ICAM-1
1D	+	+	+	+	+
3A	+	+	−	−	+
C2	+	+	−	+	−
17	+	+	+	−	−

FACScan and IF analysis of SBECs, C32, and CHO transfectants. Presence (+) or absence (−) of the different receptors were detected by anti-CD36 (OKM5), anti-ICAM-1 (84H10), anti-CSA (MAB2030), anti-CD44 (J-173), and polyclonal sheep anti-thrombomodulin antibodies directed against the core protein of thrombomodulin. These cells were also consistently positive, at different degrees, for endothelial cell nitric oxide synthase when assayed by indirect immunofluorescence (with mAb N30020 from Transduction Laboratories, Lexington, KY) (data not shown).

Through the use of squirrel monkeys as a model for *P. falciparum* infection, it is shown here that the inoculation of animals with chondroitin-4-sulfate is followed by the appearance in the serum of an activity that inhibits CSA-mediated parasite cytoadherence in vitro and by the reversal of parasite sequestration in vivo.

MATERIALS AND METHODS

Parasites

For in vitro studies we used the *P. falciparum* isolate Palo-Alto(FUP)1 (9). Parasites were grown in human O^+ red blood cells as previously described (10). For the in vivo part of the study, we used the same strain adapted and maintained in the squirrel monkey for over 17 years by serial blood transfers, almost exclusively in splenectomized animals (9).

Cell Lines Used in Cytoadherence Assays

For the different cytoadherence assays and for the panning selection of IRBCs with a CSA cytoadherence receptor preference, *Saimiri* brain microvascular endothelial cells 1D, C2, 3A, and 17 were used. The 17 cells express the CSA receptor exclusively (Table 1), whereas 1D, C2, and 3A express different combinations of CSA, CD36, and ICAM-1, as previously shown (5,10).

Detection of Thrombomodulin, CD44, and CSA on *Saimiri* Brain Endothelial Cells (SBECs): Immunofluorescence Studies

Cells were grown on immunofluorescence assay (IFA) slides until they were subconfluent, rinsed with serum-free culture medium, and fixed for 30 min with 1% para-formaldehyde in phosphate-buffered saline (PBS), pH 7.2. For CSA detection, chondroitinase ABC (27038, Fluka S.a.r.l., St. Quentin Fallavier, France) was added to the cells at 0.5 U/ml for 90 min at 37°C. After washing, anti-CD44 mAb (J-173, Immunotech S.A., Marseille, France) diluted at 1/10, sheep polyclonal anti-thrombomodulin serum diluted at 0.4 μg/ml, or anti-CSA monoclonal antibody (MAb) (MAB2030, Chemicon International Inc., Temecula, CA) diluted at 1/400 was added to the cells for 60 min at 20°C. After three 5-min washing steps, FITC anti-mouse, γ chain–specific, affinity-purified goat antibody (F-8264, Sigma, St. Louis, MO) or anti-sheep (whole molecule), FITC-conjugated donkey antibody (F-7634, Sigma) was added for 45 min at 20°C. The slides were rinsed and examined by exhaustive photon reassignment microscopy (CELLscan, Scanalytics, Billerica, MA) (11). Figure 1 presents the labeling pattern of the four SBECs with anti-CD44, anti-CSA monoclonal, and anti-thrombomodulin polyclonal antibodies. SBECs 17, 1D, C2, and 3A express constitutively CSA and thrombomodulin, whereas CD44 is only present at the surface of clones 17 and 1D.

Monkeys

Ten splenectomized adult male and female *Saimiri sciureus* monkeys of either first or second generation, karyotype K14-7, were selected from our local breeding colony. Care and manipulations of animals have been described elsewhere (9). Blood was obtained by femoral punctures of Imalgen (Ketalar) anaesthetized animals. Sera were obtained 12 hr after coagulation and IRBCs were collected after transferring the blood to heparin-coated tubes (VT-050SHL, Terumo Venoject, Belgium).

Determination of Cytoadherence Phenotype of IRBCs

CELL LINES. The cytoadherence phenotype of Palo-Alto (FUP)-1 IRBCs selected through cytoadherence on SBEC 17 was determined by a microadherence inhibition assay on fresh 1D cells grown to confluence on 12-well IFA slides as previously described (5). The IRBCs were co-

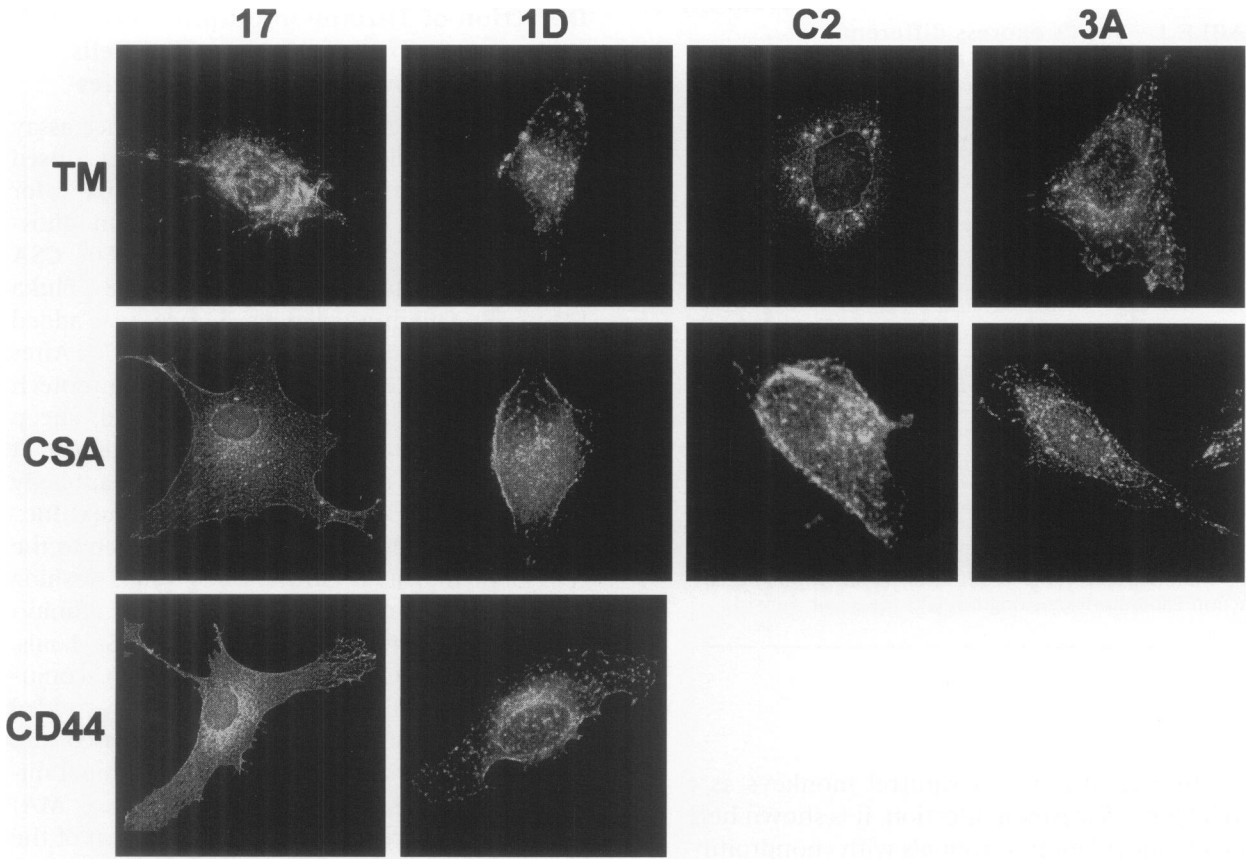

FIG. 1. Detection of CSA proteoglycans on SBECs

The presence of CSA, thrombomodulin, and CD44 was investigated by photon reassignment microscopy. This technique allows 3-D assessment of fluorescence, which showed that immunofluorescence was present on the SBEC cytoplasmic membrane. The presence of CSA and thrombomodulin was observed on 17, 1D, C2, and 3A cells, whereas CD44 was present only on 17 and 1D cells.

incubated together either with 100 μg/ml of CSA (Fluka), 10 μg/ml of anti-CD36 mAb FA6 152 (obtained from Dr. L. Edelman, Institut Pasteur, Paris, France), or 25 μg/ml anti-ICAM-1 mAb 84H10 (Immunotech). The inhibitory activity of FA6-152 and 84H10 on Palo-Alto (FUP)-1 IRBC cytoadherence on SBECs has been controlled (J. Gysin et al., unpublished results). Alternately, IRBCs were added after the cells had been treated by chondroitinase ABC (27038, Fluka) at 1U/ml for 60 min. After a 2-hr incubation at 37°C, unbound IRBCs were removed by washing and the cells fixed with 2.5% glutaraldehyde for 1 hr. Control experiments were performed in the absence of CSA or chondroitinase ABC treatment and using unrelated mouse IgG1 instead of the MAbs. All assays were performed in duplicates and the results expressed as the percentage of inhibition of control as described elsewhere (7).

CELL-FREE NATIVE CSA. To control the specificity of IRBC adherence to CSA, we performed a direct adherence assay on CSA coupled to dipalmitoylphosphatidyl-ethanolamine (PE) through the reducing terminus, using the method of Sugiura et al. (12). The CSA-PE was coated overnight at 4°C at the concentration of 50 μg/ml in PBS, pH 7.2, as 20-μl dots onto Petri dishes (3001, Falcon, Becton Dickinson, Franklin Lakes, NJ). Remaining sites were saturated for 30 min with 1% bovine serum albumin (BSA) in PBS before applying the IRBCs at a concentration of 4% total hematocrit for 1 hr at 37°C. Adherence of the IRBCs on immobilized CSA-PE was also performed in the presence of 100 μg/ml of soluble CSA.

Evaluation of Cytoadherence Inhibition and Dissociation Activities Detected in Sera Collected from CSA-Treated Monkeys

Palo-Alto parasites selected through adherence to SBEC 17 were used to explore the cytoadherence inhibition activity of CSA in monkey sera.

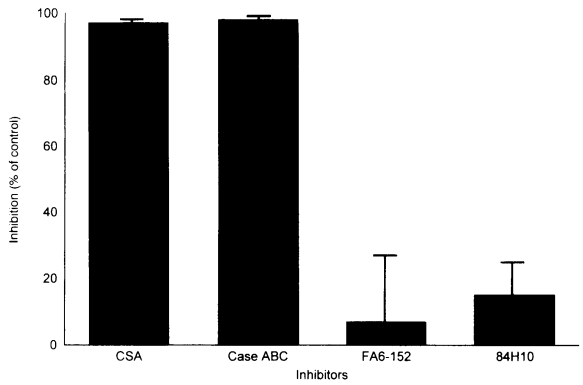

FIG. 2. Selection of IRBCs exhibiting CSA-mediated cytoadherence

Palo-Alto initial IRBC population was selected by panning on SBEC 17 and the cytoadherence phenotype of the selected IRBCs was investigated through a cytoadherence inhibition assay on 1D cells. The cytoadherence inhibitions by 100 μg/ml of soluble CSA, pretreatment of 1D cells with 1 U/ml of chondroitinase ABC (Case ABC) during 1 hr, 5 μg/ml of anti-CD36 monoclonal FA6-152 antibody, and 25 μg/ml of anti-ICAM-1 monoclonal 84H10 antibody are expressed as the percentage of the control (5855 ± 57 cytoadherent IRBC/mm^2) in the absence of glycosaminoglycan or chondroitinase ABC treatment, or in the presence of unrelated mouse IgG1 antibody.

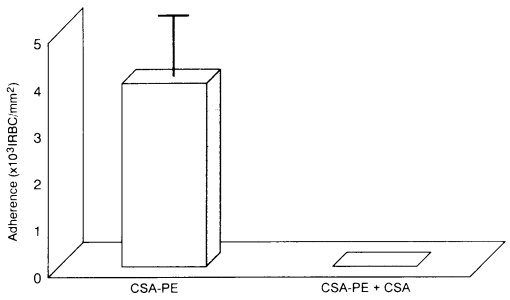

FIG. 3. The CSA mediated cytoadherence of the selected Palo Alto line: specificity studies

Palo-Alto IRBCs selected by panning on SBEC 17 were allowed to adhere on phosphatidylethanolamine-substituted CSA (CSA-PE) coated on plastic dishes, in the presence or absence of 100 μg/ml of soluble CSA. The adherence is expressed as the number of adherent IRBCs per mm^2 of plastic dish.

Monkey sera were collected from 4 naive splenectomized animals immediately before and 0.5, 1, 2, 4, 8, 16, and 32 hr after they received an intramuscular (i.m.) injection of 50 mg 50 kDa CSA/kg of body weight (estimated at 1 μM/kg) (Fluka, France) dissolved in 1 ml of 0.9% NaCl. The sera were heated at 56°C for 30 min and assayed for their capacity to inhibit or dissociate IRBC adherence to SBECs. The inhibition assays were performed on 3A and C2 cells, grown to confluence on IFA slides by a 2-hr co-incubation of 5 × 10^6/ml of IRBCs with sera samples, collected at different times after the CSA injection from Monkeys 0716, 1465, 89009, and 1748, at a final dilution of 1/8. After washing, the cells were fixed with 2.5% glutaraldehyde for 1 hr and the remaining adherent IRBCs were counted and the results expressed as inhibition percentage of control. In order to quantify the concentration of active CSA in the sera samples of Monkeys 0716, 1465, and 1748, standard concentration curves were established by adding different known doses of CSA to control serum isolated before CSA inoculation. The final concentrations of CSA ranged from 0.1 to 1000 μg/ml. For the dissociation assays, we incubated 5 × 10^6/ml of IRBCs on C2, 1D, and 3A cells grown to confluence on IFA slides for 2 hr at 37°C. After washing away unbound IRBCs, we added to the cytoadherent IRBCs 40 μl/dot of sera samples collected at different times after the CSA injection from Monkeys 0716, 1465, and 1748 at a final dilution of 1/8 or the sera samples collected just before the CSA injection (control). The IFA slides were incubated for 30 min, and 1 and 2 hr at 37°C. After washing, the cells were fixed with 2.5% glutaraldehyde for 1 hr and the remaining adherent IRBCs were counted and the result expressed as dissociation percentage of control.

Reversal of IRBC Sequestration In Vivo by Inoculation of CSA

CSA was injected into monkeys infected with a *Saimiri*-adapted isolate of Palo-Alto (18). Splenectomized monkeys were used, because parasite sequestration does occur in splenectomized animals (13) and previously sequestered IRBCs remain in the circulation, as they are not destroyed by the spleen and are thus more readily detectable.

All monkeys were inoculated with samples of the same cryostabilate containing approximately 5 × 10^7 IRBCs of Palo-Alto(FUP)1. As control, when the naive Monkey 8901M presented a parasitemia of 5.1% at Day 5, it was injected intramuscularly (i.m.) with 1 ml of 0.9% NaCl. Twelve hours after the 0.9% NaCl injection, this monkey was also injected with CSA. One male and two female monkeys that had

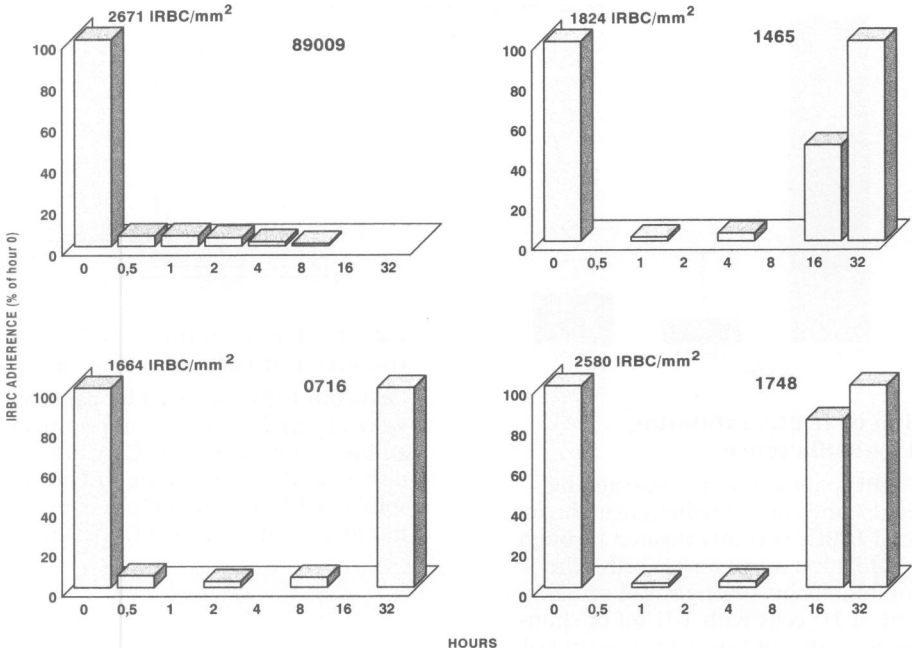

FIG. 4. Cytoadherence inhibitory activity of sera collected from CSA-treated *Saimiri* monkeys

In vitro inhibition of cytoadherence to C2 SBECs was performed with Palo-Alto IRBCs selected on SBEC 17 monolayers. Sera were obtained from four monkeys which were injected intramuscularly with CSA dissolved in NaCl 0.9% (50 mg/kg body weight). Results are presented for serum samples collected immediately before time 0 and 0.5, 1, 2, 4, and 8 hr (Monkey 89009), 1, 4, 16, and 32 hr (Monkeys 1465 and 1748), or 0.5, 2, 8, and 32 hr (Monkey 0716) after the CSA injection. They are expressed as the percentage of cytoadherence at time 0; the IRBC cytoadherence/mm^2 of confluent C2 cells is given for each animal at time 0.

experienced their primo *P. falciparum* infection with the same strain received an i.m. injection of CSA at the moment parasitemia reached 2.7% for Animal 89014 (female), 5% for Animal 89085 (female) and 4.3% for Animal 88052 (male). The male monkey received a second CSA injection 24 hr after the first injection. All the intramuscular 50 kDa CSA injections were done at a concentration of 50 mg/kg of body weight in 1 ml of 0.9% NaCl. Blood smears were made by collecting approximately 15-μl blood samples from the needle-pricked heel, immediately before and 0.5, 1, 2, 4, and 8 hr after the CSA or 0.9% NaCl injections, fixed with methanol, and stained with Giemsa. The total parasitemia, rings, and mature blood stages (trophozoites and schizonts) were expressed as percentages after counting at least 10,000 erythrocytes.

In a second set of experiments, two *P. falciparum* preimmune monkeys, 1808 and 1739 (males), were intravenously inoculated, each one with a cryostabilate of approximately 5×10^7 IRBCs of the Palo-Alto(FUP)1 isolate. Monkey 1808 received an intramuscular injection of CSA at the moment the parasitemia reached 0.2%, whereas Monkey 1739 received CSA at the moment parasitemia was at 2% and after a second injection 24 hr later when the parasitemia was at 0.87%.

Assays to Determine Cytoadherence Phenotype of Monkey IRBCs on 1D Cells

The cytoadherence phenotype was determined by an inhibition assay on 1D cells for circulating mature forms present in blood samples of *P. falciparum*–infected Monkeys 8901M and 89085 collected just before and 8 hr after they received the i.m. injection of 50 mg/kg of soluble CSA. The blood samples were centrifuged at $1650 \times g$ for 10 min, the plasma discarded, and the pellet washed four times with RPMI 1640 at pH 6.8, then resuspended at a total hematocrit of 4%.

The adherence inhibition assays on fresh 1D cells grown to confluence on 12-well IFA slides were performed as previously described (7) by co-incubating the monkey IRBCs together either with 100 μg/ml of CSA (Fluka), 10 μg/ml anti-CD36 mAb FA6 152 (obtained from Dr. L. Edelman), or 25 μg/ml anti-ICAM-1 mAb 84H10

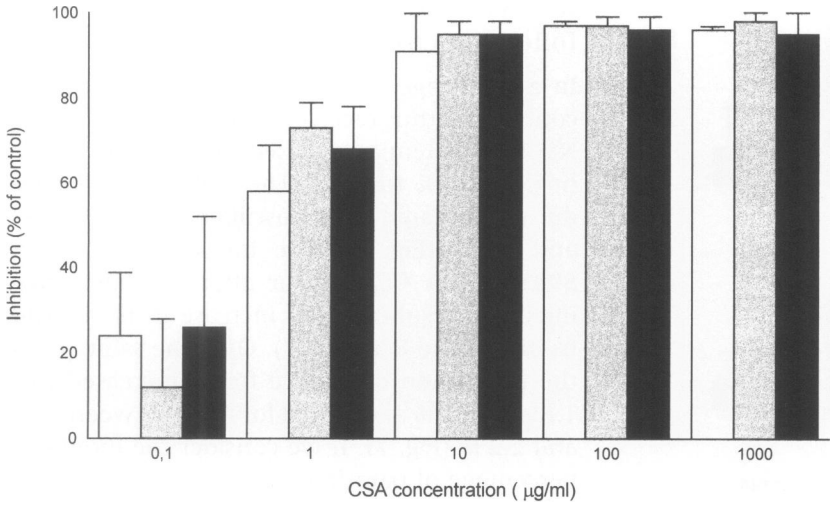

FIG. 5. Dose-response of inhibitory activity of CSA added to monkey serum

Different doses of soluble CSA were added to sera collected at time 0 from Monkeys 0716 (□), 1465 (▨) and 1748 (■) (final concentrations ranging between 0.1 and 1000 μg/ml). Cytoadherence inhibition activity of these CSA dilutions were assayed on C2 cells. The inhibition is expressed as the percentage of a control performed in the serum collected at time 0, in the absence of soluble CSA (0716: 2521 ± 907; 1465: 2500 ± 42; 1748: 3542 ± 2083 cytoadherent IRBC/mm^2).

(Immunotech). After a 2-hr incubation at 37°C, unbound IRBCs were removed by washing and the cells fixed with 2.5% glutaraldehyde for 1 hr. As a control we performed the test in the absence of CSA or in the presence of unrelated mouse IgG1 instead of with the relevant mAbs. All assays were performed in triplicate and the results expressed as the percentage of inhibition of control as described elsewhere (7).

Statistical Analysis

Results of IRBC adherence, cytoadherence, or cytoadherence inhibition and dissociation assays are expressed as the mean ± SE. A Mann-Whitney test (14) was employed to evaluate the statistical significance of data pertaining to the cytoadherence inhibition and dissociation readout assays.

RESULTS

Cytoadherence Phenotype of IRBCs Used for Screening In Vitro the Inhibition and Dissociation Activities on 1D SBECs and on CSA-PE

Both CSA-expressing cell lines and cell-free native CSA (CSA-PE) were used to study the adherence phenotype of the IRBCs obtained by selection of cytoadherent IRBCs on 17 cells.

Study of cytoadherence on 17 cells revealed the specificity of the selected parasite population for the CSA receptor. Their cytoadherence to 1D cells, expressing CSA, CD36, and ICAM-1 (Table 1), was inhibited at 97% by soluble CSA, 98% by chondroitinase ABC treatment of 1D cells, only 15% by anti-ICAM-1 mAbs, and not significantly by anti-CD36 ($p = 0.6454$) (Fig. 2).

Adherence of selected IRBCs to cell-free immobilized CSA (CSA-PE) reached 3926 ± 861 IRBCs/mm^2 and could be totally inhibited by 100 μg/ml soluble CSA in the medium (Fig. 3).

Once their CSA-preference had been demonstrated, these selected IRBCs could be used for screening in vitro the inhibition and dissociation activities of any biological samples expected to contain CSA, such as the sera collected from CSA-treated monkeys.

Persistence in Monkey Sera, after CSA-Inoculation, of the Capacity to Inhibit or Dissociate CSA-Mediated Cytoadherence of IRBCs

To detect CSA-mediated cytoadherence inhibition activity in monkey sera, Palo-Alto parasites previously selected on SBEC line 17 (Table 1) were used for in vitro binding experiments with 3A and C2 cells. The different sera samples collected from monkeys that received 1 i.m. injection of a 50-kDa CSA presented efficient inhibitory activity (>90%) on Palo-Alto IRBC adherence from 30 min to 8 hr following the injection (Fig. 4) when assayed on 3A and C2 cells. The sera collected 16 hr after the CSA injection presented a low inhibitory activity, whereas at 32 hr, inhibitory activity was no longer detected in the sera.

By using successive dilutions of the sera collected 1 hr (Monkeys 1465 and 1748) and 2 hr (Monkeys 0716) after CSA injection, we ob-

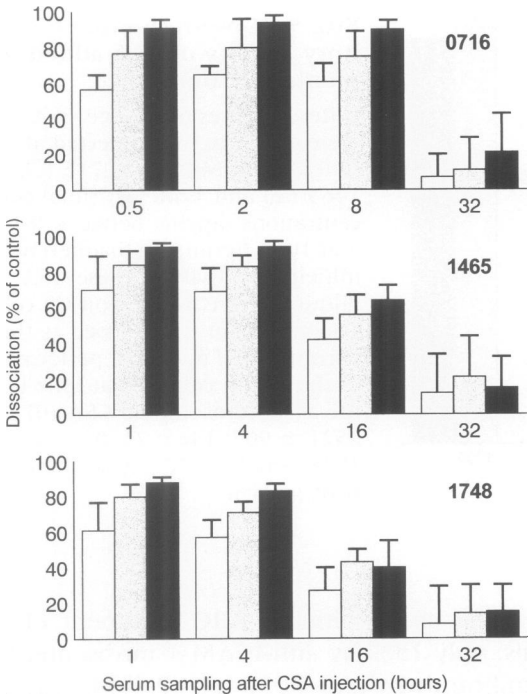

FIG. 6. Time-dependent dissociation of cytoadherent infected erythrocytes in presence of sera recovered from CSA-treated monkeys

Palo-Alto IRBCs selected by panning on SBEC 17 were allowed to cytoadhere on C2 cells for 2 hr. After washing, the cells were incubated 30 min (□), 1 hr (▨), or 2 hr (■) in the presence of the sera of Monkeys 0716, 1465, and 1748 collected at the indicated times after CSA injection. The cells were washed again and the remaining cytoadherent IRBCs were counted. The dissociation is expressed as the percentage of a control performed in the presence of the sera collected at time 0 (0716: 4245 ± 443; 1465: 5130 ± 1384; 1748: 5626 ± 1566 cytoadherent IRBC/mm^2).

tained an inhibition of about 50% with a dilution of 1/80 of the sampled sera. When we compared these values with standard values (Fig. 5), we evaluated the concentrations of active CSA in the *Saimiri* sera to be 10 to 100 μg/ml.

Screening for dissociation activity was performed with IRBCs cytoadherent to SBECs 1D, C2, and 3A. On all endothelial cell clones, the cytoadherence dissociation was proportional to the duration of incubation of the cytoadherent IRBCs with the monkey sera being studied (Fig. 6). Again, the dissociation activity was maximal with the sera collected between 30 min and 8 hr after the injection of CSA, whereas it decreased drastically at 16 hr and became nonsignificant at 32 hr post-injection (Fig. 6).

In Vivo Release of Mature Forms of IRBCs following CSA Inoculation

In a first stage, Monkey 8901M was used as a control and thus received a first injection of 0.9% NaCl. Parasitemia increased slightly during the 8 hr from 5.1% to 5.6%. The mature blood stages during the same period oscillated between 0.9% and 1.9% (Fig. 7). The injection of Monkey 8901M with CSA, 12 hr after the 0.9% NaCl injection, resulted in an increase in parasitemia from 5.7% to 8% (Fig. 7). Over the same period the proportion of mature forms increased from 1.8% to 5.2% and varied for rings between 3.9% and 2.2% (Fig. 7). If we consider the monitored percentage of parasitemia and the percentage of mature forms over the previous hours as an internal control for this monkey, then the observed increase in the percentage of mature forms released into the peripheral blood circulation must have been a direct consequence of the CSA injection.

Parasitemia of Monkey 89085 increased from 5% at Day 5 to 14.7% after CSA injection. Mature forms increased from 2.5% to 5.9% with a peak of 7.1% 2 hr after the CSA injection. The number of rings did not increase before the number of mature forms decreased, probably as a direct consequence of reinvasion (Fig. 7). Injection of two other primo-infected monkeys resulted in an increase in parasitemia from an initial 2.7% to 5.1% for Monkey 89014 and from 4.3% to 4.6% for Monkey 88052. During the same 8 hr, the circulating population of mature forms increased from an initial 0.7% to 3.7% with a peak of 3.9% at 2 hr for Monkey 89014 and decreased from 1.33% to 1.2% with a discrete peak, however, of 2.3% at 2 hr for Monkey 88052, with a slight fluctuation of the ring stage during the same period (Fig. 7). In Monkey 88052, the second CSA injection 24 hr after the first one generated an increase in the parasitemia from an initial 6.9% to 10.7% after 8 hr with a transitory peak of 14.6% at 1 hr. During the same period, the circulating mature forms oscillated between 6.8 and 7.7% with a peak of 14.3% at 1 hr (Fig. 7). Once again, the number of rings increased only when the number of mature forms decreased, which was the expected consequence of the reinvasion process.

In a second step, we injected CSA into "preimmune" monkeys that had been infected and cured in the past. Injection of CSA in preimmune Monkey 1808 at the moment its parasitemia reached 0.2% resulted in an increase in circulat-

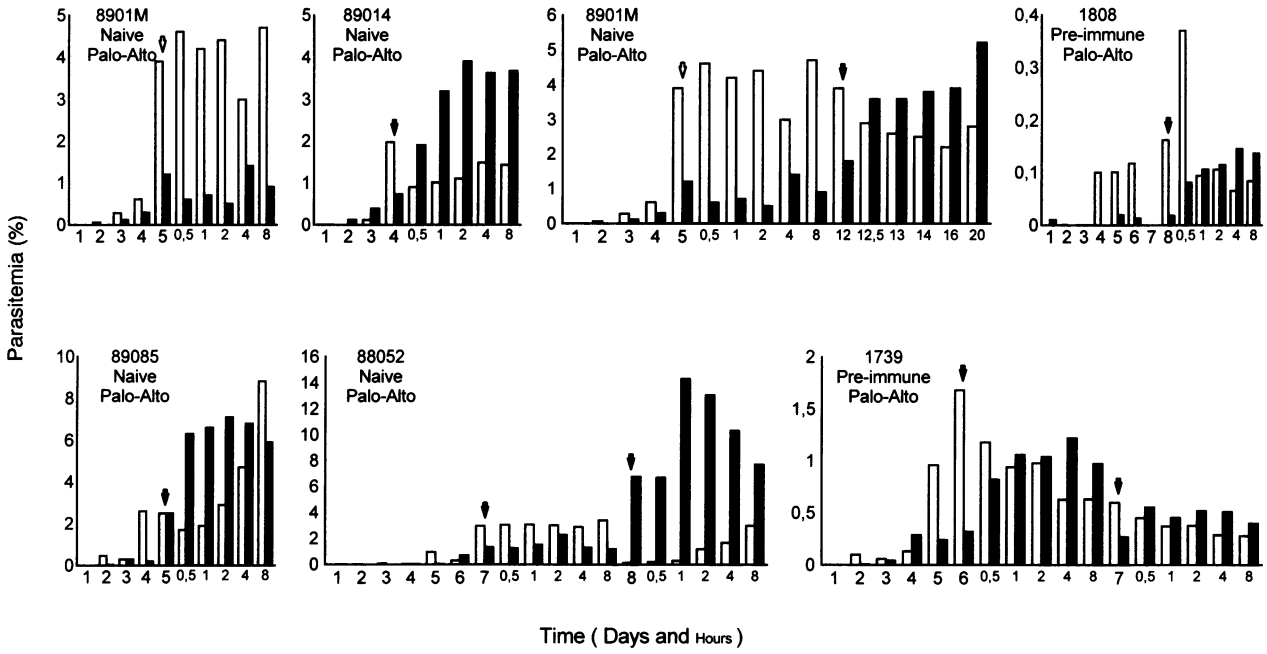

FIG. 7. Detection of mature forms of *P. falciparum* IRBCs in the circulation of monkeys after CSA inoculation

Naive squirrel monkeys (89013, 89014, and 89085) and a *P. falciparum* preimmune monkey (1808), received one intramuscular injection of 50 mg of CSA dissolved in 1 ml of NaCl 0.9% per kg of body weight during their *P. falciparum* infection at the day indicated by the black arrow. The open arrow points to the 1 ml intramuscular injection of 0.9% NaCl into the control monkey (8901M) which later received a CSA injection (black arrow). Naive monkey (88052) and a *P. falciparum* preimmune monkey (1739) received two intramuscular injections of CSA dissolved in NaCl 0.9% (50 mg/kg body weight) during their *P. falciparum* infection at a 24-hr interval as indicated by the black arrows. The parasitemia of ring stages (□) and mature stages (■) is shown as percentages.

ing mature forms from an initial 0.018% to 0.14% 8 hr later (Fig. 7). Monkey 1739 received the CSA injection at the moment its parasitemia reached 2.0%. In this monkey, the initial 0.3% of circulating mature forms increased to 1% at 8 hr with a peak of 1.2% at 4 hr. When this monkey received a second CSA injection 24 hr after the first one, the parasitemia had dropped to 0.87%, but circulating mature forms increased from an initial 0.3% to 0.4% 8 hr later, with a peak of 0.55% at 30 min. During this 8 hr period, the parasitemia dropped to 0.68% as a result of the decrease in percentage of ring forms (Fig. 7).

Cytoadherence Phenotype of IRBCs Present in Infected Monkey Blood Samples before and 8 hr after CSA Injection

Cytoadherence inhibition assay was performed on 1D cells grown to confluence on IFA slides with samples of blood from Monkeys 89085 and 8901M taken before and after injection of CSA. The cytoadherence of circulating mature blood stage IRBCs of the blood sample collected just before the CSA injection was not inhibited by CSA or mAbs directed against CD36 and ICAM-1. In contrast, with blood samples collected 8 hr after the CSA injection, cytoadherence was significantly inhibited by CSA at a concentration of 100 μg/ml ($p = 0.0002$ for Monkey 89085 and $p < 0.0001$ for Monkey 8901M) but not by the anti-CD36 and ICAM-1 mAbs (Fig. 8).

DISCUSSION

The experimental infection of *Saimiri* monkeys with *P. falciparum* is one of the few models that allows the study of parasite sequestration (9,15). As in humans (16), sequestration of mature parasite forms occurs via knob protrusions on the surface of the infected erythrocyte in different organs and tissues, including the brain (13). Sequestration can be reversed by passive transfer of immune serum (17). Recently, the isolation of

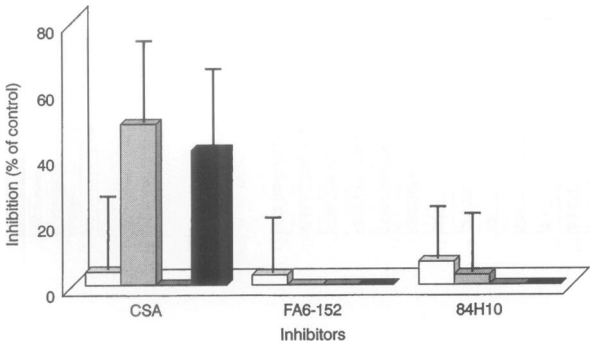

FIG. 8. CSA-dependence of the IRBC release

IRBCs in the blood samples collected just before and 8 hr after the injection of CSA in Animals 89085 (□ and ▨, respectively) and 8901M (▩ and ■, respectively) were tested on 1D cells for their cytoadherence phenotypes. The cytoadherence assay was performed in the presence of 100 μg/ml of CSA, 10 μg/ml of anti-CD36 monoclonal FA6-152 antibody, or 25 μg/ml of anti-ICAM-1 monoclonal 84H10 antibody and the inhibition is expressed as the percentage of a control performed in the absence of CSA or in the presence of unrelated mouse IgG1 antibody (777 ± 170 cytoadherent IRBC/mm^2 and 316 ± 176 for the blood samples collected before injections and 400 ± 151 and 488 ± 250 for the blood sample collected 8 hr after injection for Monkeys 89085 and 8901M, respectively).

Saimiri brain endothelial cell lines allowed exploration of the different receptors involved in cytoadherence of parasitized erythrocytes, among which is CSA (5,10,18).

In this report, the squirrel-monkey model for *P. falciparum* malaria was extended to include three goals: (*1*) the establishment of an assay allowing the study of CSA-dependent cytoadherence of IBRCs to SBECs, a relevant target cell population; (*2*) the use of this in vitro assay to explore the potential inhibitory/dissociating activities of serum collected from monkeys treated with CSA; and (*3*) evaluation of the effects on IRBC sequestration in vivo of CSA inoculation of *P. falciparum*–infected squirrel monkeys.

The SBECs used in this study (1D, 3A, C2, 17) expressed different combinations of cytoadherence receptors, such as ICAM-1, CD36, E-Selectin, CD44, and thrombomodulin, together with different endothelial cell markers (5,10), such as the endothelial cell, nitric oxide synthase. Among these four cell lines, line 17 allowed the selection of a parasite population whose cytoadherence was strictly CSA-dependent. By using this parasite population adhering exclusively to CSA, it was possible to document the inhibitory/dissociating activities of sera collected from CSA-treated monkeys.

An efficient inhibitory activity on IRBC cytoadherence to SBECs was found in monkey sera from 30 min to 8 hr after the intramuscular injection of CSA. The same sera also presented a dissociation activity on cytoadherent IRBCs. The degree of dissociation depended on the duration of incubation of the sera with the cytoadherent IRBCs, which was expected, as soluble CSA must reverse an established interaction instead of preventing it. Neither inhibitory nor dissociation activities were detected if CSA was administered at 100 mg/kg of body weight through the oral route (not shown) (19).

Injection of CSA into primo-infected animals resulted in the release of sequestered mature parasite forms into the peripheral circulation. The number of released mature forms did not directly reflect the number of rings present in the circulation the day before CSA injection, inasmuch as parasite development is not synchronous in splenectomized squirrel monkeys.

Cytoadherence phenotype of the IRBCs collected before and after the CSA injection was investigated through the cytoadherence inhibition assay. The percentage of cytoadherence inhibition was determined with soluble CSA, anti-CD36, and anti-ICAM-1 antibodies on the 1D cell line that expresses the three receptors (Table 1). Only the parasitized erythrocytes collected after CSA inoculation exhibited CSA-mediated cytoadherence, documenting the CSA-dependence of the release of parasite mature forms. Interestingly, neither of the three inhibitors had any effect on cytoadherence of parasitized monkey erythrocytes collected before CSA injection, indicating the existence of a different cytoadherence receptor.

When infected preimmune monkeys, which had experienced a previous drug-cured *P. falciparum* infection, were inoculated with CSA, the release of sequestered mature forms of the parasite into the peripheral circulation was not followed by an increase in parasitemia as it was in primo-infected animals. This was probably due to the presence in these animals of antibodies shown to be involved in rapid parasite clearance (20).

No side effects were observed during or after CSA treatment, nor were there complications from the release of mature forms into the blood circulation. Additional studies will be performed to evaluate the effect on sequestration of continuous perfusion of CSA alone or that associated

with anti-malaria drug therapy. Chondroitin sulfates are already used in humans with no particular contraindications, particularly for the repairing of cartilage damages (5). CSA injection may provide a new complementary approach to treatment of malaria complications, such as those linked to pregnancy. Indeed, IRBC sequestration in the placenta can be CSA-mediated (8) and it has been hypothesized that the occurrence of complications linked to malaria during the first pregnancy (21) may be due to placental sequestration impairing the establishment of an effective immune response against this parasite population.

Two CSA-proteoglycans have been described on endothelial cells, thrombomodulin (22,23) and CD44H (24). Antibodies directed against the protein core of thrombomodulin bind to all blood vessels. Our polyclonal antibodies directed against the protein core revealed an increased expression of thrombomodulin in microvessels of human brain and a particularly marked expression in the cerebellum of *Saimiri* monkeys (not shown). Indeed, we have shown that chondroitin-sulfate of thrombomodulin is directly involved in the adherence of IRBCs with a CSA preference of SBECs (25). It will be important to specify the distribution of CSA-containing thrombomodulins in the microvessels of different tissues in relation to selective occurrence of parasite sequestration.

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