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Synthetic peptides based on motifs present in human band 3 protein inhibit cytoadherence/sequestration of the malaria parasite *Plasmodium falciparum*

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ABSTRACT **Synthetic peptides patterned on the amino acid sequences found in two exofacial regions of band 3 protein (residues 824–829 of loop 7 and residues 547–553 of loop 3) blocked, in a dose-dependent fashion, the *in vitro* adherence of *Plasmodium falciparum*-infected erythrocytes to C32 amelanotic melanoma cells. Intravenous infusion of these synthetic peptides into *Aotus* and *Saimiri* monkeys infected with sequestering isolates of *P. falciparum* resulted in the appearance of mature forms of the parasite in the peripheral circulation. The finding that the peptides were effective as adhesion blockers in the micromolar range suggests that cerebral malaria could be managed through antiadhesion therapy.**

The hallmark of *Plasmodium falciparum* infections is sequestration—that is, the attachment of erythrocytes infected with mature-stage parasites (trophozoites/schizonts) to the endothelial cells lining the postcapillary venules (1). In humans, the principal organs in which sequestration takes place are the heart, lung, kidney, and liver (2). Sequestration in the brain microvessels—a special pathology of *P. falciparum* infections called cerebral malaria—may occlude blood flow and result in confusion, lethargy, and deep coma (3). Although the plasmodial molecules on the surface of the malaria-infected erythrocyte that are responsible for binding to the endothelium have not been identified, a parasite-encoded protein, *P. falciparum* erythrocyte membrane protein 1 (PfEMP 1; ref. 4), similar to a recently described protein called sequestrin (5), has been correlated with cytoadherence. However, the precise role of PfEMP 1 (or sequestrin) in erythrocyte sequestration has not been determined.

Earlier work (5–7) indicated that infection of erythrocytes by the malaria parasite *P. falciparum* leads to truncated forms of band 3 protein in the erythrocyte membrane and that these were involved in the cytoadherent behavior of the infected erythrocyte. Further, several monoclonal antibodies directed against exofacial loops 3 and 7 of band 3 protein blocked cytoadherence (I.C. and I.W.S., unpublished results). In an attempt to further define the regions of band 3 protein responsible for the cytoadherent behavior of infected erythrocytes, peptides patterned on the surface-exposed domains of band 3 were synthesized, and their ability to inhibit the *in vitro* and *in vivo* adherence of *P. falciparum*-infected erythrocyte was determined.

The following observations were used to determine which sequences in the exofacial loops (Fig. 1) might contain the adhesive region: (*i*) Cytoadherence is strongly influenced by pH (16, 17), consistent with a protonated histidine residue being near to, or part of, the adhesin site. (*ii*) Cytoadherence is strongly inhibited by the iodination of surface proteins of a *P. falciparum*-infected erythrocyte (18), implying that a tyrosine residue is near to, or part of, the adhesin site. (*iii*) The adhesin is trypsin sensitive (18), suggesting that a trypsin site (a lysine or arginine) is close to, or part of, the adhesive region. Because free lysine (at millimolar concentrations) inhibited cytoadherence, whereas free arginine and some other amino acids did not (Table 1), a lysine residue was expected to be in the adhesive region.

Since amino acid residues 546–555 of human band 3 protein appeared to meet most of these criteria (Fig. 1*B*), synthetic peptides based on this sequence were initially screened for their ability to inhibit cytoadherence. In addition, a review of the primary structure of band 3 protein revealed similarities of some of the amino acid sequences of loops 3 and 7; consequently, synthetic peptides based on the latter sequence (and predicted to be in the exofacial region of loop 7) were also assayed for their capacity to block the adhesion of infected erythrocytes both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Peptides. Peptides synthesized using the *t*-butoxycarbonyl method followed by HF release were obtained from Multiple Peptide Systems (San Diego) or Coast Scientific (San Diego). All peptides were >97% pure as determined by HPLC and mass spectrometry.

Cytoadherence Assays. *In vitro* cytoadherence assays were as described by Udeinya *et al.* (19), with modifications described by Crandall *et al.* (17) using the C32 amelanotic melanoma cell line.

Parasites. *P. falciparum* was cultured according to Trager and Jensen (20). The medium used for adherence assays has been described (17). Data points (see Fig. 2) represent the average of duplicate determinations, and IC_{50} values were determined by extrapolating the linear portion of inhibition curves to determine the 100% inhibition value. All *in vitro* assays were carried out with the Gambian FCR-3 isolate. *In vivo* experiments used the FVO/DNAX and IPC/RAY isolates in *Aotus* and *Saimiri* monkeys, respectively.

Inhibition of Sequestration. *Saimiri sciureus* and *Aotus nancymai* monkeys were kept as described (21, 22). Nonimmune monkeys were inoculated with *P. falciparum* isolates, and the course of the infection was monitored by Giemsa-stained blood films. Once the infection was established, peptides were injected intravenously and blood films were taken at regular intervals. Microscopic examination of such films was used to determine parasitemia and stage distribution.

RESULTS

Inhibition of Cytoadherence. Peptides 3a, 3b, 7a, and 7b (see Fig. 2 and Table 1) inhibited cytoadherence of infected erythrocytes to melanoma cells, whereas peptides 3c, 7e, and 3ds were without effect; peptide 4 reduced adherence, but not to a significant degree (Table 1). Inhibition of adherence was directly related to peptide concentration and was consistent with the peptides competing with adhesin sites on the surface

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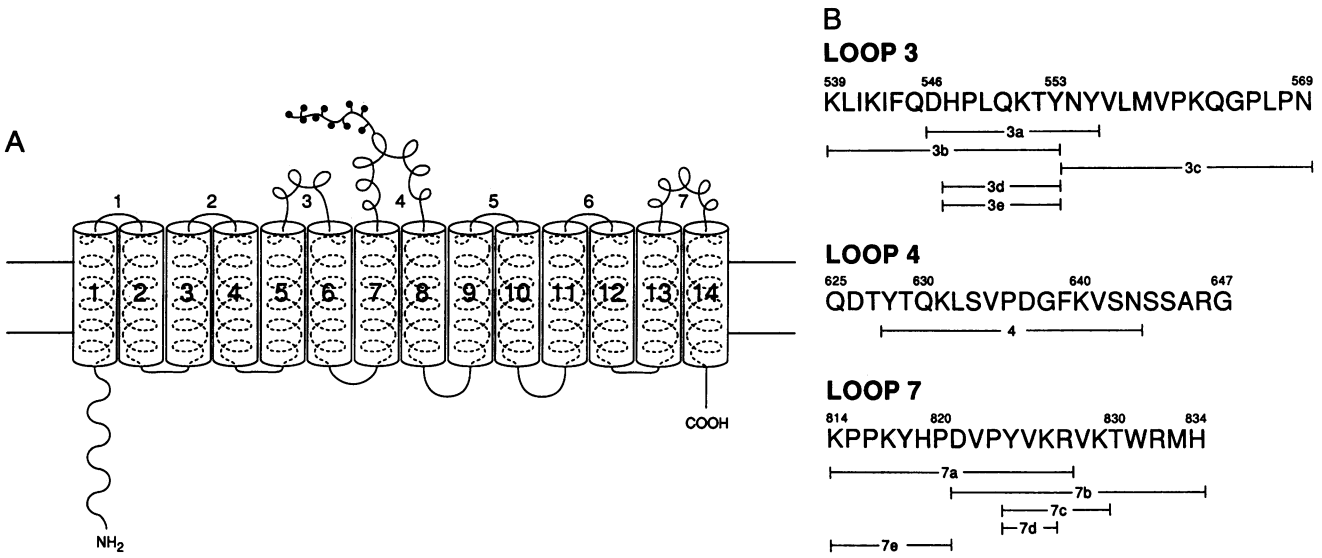

FIG. 1. Relationship between synthetic peptides and amino acid sequence of band 3. Examination of the amino acid sequence (8, 9) of band 3 coupled with information about which residues are reactive in intact cells (9–13) was used to predict the regions of the band 3 molecule that are exofacial. Division of sequences into external loop regions was based on hydropathy plots (9) and published information (14, 15). The initial choice of peptide sequence was based on the observation that a histidine, a lysine, and two tyrosine residues [residues thought to be involved in cytoadherence (16, 17)] were present in the region encompassing amino acids 547–555. Loop 7 contained the sequence Lys-Tyr-His (residues 817–819); however, because of the similarity in the spacing of lysine and proline residues in loops 3 and 7, the stretch from 539–553, a region that contains a tyrosine residue, two lysine residues, and a histidine residue, was also tested.

of the malaria-infected erythrocyte. When peptide 3b and peptide 7a were present in combination, inhibition of cytoadherence did not increase beyond that observed for peptide 7a alone (Fig. 3*A*). Therefore, it would appear that synthetic peptides based on specific regions of loops 3 and 7 compete for the same receptor sites on the target cell. The active synthetic peptides inhibited adherence in the micromolar to nanomolar range, indicating that these peptides mimicked a sequence similar to, or identical with, the adhesive region on the surface of the infected erythrocyte. Iodination of the active peptides prior to their addition to the cytoadherence assay abolished their inhibitory properties (data not shown).

Table 1. IC_{50} values for synthetic peptides and free amino acids

Peptide or amino acid	Sequence	Residues	IC_{50}
3a	DHPLQKTYNY	546–555	9 μM
3b	KLIKIFQKHPLQKTY	539–553	7 μM
3c	NYNVLMVPKPQGPLPN	554–569	>500 μM
3d	GHPLQKTY	546–553	8 μM
3e	YTKQLPHG	553–546	4 μM
3ds	LYPQHKT		>500 μM
4	YTQKLSVPDGFKVSN	628–642	≈300 μM
7a	KPPKYHPDVPYVKR	814–827	3 μM
7b	DVPYVKRVKTWRMH	821–834	1 μM
7c	YVKRVK	824–829	6 μM
7d	YVK	824–826	26 μM
7e	KPPKYHP	814–820	>500 μM
Lys			25 mM
Arg			>100 mM
Tyr			30 mM

All peptides were purchased from Coast Scientific (San Diego) except peptide 3a, which was purchased from Multiple Peptide Systems (San Diego). All peptides were >97% pure. IC_{50} values were determined by linear regression determination of the *x* intercept value using data points derived from the initial slope of the inhibition curve. Peptides 3d and 3e have had glycine residues added for synthetic considerations. Peptide 3ds is a scrambled version of peptide 3d. Free amino acids were assayed under isotonic buffer conditions.

Addition of the active peptides after infected cells had already adhered to the target cells resulted in the release of ≈60% of the *Plasmodium*-infected erythrocytes; however, the reversal of adhesion (i.e., addition of peptide for 30 min after the infected cells were already attached to the melanoma cells) was always less than the degree of inhibition observed when the peptide was present during the initial 90 min of incubation (Fig. 3*B*).

Although the topographical features shared by the active peptides based on exofacial loops 3 and 7 are not immediately apparent, some common features do exist (Fig. 4). It would appear that the amino acid side chains contain the necessary topographical features, since peptides synthesized in the reverse sequence [i.e., 3d (GHPLQKTY) vs. 3e (YTKQLPHG)] were equally effective in blocking adhesion.

To determine whether both composition and sequence were critical factors that affected the capacity of a peptide to

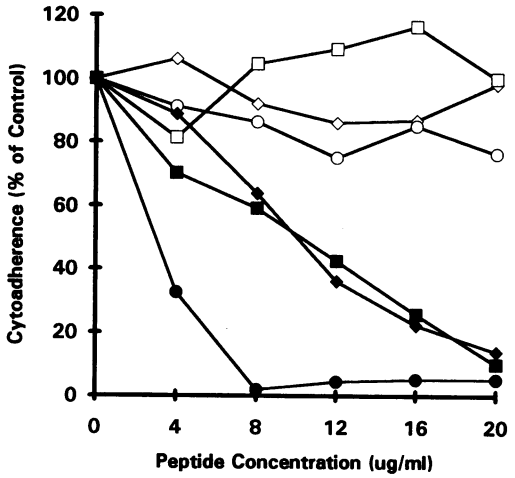

FIG. 2. Inhibition of cytoadherence with synthetic peptides. Synthetic peptides were added to the incubation medium of a cytoadherence assay (9, 18). Human erythrocytes were infected with the isolate FCR-3. The peptides used were 3ds (◇), 4 (○), 3c (□), 3a (■), 7a (◆), and 7b (●). Results are the average of duplicate determinations.

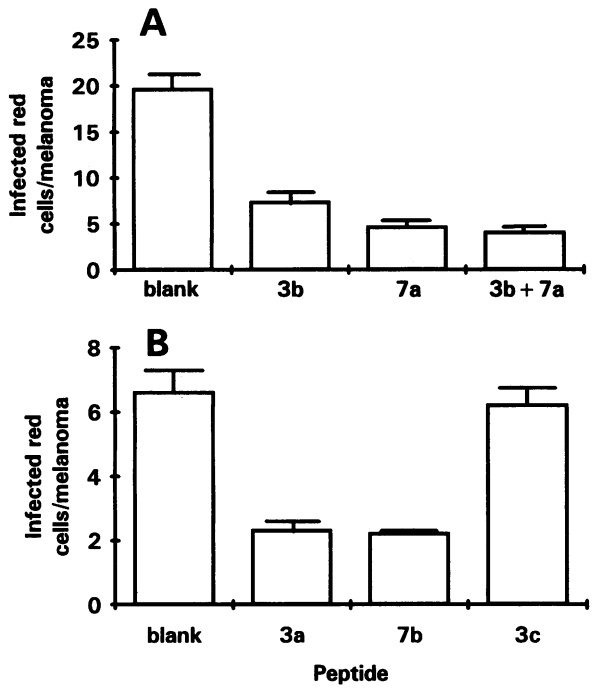

FIG. 3. (*A*) Effect of combining peptides from two adhesive regions. Peptides 3b and 7a were added to a final concentration of 15 μM either individually or in combination (3b+7a contains each peptide at a concentration of 15 μM). The blank sample does not contain either peptide. (*B*) Reversal of cytoadherence by the addition of peptides. Infected erythrocytes were allowed to adhere to amelanotic melanoma cells for 90 min before peptides were added at 20 μg/ml (≈10 μM); incubation was then continued for 30 min. Results are expressed as the mean of triplicates ± SD. The control (100% value) was seven infected cells per melanoma cell.

block adhesion, two peptides containing residues thought to be critical to binding were assayed. Peptide 7e (a sequence found in native band 3 and with lysine, tyrosine, and histidine residues) and peptide 3ds (a scrambled version of 3d that also contains these three amino acids) were both without inhibitory activity (Table 1). This suggests that the order of the amino acid residues is critical to the adhesion-blocking activity of the peptide.

Inhibition of Sequestration. To determine whether the synthetic peptides that were able to block cytoadherence *in vitro* were capable of affecting sequestration, peptides were administered intravenously to *P. falciparum*-infected *Aotus* and *Saimiri* monkeys.

An initial experiment using *Aotus* monkeys involved infusion of peptide 3d at two levels (Fig. 5 *A* and *B*). Since a previous report using hyperimmune serum had indicated that release of mature forms into the peripheral circulation might occur <2 hr after infusion (23), the parasitemia of the peripheral blood was followed during this time period. With the exception of peptide 3d at the higher dose (9 mg per animal), no significant effect on sequestration was seen during this time period. However, a significant percentage of mature forms were observed ≈24 hr after infusion of peptides at both dosages (Fig. 5). The effect of peptide infusion on parasitemia is indicated in Fig. 5*E*.

A second trial was conducted using splenectomized *Aotus* infected with *P. falciparum*. The results of the second trial were similar to the previous experiment (Fig. 5 *C* and *D*). The effect of a second infusion 24 hr after the first was determined by using one animal. The infused peptides for this animal were 3d (at 0 hr) and 3e (at 24 hr), which are the reversed sequence pair (Fig. 5*D*).

Intravenous infusion of peptides into *Saimiri* monkeys with *P. falciparum* infections gave similar results (i.e., there was a dramatic increase in the number of mature-stage parasites in the peripheral circulation ≈24 hr after the injection of peptide 3a) (Fig. 6). All four animals (especially the splenectomized ones) presented a marked autoagglutination of the erythrocytes bearing trophozoite and schizont stages of the parasite (Fig. 7).

No ill effects were observed in any of the animals after peptide infusion.

DISCUSSION

Based on the foregoing results with synthetic peptides, as well as the ability of monoclonal antibodies prepared against *P. falciparum*-infected erythrocytes to react with the surface of live infected cells (6, 24), it appears the human band 3 protein contains at least two exofacial regions that can serve as putative adhesins; we presume that these regions are cryptic in the uninfected erythrocyte and become exposed in the *P. falciparum*-infected erythrocyte upon parasite maturation.

Earlier work from our laboratory has shown that monoclonal antibodies that block cytoadherence also recognize modified forms of band 3 protein in *P. falciparum*-infected erythrocytes (6, 24). It is conceivable that an exhaustive examination of all sequences found in the human band 3 protein would identify putative adhesive regions; however, this tedious and time-consuming evaluation has not been undertaken to date. Rather we elected to restrict our analysis to those residues that are potentially exofacial regions of the band 3 molecule and that react with anti-falciparum monoclonal antibodies that block cytoadherence (6, 24). The adhesive sequences of band 3 involve amino acids 546–555 and amino acids 821–834. Synthetic peptides based on these sequences were able to inhibit the adhesion of *P. falciparum*-infected erythrocytes *in vitro* and to affect sequestration.

Iodination of the active peptides resulted in a loss of inhibitory activity; this suggests that the tyrosine residue is near, or in, the binding region and that the introduction of a bulky iodine atom onto this residue is not tolerated by the receptor molecule. The tripeptide YVK (7d) was inhibitory at a concentration 1000 times less than tyrosine in solution alone (Table 1), indicating that more than a single residue is required to block binding.

Peptide 3b (which was prepared with an accidental substitution of a lysine for an aspartic residue at position 546) had an IC_{50} value of 7 μM, a value not significantly different from the aspartic-containing peptide 3a (9 μM). This implies that the residue at position 546 is probably not critical to the adhesin–receptor interaction.

Since reversing the order of the residues (i.e., peptide 3d vs. 3e) did not result in a significant loss of activity, the peptide backbone appears not to participate to a significant extent in the adhesin–receptor interaction. All the active sequences we used contained as a minimum the subsequence YXK; however, because of the pH-sensitive nature of adherence (16, 17), we assume that a positive charge on another residue may also be necessary (His-547 and Lys-829?) for

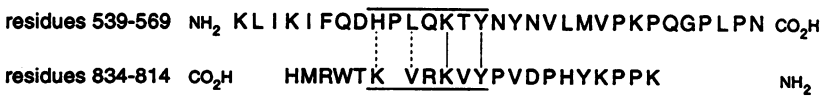

FIG. 4. Sequence comparison of exofacial loops 3 and 7, which include regions involved in adhesion blocking. Identity is denoted by a solid vertical line, and conservative substitutions are indicated by a dashed line. The minimal active sequences are bracketed by the horizontal lines.

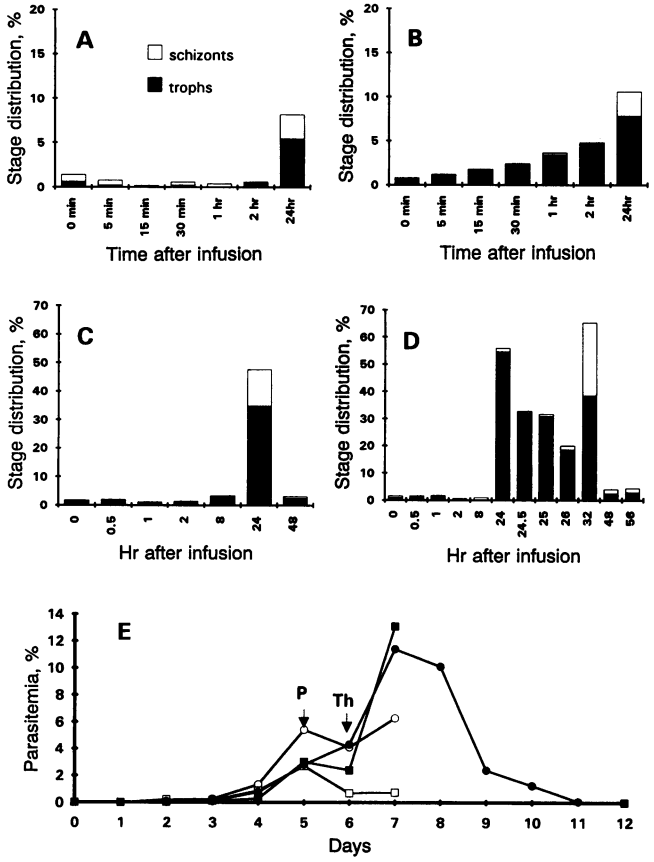

FIG. 5. *In vivo* action of peptides: appearance of mature forms in the peripheral blood after infusion of peptide into intact and splenectomized animals. *Aotus* monkeys infected with *P. falciparum* FVO/DNAX were intravenously injected with peptide suspended in phosphate-buffered saline. Blood smears were made at intervals, and the distribution of the developmental stages of the parasite was determined. The *y* axis of *A–D* indicates the percentage of infected cells that contained rings (not shown), trophozoites (trophs), or schizonts (rings + trophozoites + schizonts = 100%). Peptide 3d was infused at 1 (*A*) or 9 (*B*) mg per animal into monkeys with an intact spleen. (*C* and *D*) Ten milligrams of peptide 3d was administered at 0 hr to splenectomized animals. In *D*, a second infusion of peptide (using peptide 3e) was given at 24 hr to determine whether an additional dose would produce desequestration. (*E*) Parasitemia of animals inoculated on day 0 with *P. falciparum*. On day 5, they were injected with peptides, and on day 6 they were treated with quinine and mefloquine. □, 1 mg of peptide 3d to intact animal (from *A*); ○, 9 mg of peptide 3d to intact animal (from *B*); ■, 10 mg of peptide 3d to splenectomized animal (from *C*); ●, 10 mg of peptide 3d to splenectomized animal (from *D*). P denotes administration of peptide; Th denotes administration of anti-malarial therapy. The isolate FVO/DNAX normally produces increasing parasitemia until death results.

binding. Indeed, short peptides that included a second protonatable residue were more effective in blocking adhesion than peptides lacking such a residue. It is interesting to note that there are three residues between His-547 and the YXK sequence (residues 553–551) but only two between Lys-829 and its YXK sequence (residues 824–826); however, the physical distance between the positive charge and the YXK sequence may be very similar due to the length and flexibility of the lysine residue's side chain and the relative compactness of the Pro-His pair.

Although peptides from two regions of band 3 protein block adhesion, it appears that the cytoadherent behavior of *P. falciparum*-infected erythrocytes is primarily due to the exofacial adhesive region found in a part of loop 3. We base this on the observation that the pH dependence curve of

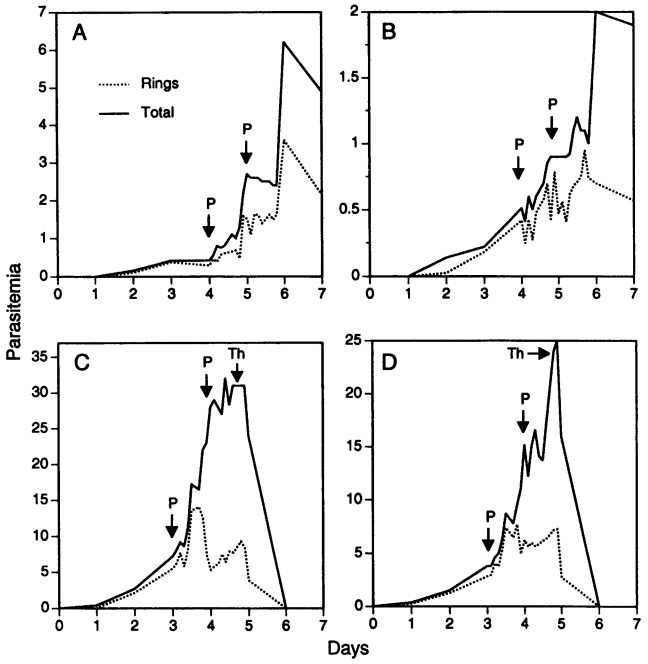

FIG. 6. *In vivo* action of peptide 3a in intact and splenectomized *Saimiri* during the *P. falciparum* infection (IPC/RAY isolate). *Saimiri* were infused with peptide (3 mg per animal) on the days indicated. The difference between the total parasitemia and that of the ring-stage parasitemia indicates the appearance of mature-stage parasites in the peripheral circulation. (*A* and *B*) Intact animals. (*C* and *D*) Splenectomized animals. P denotes administration of peptide; Th denotes administration of antimalarial therapy.

cytoadherence is consistent with a protonated histidine residue being a component of the adhesive site (16, 17) and only the adhesive region on loop 3 contains such a residue.

In vivo, the appearance of mature forms in the peripheral circulation differs from the <2-hr appearance of such forms after infusion of hyperimmune serum (23). If there was direct competition between the infused peptides and the surface adhesin of infected erythrocytes, then mature forms of the parasite should have been observed in the peripheral circulation shortly after the peptide was administered, as was the case with hyperimmune serum. However, after peptide infusion, a delay of ≈24 hr in the appearance of mature forms was seen. The precise mechanism underlying this delay is not known.

The delay may result from the peptides being infused at a dosage too low to produce the release of infected erythrocytes already bound to the postcapillary venules (= desequestration). Only at high doses was release of mature forms over a short term observed (Fig. 5*B*); this may represent a dose sufficient to initiate desequestration. In all the other *in vivo* experiments, the dose of peptide may have been sufficient to occupy receptor sites on the endothelial cell, thereby preventing attachment; however, the peptide concentration was below the level needed to achieve desequestration through direct competition. If this was the case, then the peptides would not only have to persist in the circulation long enough to occupy the receptor sites, but such endothelial sites would have to be occupied for 12–24 hr. A consequence of this would be that after peptide infusion the next generation of parasites (trophozoites/schizonts) would accumulate in the peripheral circulation. This was what was typically observed.

A second possibility for the delay in appearance of mature forms in the peripheral blood is that the peptides may affect the parasite at the ring stage of development, and this results in nonadherent or altered mature forms. However, when

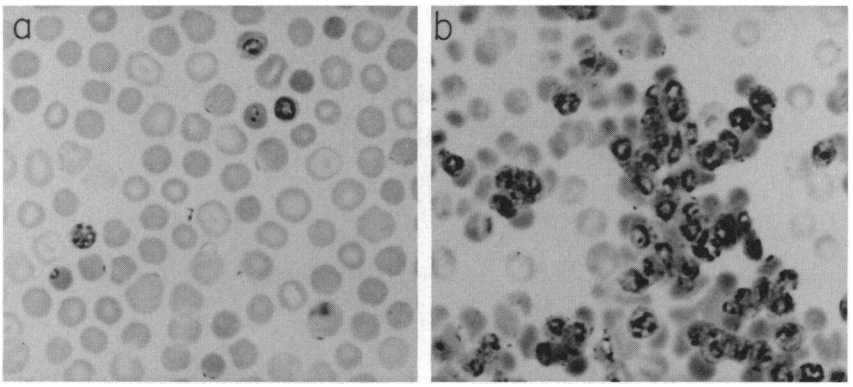

FIG. 7. Appearance of peripheral blood in infected *Saimiri*. (*a*) Preinfusion. (*b*) Approximately 24 hr after peptide infusion.

peptides were added to *in vitro* cultures of parasites, there was no effect on the growth and development of the parasites, and no toxic effects were seen (I.C., unpublished observation). Since the peptides had a clear effect on the adhesin–receptor interaction *in vitro*, it suggests that the effects observed from *in vivo* infusion of peptides result from a direct disruption of the interaction of adhesin with receptor.

A third possibility is that the introduction of free peptide into the circulation affects the number of receptor molecules expressed on the endothelial cells lining the postcapillary venules (i.e., the presence of the peptide causes depletion of an endothelial receptor). As of yet, there is no evidence to support or refute this.

We cannot rule out the possibility that the immune system of the animal is involved in the desequestration observed after infusion of the peptides, but the short latency period (24 hr) appears to be too brief for an antibody response. However, it is interesting to note that the mature forms in the peripheral blood autoagglutinate.

Inhibition of adherence/sequestration was observed for several isolates of diverse geographic origin (The Gambia, Vietnam, and French Guiana); this indicates that band 3-related adhesins are not isolate specific but are common to many or all *P. falciparum* isolates. Indeed, before we attempted *in vivo* experiments, blood smears of infected *Aotus* and *Saimiri* were examined for reactivity with the monoclonal antibody 1C4 and were found to be positive. Previously this antibody was found to react with the human band 3 protein (24), and this antibody recognizes every *P. falciparum* isolate on which it has been tested (24).

Putative receptors on the endothelial cell for the *P. falciparum*-infected erythrocyte have been reported to be CD36, intercellular adhesion molecule 1, and thrombospondin (4, 25, 26). The identity of the receptor for the band 3-related adhesin is not known; however, if, as has been suggested, multiple adhesive mechanisms are involved in sequestration, then the receptor affected by synthetic peptides based on band 3 motifs must constitute a major component of adhesion/sequestration, since peptide infusion is sufficient to result in large-scale appearance of mature forms in the peripheral circulation. Given the CD36-preferring nature of the FCR-3 line, it is likely that at least for this line the receptor for the band 3-related adhesin is CD36. Indeed, in a preliminary trial with a rat anti-idiotype to monoclonal antibody 4A3 [which recognizes the surface of *P. falciparum*-infected cells and partially blocks cytoadherence (6)], we found it to react with CD36 immobilized on plastic, but not with intercellular adhesion molecule 1 or infected erythrocytes.

Finally, since low concentrations (in the micromolar range) of peptides block adherence, it may be possible to develop mimetics of these peptides that can serve as an antiadhesion therapy for cerebral malaria.

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