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Membrane knobs are required for the microcirculatory obstruction induced by *Plasmodium falciparum*-infected erythrocytes

(malaria/parasitology/rheology)

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ABSTRACT **We have studied the pathophysiology of the vascular obstruction induced by *Plasmodium falciparum*-parasitized erythrocytes with the use of an *ex vivo* microcirculatory preparation perfused with red cells infected with knobless and knobby clones of the FCR-3 strain. We find that parasitized erythrocyte membrane knobs are indispensable for the generation of the circulatory obstruction. Uninfected erythrocytes incubated in culture and erythrocytes infected with early or late forms of the knobless clones or the early forms of the knobby clone all failed to obstruct the microcirculation, although exhibiting various effects on bulk viscosity and peripheral resistance during flow. In contrast, late forms of the knobby clone produced significantly higher peripheral resistance during flow and significant obstruction as detected by changes in time of pressure flow recovery as well as by direct videorecorded microscopic observation. Optical and electron microscopy showed that the adherence of parasitized cells to the endothelium was limited to the venules and involved the knobs in junctions. In addition, we were able to follow the sequence of events during obstruction: initial red-cell adherence to the venular endothelium (sometimes only transitory) followed by progressive recruitment at the venule surface, finally leading to total obstruction that involved parasitized and nonparasitized erythrocytes. Sometimes, retrograde aggregation would extend the obstruction to the capillaries or even precapillary arterioles. These results show that knobs are necessary and sufficient to produce vascular obstruction and that other factors (spleen, immunological, etc.) can only have a modulating role. These results also exclude the possibility that the exclusive adherence to venules is the consequence of "plasma factors" found in the malaric patients.**

The infection caused by *Plasmodium falciparum* produces the most severe form of human malaria (1) and the potentially lethal course of this infection is due, among other factors, to complications caused by sequestration of parasitized red cells in small vessels. The presence of masses of aggregated erythrocytes in the microcirculation may lead to hemorrhage and necrosis in the perivascular areas of the brain, myocardium, liver, intestinal mucosa, skin, and other organs (2). This process is accompanied by profound anoxia which can result in shock and death (3).

The mechanism of the *P. falciparum*-induced microvascular obstruction is not clearly understood; both the capability of malaria parasite-infected erythrocytes to produce aggregates through cell–cell interactions (4) as well as parasitized erythrocyte–endothelial cell interaction (5, 6) have been suggested as mechanisms for the vascular entrapment of parasitized cells. Knobs found in the membranes of malaria-infected erythrocytes (7) have been proposed as the specific structures involved in both possible mechanisms. Decreased red cell deformability secondary to increased viscosity of parasitized red cells may also play a role in obstruction (8).

The work presented here attempts to dissect the differential contribution of erythrocyte membrane knobs, decreased deformability, and aggregation (clumping of parasitized and nonparasitized cells) to the pathophysiology of the microcirculation obstruction induced by *P. falciparum* malaria infection. Our experimental design makes use of an isolated vasculature preparation of the rat mesoappendix [Baez preparation (9)] artificially perfused with *P. falciparum*-infected erythrocytes bearing either the knobby or knobless clone of a Gambian isolate (FCR-3).

We find that the presence of knobs is indispensable for the blockage of the microvasculature by mature parasites. We also find that increased erythrocyte viscosity may be a contributing but not a primary factor in the induction of the obstruction. Finally, the occluding aggregation or clumping of erythrocytes is an event secondary to the knob-mediated red-cell attachment to the endothelium.

MATERIALS AND METHODS

Malaria Parasites. *Culture conditions.* The FCR-3 strain of *P. falciparum*, as well as the knobby [A2 (K+)] and the knobless [D4 (K−)] clones of FCR-3 were kindly provided by W. Trager (10) and maintained in our laboratory in 5% erythrocyte suspensions under the candle-jar conditions (11). For the perfusion experiments, parasites were cultivated synchronously in 1% red cell suspensions (12) to obtain a high proportion of parasitized erythrocytes.

Preparation of cells. Different levels of parasitemia were obtained by gelatin sedimentation (13) of the FCR-3 strain and used for the viscosity and hemodynamic studies. As a control, we used uninfected cells that were maintained in culture and exposed to the same manipulations. These cells were also used to dilute the parasitized cells to obtain intermediate parasitemias. The knobby and knobless clones for hemodynamic studies were obtained directly from cultures to avoid the manipulations involved in the gelatin sedimentation. In all studies, erythrocyte suspensions were washed once in culture medium without serum, followed by two washes in nonbicarbonate Ringer's solution (154.3 mM NaCl/5.63 mM KCl/2.16 mM CaCl/0.6 mM $MgCl_2$) containing 0.5% (for viscometry) or 1.5% bovine serum albumin (for hemodynamic studies), and finally resuspended in Ringer's solution.

Viscometry. Viscosity measurements were made at 37°C on a Wells–Brookfield microcone-plate viscometer (model LVT, Brookfield Engineering Laboratories, Stoughton, MA). Cone and cup were calibrated frequently with a silicone oil standard [viscosity 5 centipoise (cP; 1 P = 0.1 Pa·sec)]. The sample (1.10 ml) was pipetted into the center of the cup and viscosity measurements were taken in duplicate at shear rates ranging from 11.5 to 230 sec^{-1}.

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Abbreviations: PRU, peripheral resistance unit(s); t_{pf}, pressure/flow recovery time.

Hemodynamic Measurements. Perfusion studies were performed in the isolated, acutely denervated, artificially perfused rat mesoappendix (mesocecum) vasculature according to the method of Baez (9) as modified for the study of erythrocytes (14). Briefly, in anesthetized (sodium pentobarbitol, 30 mg/kg) 120–150 g Wistar rats, the right ileocolic artery and vein were cannulated with heparinized (100 units of sodium heparin/ml of normal saline solution) silastic tubing at a site 3 cm from the ileocolic junction. Under steady-state perfusion with Ringer's solution containing 1.5% bovine albumin, the ascending colon and terminal ileum (3 cm each) were sectioned between ties. After hemostatic ties for all vascular connections were achieved, the tissue was isolated. The isolated mesoappendix was gently spread on a translucent Lucite block on a special microscope stage.

Initially, the vasculature was perfused for 12–15 min to allow stabilization of the tissue and clear the vessels of the remaining blood cell elements of the host animal. For observation and measurements, we used a triocular Bausch and Lomb microscope equipped with a television camera and video monitor (Sony). Simultaneous videotaping of vaso-occlusive events was carried out with a Panasonic videorecorder (Model NV9300A).

Arterial perfusion pressure (P_a), which was rendered pulsatile (310 cycles/min) with a peristaltic pump, and venous outflow pressure (P_v) were monitored with Statham–Gould P-50 transducers. P_v was kept at 3.8 mm Hg (1 mm Hg = 133 Pa) and the venous outflow rate (F_v) was monitored with a photoelectric drop-counter and expressed in ml/min. After control measurements of P_a and F_v, a bolus of erythrocyte suspension (control or parasitized) was gently delivered through an injection port 15 cm distal to the site of arterial cannulation, and the changes in P_a and F_v were recorded. Peripheral resistance units (PRU) were determined as described (15) and expressed in mm $Hg \cdot ml^{-1} \cdot min^{-1} \cdot g^{-1}$. PRU = P/Q, where P is the arteriovenous pressure difference and Q is the rate of venous flow (F_v) per gram of the tissue. Hemodynamic parameters (i.e., P_a and F_v) were recorded on a physiograph (model DMP-4B, NARCO Bio-systems, Houston, TX).

In each experiment, pressure flow recovery time (t_{pf}) was determined following the bolus infusion of samples. t_{pf} is defined as the time (sec) required for P_a and F_v to return to their base-line levels after the delivery of the bolus sample. t_{pf} represents total transit time through the mesoappendix vasculature.

Electron Microscopy. The rat mesoappendix was fixed by overnight immersion of the tissue in Karnovsky fixative (16), followed by three washes over the next 24 hr with 0.1 M cacodylate buffer (pH 7.4) containing 1% sucrose. Areas of the vasculature with large numbers of entrapped cells as well as areas with apparently clean vessels were dissected under a stereoscopic microscope and selected for processing for electron microscopy.

Tissue was post-fixed with 1% osmium tetroxide/0.1 M cacodylate buffer (pH 7.4) for 1 hr followed by 1% uranyl acetate for 1 hr, dehydrated in a graded series of ethanol solutions followed by propylene oxide, and embedded in LX112 resin (Ladd Research Industries, Burlington, VT).

Thick (0.5-μm) sections were stained with 1% toluidine blue to select cross-sections of blocked and empty vessels for thin sectioning. Thin (80-nm) sections were cut using an LKB III Ultramicrotome, stained with uranyl acetate followed by lead citrate, and viewed in a JEM 100CX electron microscope (Japan Electronic Optics Laboratory USA) at an accelerating voltage of 80 kV.

RESULTS

Viscosity Measurements. Control (uninfected) erythrocytes maintained in culture for 0–6 days underwent a gradual but small increase in viscosity starting at 24 hr. After 6 days in culture, the increase in viscosity from initial values was 15.5 and 18.0% at shear rates of 230 and 23 sec^{-1}, respectively. Fig. 1 depicts the influence of varying degrees of parasitemia (schizont stage) on bulk viscosity at a shear rate of 230 sec^{-1} and constant hematocrit (30%). In each case, increasing the percentage of parasitized cells resulted in a progressive elevation of the bulk viscosity.

Hemodynamic Observations. In these experiments, the isolated mesoappendix vasculature was first perfused with Ringer's solution at a P_a of 80 mm Hg, followed by a bolus infusion of infected or uncloned FCR-3 infected red cells (hematocrit 30–40%). Table 1 shows the resulting changes in PRU along with t_{pf}. In the first three experiments (hematocrit 30%), the infusion of parasitized cells (38–50% parasitemia) caused a significant increase in PRU as compared to the control cells. The passage of control cells was followed by complete recovery of pressure and flow, whereas the passage of parasitized cells was accompanied by delayed or partial recovery of pressure and flow. The partial recovery and prolonged t_{pf} indicates microvascular blockage by parasitized cells, an event confirmed by simultaneous television monitoring and video-replay analysis.

Next, we compared hemodynamic changes caused by knobless and knobby clones of parasitized cells (schizont stage) at 30% parasitemia and 40% hematocrit (Table 1, Exp. 4). The infusion of the knobless strain induced 42.7% higher PRU but only a slightly longer t_{pf} than the control infusion. In contrast, the erythrocytes parasitized with the knobby clone caused pronounced hemodynamic changes, evidenced by a 79.2% increase in PRU and only partial recovery of pressure and flow. In addition, other experiments done exclusively with knobless strain (3 experiments) or knobby strain (6 experiments) confirm these results entirely.

Direct Observation of Microcirculatory Flow and Electron Microscopy. Perfusion with uninfected human erythrocytes or with cells parasitized by the knobless clone or by ring stages from either the knobby or the knobless clone was characterized by eventual complete clearance of the red cells from the vessels. Careful examination of the microcirculation showed that no cells remained attached to the endothelium.

When schizont-stage parasites (containing some trophozoite forms) of the knobby clone were perfused, blockage of the vessels was observed, resulting in slow flow. Fig. 2 depicts the events following the injection of a bolus of infected

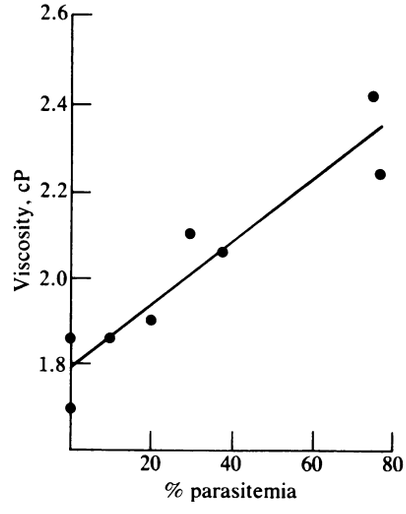

FIG. 1. Bulk viscosity versus percentage of parasitized red cells (FCR strain). Shear rate was 230 sec^{-1}. The regression line fits the equation $y = 1.791 + 0.0072x$; $r^2 = 0.893$, $P < 0.0001$.

Table 1. Perfusion experiments with *P. falciparum* (A2 K+ clone)-parasitized cells

Exp.	Cells	% parasitemia	PRU, mm $Hg \cdot ml^{-1} \cdot min^{-1} \cdot g^{-1}$		% PRU increase	t_{pf}, sec
			Ringer's	Cells		
1	Control		9.45	12.7	34.4	120
	Parasitized (FCR-3 uncloned)	50	9.83	25.7	161.7	Partial recovery
2	Control		17.27	25.0	45.0	65
	Parasitized (FCR-3 uncloned)	38	18.80	49.8	180.0	Partial recovery
3	Control		10.9	15.4	41.2	55
	Parasitized (FCR-3 uncloned)	41	12.7	23.1	82.0	90
4	Control		4.43	5.08	14.7	50
	Parasitized knobless clone	30	4.75	6.78	42.7	60
	Parasitized knobby clone	30	4.80	8.60	79.2	Partial recovery

Exps. 1, 2, and 3 were done with parasites grown in culture for 96 hr and concentrated through a gelatin-sedimentation procedure. Exp. 4 was done with parasites obtained directly from culture after 96-hr incubation. Isolated preparations obtained from different animals normally show variations in the venous outflow rates due to anatomical and vascular topographical variations. These differences are reflected in PRU values for Ringer's solution.

erythrocytes. The Ringer's solution-perfused microvasculature exhibits clear vessel lumens (Fig. 2*A*). Following the infusion of infected blood, a fast normal flow was observed in arterioles and a slow flow in venules, with some cells becoming attached to the vessel walls (Fig. 2*B*). Not all the cells that initially adhered to the vessel wall remained attached. Some cells were observed detaching and rejoining the flow. Nevertheless numerous cells remained permanently attached to the endothelium even under the continuous flow of Ringer's solution (Fig. 2*D*).

The specificity of venular attachment (5, 17) was confirmed by scanning the preparation. An example is shown in Fig. 2*E*, where a venule exhibits several cells attached to the walls, while the arteriole remains clear. A more striking feature is the complete blockage at several levels of the vessel as seen in Fig. 3.

Single cells found attached in otherwise clear vessels were always found to be parasitized (Fig. 4*A*). Furthermore, the sites of interaction between the endothelial cell and the parasitized erythrocyte were always at the knobs (Fig. 4*B*). In vessels where areas of complete blockage were found, nonparasitized cells were also observed entrapped, without any morphological interaction with the endothelium (Fig. 5*A*). In contrast (Fig. 5*B*), when knobs were present, junctions (6) were found (arrows), accompanied many times by a discontinuous membrane structure of the endothelial cell. These characteristics were observed even when early trophozoites with less developed knobs (Fig. 5*C*) attached to the endothelial cells.

The morphology of the rat mesoappendix (18, 19, 20) was studied by fixing the intact vasculature, both *in situ* and after perfusion with Ringer's solution, and examining it with the electron microscope. Rat erythrocytes were seen in the lumen of the vessels in the intact mesenterium. These cells were removed by perfusion with Ringer's solution, without causing any morphologically detectable alteration of the vasculature. Normal morphological features of the endothelium were found in all the cases where the perfused cells did not show attachment to the endothelial cells (data not shown).

In certain occluded vessels, effects that resemble the damage of anoxia were detected. Altered endothelial cells and abnormal collagen fibers, associated with the extracellular matrix of these vessels, were observed (data not shown).

DISCUSSION

To date there has been no conclusive evidence of the exact role of the membrane knobs observed in the late stages of *P. falciparum*-infected red cells. There is, nevertheless, a long-standing belief, based on morphological observations, that these knobs are active participants in the attachment to the endothelium during the deep-vascular, schizogonic phase of

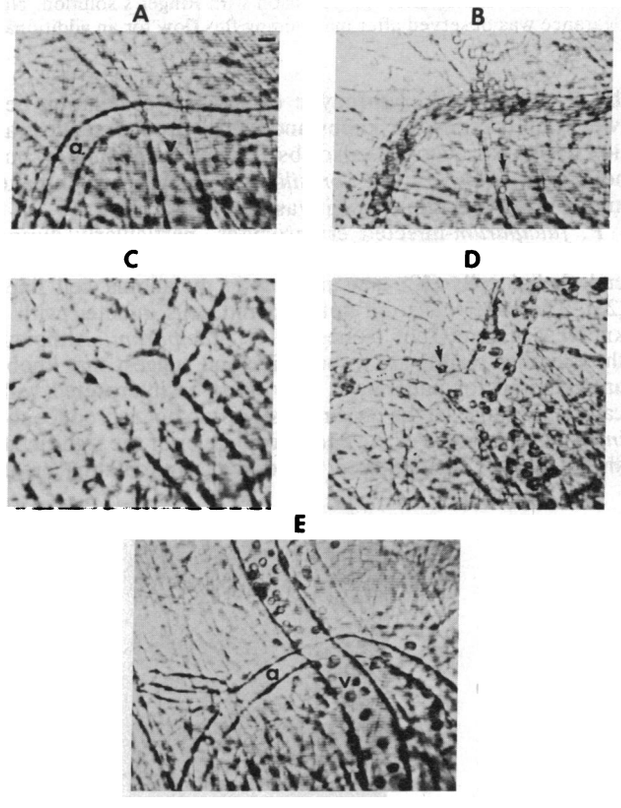

FIG. 2. Flow properties of erythrocytes infected with *P. falciparum* clone A2 (K+). (*A*) Clear vessels (a, arteriole; v, venule) seen during perfusion with Ringer's solution. (*B*) During perfusion with infected erythrocytes a fast flow is seen in the arteriole (no individual cells could be identified), whereas the venule shows a slow flow. Short arrow points to a cell that initially adhered but that later rejoined the flow. The long arrow points to a cell permanently adhered. (*C* and *D*) Venules seen before (*C*) and after perfusion (*D*), respectively. Numerous cells (e.g., arrow) remain attached to the walls even under the continuous perfusion of the Ringer's solution. (*E*) Venule (v) and arteriole (a) after perfusion with parasitized erythrocytes. The attachment is limited exclusively to venules. [Bar = 20 μm (see *A*)].

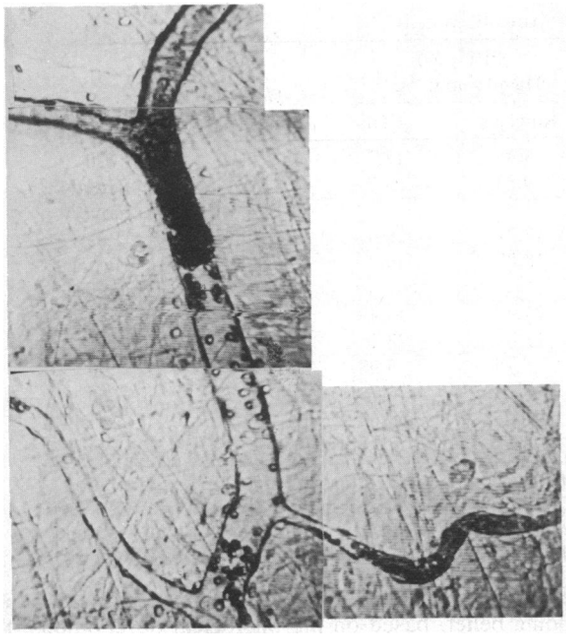

FIG. 3. Microcirculatory obstruction by *P. falciparum* (A2 K+ clone)-infected erythrocytes. Two areas of complete obstruction found in the venules after the perfusion with Ringer's solution. No clearance was observed after maintaining this flow for an additional 5 min.

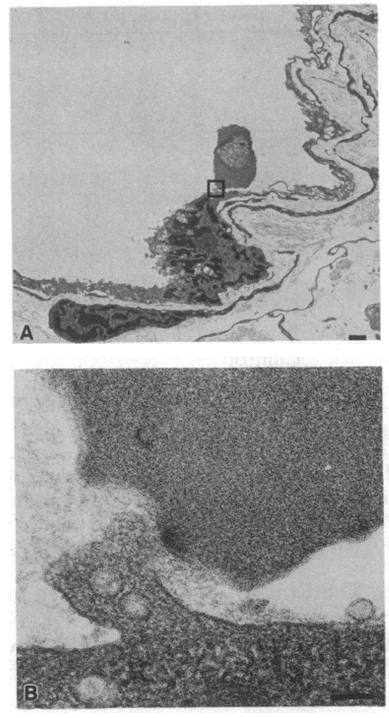

FIG. 4. Characteristics of individual cells attached to otherwise clear vessels. (*A*) Individual cells attached to the vessel walls were always parasitized. (Bar = 1.0 μm.) (*B*) Enlargement of the area within the square in *A*. Contact site showing a knob-mediated interaction between the endothelial cell and the parasitized red cell. (Bar = 0.1 μm.)

the *P. falciparum* erythrocytic cycle. The correlation between the presence of knobs and deep vascular sequestration during schizogony is also observed in *P. coatneyi* (4) but not in *P. malariae* and *P. brasilianum*, both of which have knobs (21) but do not exhibit vascular entrapment.

P. falciparum-infected erythrocytes, particularly during the late stages of development, adhere to cultured human endothelial cells (22) and amelanotic melanoma cell lines (23). The latter cells did not bind red cells infected with a knobless variant (not cloned) of *P. falciparum*. Although these earlier results are informative and suggest that knobs are required for adherence, they must be interpreted with caution because (*i*) the culture systems do not reproduce the *in vivo* architecture of the endothelial layer or the significant shear stresses encountered by red cells (circulating or attached) during blood flow and (*ii*) the results do not account for the *in vivo* specificity of the binding to the venules.

Through the use of cloned knobby and knobless strains of *P. falciparum*, we have determined that the knobs are indispensable for the attachment of parasitized erythrocytes to the endothelium of blood vessels. Other abnormalities, such as the increase in viscosity and consequently reduced deformability of parasitized erythrocytes [also observed by others (24)], may have only a secondary or contributing effect on the obstruction of the microcirculation. These abnormalities might, however, have a primary function in the spleen se-

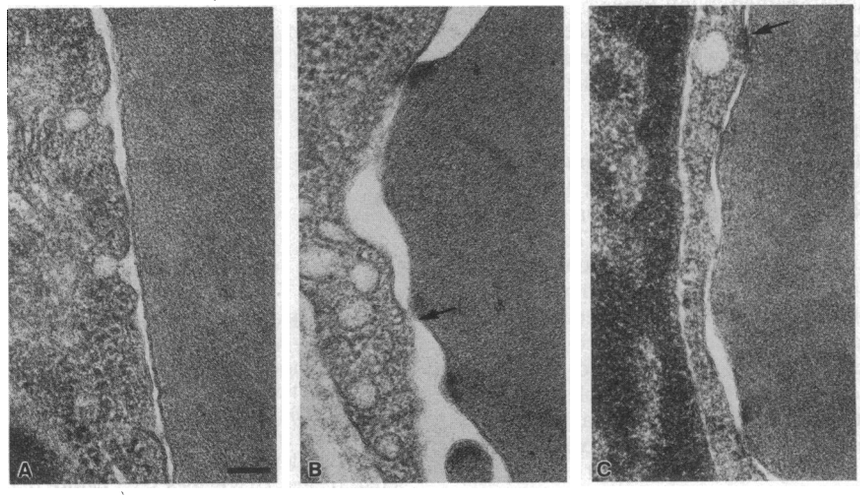

FIG. 5. Interaction between parasitized and nonparasitized cells in occluded vessels. (Bar = 0.1 μm.) (*A*) Noninfected erythrocytes show no direct interaction with endothelial cells and both cells retain their bilayer membrane structures. (*B*) Parasitized cells at late stage of development (trophozoites and schizonts) exhibit junctions (arrows) between endothelial cells and red-cell knob protrusions. (*C*) Less-developed knobs found in early trophozoites are also able to interact with the endothelial cell in a manner similar to that seen in *B*.

questration of parasitized erythrocytes (25).

The data presented here also show that the process of attachment of knobby late-stage parasitized red cells to the endothelium can take place without the help of the spleen, "plasma factors," specific antibodies, or other potential participants that can be found in the blood of malaria patients. Red cell knobs and a venular endothelium seem to be necessary and sufficient elements in the adherence phenomenon. Of course, these experiments do not exclude the possibility that splenic or plasma factors (26) could modulate the process by either enhancing or inhibiting the normal parasitized erythrocyte–endothelium interaction. This conclusion has to be understood to be in the context of "sticky" knobs, as mutants have been found in which anatomical knobs did not adhere (27).

Television monitoring and simultaneous videotaping during the perfusion of the Baez mesoappendix preparation with knobby, late-stage parasitized erythrocytes allowed us to record the sequence of events that characterizes the microcirculation obstruction observed with *P. falciparum*-infected red cells. First, a few cells became attached to the endothelium (Fig. 2*B*), some of which were subsequently dislodged under the shear stress of the blood flow. Erythrocyte attachment was restricted almost exclusively to the endothelial walls of venules. With time, a considerable number of cells became permanently adhered to the endothelium wall of some of the venules, an event that was followed by cellular aggregation which, in turn, ended in the complete obstruction of the microcirculation (Fig. 3). In some instances, by retrograde accumulation, the red-cell plug extended to the capillaries and even the precapillary arterioles. Electron microscopy of these plugs demonstrated, not unexpectedly, that the obstruction comprised a mixture of parasitized and non-parasitized erythrocytes.

Of considerable interest is the fact that the pattern of erythrocyte attachment in our *ex vivo* preparation perfectly mimicked observations made on autopsy material of human *P. falciparum* malaria (5, 17); the erythrocyte adherence and aggregation is almost exclusively limited to the venules. The mechanism for this selectivity is not known. Three possibilities need to be considered. The first is that red-cell attachment to the endothelial wall of the venules is due to the relatively low shear stresses and low flow rates characteristic of these vessels. Second, the venous endothelium may be the only one that has the specific binding sites (or receptors) for the *P. falciparum*-induced erythrocyte knobs. Finally, "factors" present in the malarial blood may inhibit or promote the binding of parasitized red cells so as to localize the interaction exclusively in the venules. Our data argue strongly against the last possibility. Further work is necessary to distinguish between the other alternatives.

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