Resealed ghosts of human RBCs, containing diluted cytosol, are susceptible to invasion by Plasmodium falciparum. If ATP is present, a dilution of up to about 30-fold, corresponding to an intracellular hemoglobin concentration of ~10 mg/mL, can be tolerated without total loss of susceptibility to invasion. Up to a dilution of about one-half this, the parasites also develop normally. When the cytosol is diluted by more than the critical amount, invasion of the resulting resealed ghosts falls off abruptly. If the diluent buffer is replaced by extraneous concentrated hemolysate, an indefinite dilution is possible without loss of invasion. There is thus an intracellular constituent, which must be present at a concentration above some critical level if the parasite is to enter the cell. The factor in question is not dialyzable. It is largely inactivated when the hemolysate is kept for ~1 day in the cold or for ~20 minutes at 45°C. The inability of a heat-treated hemolysate to support invasion is not due to the generation of inhibitory products, because such a solution can be used as a diluent of a fresh hemolysate without inhibition of invasion. When the inactivated hemolysate is present as a major component, however, the parasites fail to develop to the trophozoite stage. The invasion-linked factor remains in the strongly adsorbed nonheme fraction when a batchwise separation from hemoglobin on an anion exchanger is made and is thus probably acidic in character; the adsorbed fraction, recovered from the ion-exchanger, substantially restores capacity for invasion when sealed into ghosts. Its activity is destroyed by treatment with trypsin. The adsorbed fraction contains many proteins. When fractionated on a gel filtration column by fast liquid chromatography, active material eluted at a volume corresponding to a mol wt for a globular protein in the region of 10,000. A component of apparent subunit mol wt of 13,000 was observed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of this eluate fraction.

White ghosts prepared from simian RBCs do not support invasion by merozoites of Plasmodium knowlesi, even though the initial recognition process still appears to operate. It has, however, proved possible to prepare resealed ghosts that are susceptible to invasion by ensuring a sufficient ATP concentration in the internal solution. There are indications that the ATP is required for phosphorylation of membrane-associated proteins, possibly spectrin. To prepare resealed ghosts, we dialyzed RBCs against a hypotonic medium, including in the solution any solutes to be introduced into the cells; the dialysate is then restored to isotonicity and dialysis is continued at 37°C to bring about resealing. When ATP is present, the hemocrit of the original cell suspension can be reduced to 5% to 10% without eliminating the capacity for invasion of the resulting resealed ghosts; that is, a hemoglobin concentration of ~30 mg/mL inside the cell is sufficient to allow invasion and indeed development of the parasites. However, a dilution is eventually reached at which no invasion will occur. Thus a component in the cytoplasm, other than ATP, is evidently required, the concentration of which must not fall below some critical level, if the membrane is to remain capable of penetration by the merozoite. We report here the results of our attempts to establish the nature of this component.

MATERIALS AND METHODS

P falciparum was maintained in continuous culture in adult human RBCs. Schizonts were isolated by a procedure based on that of Saul and colleagues, involving centrifugation on a cushion of Percoll. Resealed ghosts, containing 1 mmol/L of magnesium ATP, were prepared as described previously. In brief, washed intact cells were suspended at the required hematocrit in a solution of the required composition and dialysed at 4°C for 2 hours against 100 vol of 5 mmol/L of phosphate buffer, pH 7.4, containing in addition any diffusible solutes present in the suspending solution inside the dialysis bag. The dialysate was then restored to isotonicity (0.15 mol/L of sodium chloride, 5 mmol/L of potassium phosphate), and dialysis was resumed for 45 minutes at 37°C. The resulting resealed ghosts were washed three times in culture medium (RPMI 1640, containing 10% vol/vol human serum), and were finally suspended at 30% hematocrit in the same solution. The hemoglobin content of the cells was determined by lysing an aliquot after the buffer wash with an equal volume of the 5 mmol/L of phosphate buffer and determining the absorbance at 577 nm, the millimolar absorptivity per heme being taken as 15.2. The Visking dialysis tubing was prepared by boiling in three changes of a solution of 0.1 g/L of sodium bicarbonate and 0.1 mmol/L of EDTA.

Undiluted fresh hemolysate was prepared by freezing and thawing packed washed cells three times and removing stromal material by centrifugation at 42,000 g for 20 minutes. Heat denaturation experiments were conducted by warming the hemolysate in a water bath at the desired temperature for 15 or 20 minutes and clarifying the solution by centrifugation as before. Small volumes were rapidly stripped of dialyzable components on centrifugal columns, packed with Sephadex G-25 in isotonic buffer. The columns (supplied by Pharmacia Fine Chemicals, Uppsala, Sweden) were 10 cm long, and contained ~1.8 mL of Sephadex. After centrifugation of the columns packed with Sephadex at 200 g in a bench-top centrifuge for 2 minutes, 0.1 or 0.2 mL of the hemolysate was applied to each column and brought through by a further centrifugation. Hemoglobin concentrations were determined spectrophotometrically, and ATP was determined by luciferin-luciferase assay, using an LKB Lumino-neter 1250 (LKB Instruments, Bromma, Sweden).

Batch-fractionation of the hemolysate was accomplished on diethylaminoethanol (DE) 52 cellulose (Whatman, Maidstone, UK) following the general procedure of Cheung and colleagues. The ion-exchanger, equilibrated with 10 mmol/L of sodium phosphate, pH 7.5, was added to a hemolysate (1 g to 5 mL), prepared by lysis of

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packed, washed cells with 2 vol ice-cold water, followed by centrifugation at 42,000 g for 20 minutes to remove stroma. The mixture was gently stirred for 30 minutes at 4°C and centrifuged at low speed. The supernatant, containing the hemoglobin, was removed, and the cellulose was washed with 5 mmol/L of phosphate buffer, pH 7.4. The adsorbed protein was liberated from the cellulose by addition of potassium chloride 1 mL of 0.5 mol/L/g of cellulose, followed by stirring for 1 hour on ice. The supernatant was collected after brief centrifugation and concentrated by passage through a centrifuge tube packed with dry Sephadex G-25. It was finally reequilibrated with isotonic phosphate-buffered saline (PBS), using the centrifugal Sephadex column, as above. A nominal protein concentration was obtained by the method of Bradford, and was generally ~1 mg/mL. This preparation could be stored frozen for some days without loss of activity.

A hemoglobin preparation, free of most minor cytoplasmic proteins, was also prepared by chromatography of centrifuged hemolyseate on a column (2.5 x 90 cm) of Sephadex G-75.

To examine the effect of proteolysis, trypsin (10 μg/mL final concentration) was added to the solution of nonheme protein, prepared as above. After 5 minutes at 37°, soybean trypsin inhibitor was added from a stock solution to give a final concentration of 100 μg/mL. As a control, samples were treated with a mixture of trypsin and trypsin inhibitor in the same final proportions.

The first attempt at further fractionation of the active nonheme hemolysate fraction made use of a triacetate ultrafiltration membrane (Sartorius Centrisart; Sartorius, Göttingen, FRG) with a nominal mol-wt cut-off of 20,000. To characterize and isolate the active material, fast liquid chromatography (Pharmacia FPLC) was used, with two Superose 12 columns (1 x 50 cm) in series. Samples of 0.5 mL were applied, and the columns were eluted with PBS, pH 7.4, at a flow rate of 0.7 mL/min. The column was calibrated with the following proteins as mol-wt markers: β-galactosidase, human immunoglobulin G, transferrin, bovine serum albumin (BSA), ovalbumin, trypsinogen, bovine β-lactoglobulin, soybean trypsin inhibitor, bovine pancreatic ribonuclease, horse heart cytochrome c, and bovine pancreatic trypsin inhibitor. Potassium nitrate was used as a marker for the included volume, and blue dextran (Pharmacia) was used for the void volume. Fractions of 1.5 mL were collected and stored frozen. The fractions were concentrated to 0.3 to 0.4 mL with dry Sephadex G-25, as described. For invasion assays, the material was incorporated into resealed ghosts: the hematocrit was chosen to give a low basal invasion level (Fig 1). Thus, lysis and resealing was done in a mixture containing 12 μL of packed cells (sampled with a positive-displacement pipette), 10 μL of 20 mmol/L of magnesium ATP, 100 μL of the concentrated fraction, made up to 200 μL with buffer; three or four replicate assays were performed on each fraction.

Gel electrophoresis with sodium dodecyl sulfate (SDS) was performed in vertical polyacrylamide slab gels in the system of Swank and Munke. which gives good resolution in the low-mol-wt range. The gels were stained with Coomassie brilliant blue or silver. Experiments were also performed with resealed ghosts containing added enzymes, ie, glyceraldehyde 3-phosphate dehydrogenase, glucose 6-phosphate dehydrogenase, aldolase, phosphofructokinase, catalase, carbonic anhydrase, and superoxide dismutase (Sigma Chemicals, St Louis). These were added to the suspending solution before reversible lysis at the concentration corresponding to that present in the normal RBC.

For invasion assays, purified schizonts (80% to 90% purity) were added to the RBC or ghost suspension; after incubation, parasitemia levels were determined by counting 10⁶ cells on thin smears, stained with Giemsa's solution. Invasion efficiency is expressed in terms of the invasion ratio, which is defined as the ratio of the proportion of parasitized cells at a given time to that at the time of inoculation (0 hours).

RESULTS

Figure 1 shows the dependence of invasion by merozoites on the dilution of the cell contents. ATP at 1 mmol/L, added as the magnesium salt, was present throughout. There was no major or consistent effect of 2,3-diphosphoglycerate or glutathione (GSH) on the invasion levels, although occasionally (presumably when some oxidation of membrane protein thiol groups had occurred) glutathione increased invasion perceptibly (data not shown). The profile of Fig 1 shows a rather abrupt decline of invasion at a critical dilution of the hemolysate, which varied somewhat from preparation to preparation, but always fell to zero at 20- to 50-fold. In the experiment shown (Figs 1 and 2), there was still detectable invasion at 30-fold dilution (97% diluent buffer in Fig 1), corresponding to an intracellular hemoglobin concentration of ~10 mg/mL. Under these conditions, the staining of the cytosol is so weak that phase contrast is required to see the cells clearly. At a 16-fold dilution (hemoglobin concentration ~20 mg/mL), the parasites continued to develop, and ring forms could be observed, whereas at higher dilution there was little indication of maturation (Fig 2).

The implication of the profile of invasion against dilution, seen in Fig 1, is that a cytosolic component other than ATP, diphosphoglycerate, or glutathione is required for invasion and that its concentration must exceed ~1/30 of its normal concentration in the circulating RBC. This factor may bind to the membrane and be progressively dissociated on dilution. To confirm that an intracellular factor exists, and that the decline of the capacity for invasion is not due to an irreversible change in the membrane, resulting from dilution, the same experiment was made with concentrated freshly prepared hemolysate in place of isotonic buffer. A dilution of
Fig 2. Infected resealed ghosts, viewed in phase contrast (original magnification ×1,500; current magnification, ×1,000). R, rings; T, trophozoites. (A) Ghost contents are cytosol diluted 16-fold with isotonic buffer (hemoglobin concentration ~20 mg/mL), 21 hours after inoculation; (B) the same 45 hours after inoculation; (C) ghost contents are cytosol diluted by a factor of 32 (hemoglobin concentration ~10 mg/mL) 21 hours after inoculation; (D) the same 45 hours after inoculation. Trophozoites are poorly developed in (D) as compared with (B).

1:1 of the fresh hemolysate (prepared by freezing and thawing of packed cells, followed by centrifugal removal of stromal material) was selected, being more effective in promoting invasion than the undiluted hemolysate. The results of such an experiment are shown in Fig 1, from which it is clear that there is no longer any decline of invasion at some critical dilution of the membranes during lysis and resealing. Thus, an essential constituent for invasion is indeed present in the cytosol. The slightly lower basal level of invasion when undiluted hemolysate was used was always observed and remains unexplained; we conjecture that it may be related to the effect of internal viscosity on the membrane properties.15

To establish whether the unknown component is a dialyzable factor or a macromolecule, the hemolysate was dialyzed for 24 hours against isotonic buffer containing 1 mmol/L of ATP. When this was used in a lysis and resealing experiment, the resulting cells were poorly invaded. However, a comparable loss of invasion capacity occurred when the hemolysate was simply allowed to stand at 4°C for a similar period. Thus, the factor in vitro is substantially inactivated within ~1 day in the cold. The activity could not be restored with 1 mmol/L of glutathione. A rapid method for removal of dialyzable solutes was therefore used. Small centrifugal columns packed with Sephadex G-25 proved satisfactory. About 100 μL of fresh concentrated hemolysate could be applied to each, and the hemoglobin was recovered, diluted by not more than 25%. As judged by analysis of the ATP content of the fresh hemolysate and the centrifugal eluant, removal of ATP and, by implication, of other dialyzable solutes, was complete. This procedure required only a few minutes. The resulting stripped hemolysate retained most of its capacity to restore the invasion of resealed ghosts (Table 1). We infer that the factor is macromolecular.

If the molecule in question is a protein, and is inactivated on standing in the cold, one might expect that it would be sensitive to denaturation generally. The hemolysate was therefore subjected to heat treatment. With increasing temperature, appreciable turbidity was apparent in the solution after dilution for spectrophotometry. Moreover, ghosts resealed with hemolysate, which had been treated at ≥50°C for 15 minutes and centrifuged, separated into two layers after sealing, the lower one darker than the upper. Thus, at high temperature, complicating factors arise. However, inactivation of the hemolysate, with respect to its capacity to restore susceptibility to invasion of sealed ghosts, was largely complete after heating at 45°C for 15 minutes (Fig 3, inset). Under these circumstances the hemoglobin was undamaged, as judged by its absorption spectrum, which was that of oxyhemoglobin, both in the visible and Soret regions, with no
indication of the formation of a hemichrome or of methemoglobin and in the appearance of a band at longer wavelengths.\textsuperscript{16} 

The fall in invasion could be interpreted in terms of heat-inactivation of the hemolysate or of the formation of a product of heating (possibly aggregated protein)\textsuperscript{17} that could damage the membrane. To test this, mixtures of fresh and heated hemolysates, matched for hemoglobin concentration, were made up and assayed for their activity in restoring invasion. Within the limits of experimental error, dilution of fresh with heated hemolysate caused no greater reduction of invasion than did dilution with isotonic buffer (Fig 3). We thus conclude that the active factor is destroyed by relatively mild heat treatment. A striking feature of these results, however, was that when the proportion of heated hemolysate was substantial, the parasites, though present in the cells in undiminished numbers, failed to develop to the trophozoite stage. Thus, heat treatment either reduced the concentration of a constituent required for parasite maturation to below some critical level or generated a species toxic to the intracellular parasite. Hemoglobin, freed of most cytoplasmic proteins, introduced into ghosts, together with magnesium-ATP, gave almost no invasion (Table 1 and Fig 4D).

We attempted to obtain an active fraction from concentrated fresh hemolysate by a batch separation on the anion exchanger, DE52 cellulose.\textsuperscript{9} The supernatant from this procedure had low activity with respect to restoration of invasion of resealed ghosts, whereas the adsorbed nonheme fraction, recovered from the ion-exchanger, showed significant activity, both when mixed with an inactivated hemolysate or with diluent buffer and then introduced into ghosts (Table 1 and Fig 4E and F).

As a further indication that the active constituent is a protein, samples were exposed to trypsin (10 \textmu g/mL for 5 to 10 minutes); the reaction was terminated by addition of an excess of soy bean trypsin inhibitor. Such treatment reduced the invasion of cells containing the preparation (with or without additional DE52-stripped hemolysate) by 50\% to 60\%. Controls, treated with a trypsin–trypsin inhibitor mixture, showed no such reduction.

To examine whether any of the common enzymes of the RBC cytosol might be implicated, glyceraldehyde 3-phosphate dehydrogenase, glucose 6-phosphate dehydrogenase, aldolase, phosphofructokinase, catalase, carbonic anhydrase, and superoxide dismutase were incorporated in the diluent buffer used to prepare resealed ghosts, at concentrations corresponding to those in the native cell.\textsuperscript{18} In no case was the efficiency of invasion affected (data not shown).

As a first step toward a characterization of the active factor, the active fraction from the anion exchanger was passed through a centrifugal cut-off filter, with a nominal mol-wt exclusion of 20,000 for globular proteins. The activity was recovered in the filtrate. A protein of low mol wt was thus indicated.

The active hemolysate fraction was applied to a Superose

### Table 1. Invasion of Resealed Ghosts Containing Different Hemolysate Fractions

<table>
<thead>
<tr>
<th>Contents of Ghosts*</th>
<th>Invasion Ratio†</th>
<th>No. of Experiments</th>
<th>Key in Fig 4†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilute Cytosol (16-fold dilution with buffer§)</td>
<td>0.29 ± 0.10 (SD)</td>
<td>16 A</td>
<td></td>
</tr>
<tr>
<td>Concentrated cytosol (extraneous hemolysate, diluted with equal volume buffer)</td>
<td>2.10 ± 0.93</td>
<td>13 B</td>
<td></td>
</tr>
<tr>
<td>Stripped hemolysate (as previous, but after removal of dialyzable solutes)</td>
<td>1.40 ± 0.16</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Heated hemolysate (fresh hemolysate, heated at 45°C and diluted with equal volume buffer)</td>
<td>0.73 ± 0.35</td>
<td>7 C</td>
<td></td>
</tr>
<tr>
<td>Unadsorbed fraction from DE 52 (diluted with equal volume buffer)</td>
<td>0.47 ± 0.08</td>
<td>6 D</td>
<td></td>
</tr>
<tr>
<td>Adsorbed fraction from DE 52 diluted threefold with heat-inactivated hemolysate</td>
<td>2.00 ± 1.1</td>
<td>4 E</td>
<td></td>
</tr>
<tr>
<td>Adsorbed fraction from DE 52 diluted threefold with buffer</td>
<td>1.05 ± 0.24</td>
<td>6 F</td>
<td></td>
</tr>
</tbody>
</table>

*RBCs were lysed and resealed at 6\% hematocrit in the specified solutions
†Invasion ratio is parasitemia after one invasion cycle (~24 hours) to that at inoculation (starting parasitemias in range 1\% to 5\%).
§Phosphate-buffered saline, containing 1 mmol/L of magnesium-ATP.

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**Fig 3.** Invasion ratio (ie, parasitaemia ~24 hours after inoculation, relative to that at time of inoculation) in resealed ghosts, containing fresh hemolysate, diluted with varying proportions of isotonic buffer (O) and with heat-inactivated hemolysate (O) (1 mmol/L of ATP present throughout). Inset: Effect of temperature of heating on activity of a fresh hemolysate with respect to its ability to restore susceptibility of resealed ghosts to invasion. The controls were resealed ghosts, containing a 16-fold dilution of hemolysate that had been incubated at 37°C for 15 minutes.
12 gel filtration column in an FPLC apparatus. The column was calibrated with a series of globular proteins (Fig 5, inset). Fractions were assayed for activity in promoting malarial invasion. They were first concentrated about fourfold and added to the buffer in which the resealed ghosts were prepared. A hematocrit of 6% (16-fold dilution of cell contents after lysis and resealing) was selected to give a low basal parasitemia (Fig 1). Not all the elution profile could be assayed, because in each of three experiments several fractions caused the resealed ghosts to become misshapen and vesiculate. The reason for this was not discovered; the effect could not be reversed by the addition of 10 mg/mL of fatty acid-free BSA to adsorb lysolipids, or by inclusion of 0.2 mmol/L of EGTA in the buffers used in the course of the preparation up to the column stage, to exclude calcium ions. Three separate elutions allowed assay of most of the profile: only seven fractions (including the void volume) caused vesiculation in all experiments. An elution curve, with activity essays, is shown in Fig 5. In all experiments, activity was discovered within the elution volume range of 38 to 42 mL. This corresponds to a mol wt for a globular protein of ~10,000, although the curvature of the calibration curve in this region limits the precision of the estimate. As Fig 5 also shows, the protein concentration, as judged by the absorbance at 280 nm in this region of the elution profile, is low. Gel electrophoresis in the presence of SDS revealed a component with an apparent subunit mol wt of ~13,000 and also various weaker bands.

**DISCUSSION**

Several unexpected features emerged from these studies. First, resealed ghosts, depleted of the bulk of the hemoglobin and other cytosolic contents, are still susceptible to invasion by *P falciparum*. Second, only ~6% of the hemoglobin concentration (ie, ~20 mg/mL) is required for the intracellular parasites to develop to the schizont stage. This implies that the amount of hemoglobin needed for the metabolic processes of maturation and egress from the cell is much lower than had been supposed (for review, see ref. 19). After a larger (30-fold) dilution, the capacity of the cytosol to support normal maturation of parasites is altered; dilution of the fresh hemolysate with heat-treated hemolysate has a similar effect. The factor required for maturation could well be a host cell enzyme, such as superoxide dismutase, on which the parasite is thought to be dependent.20

The invasion-related factor must be supposed normally to bind to or to modify the membrane enzymically in such a manner as to affect its structure, as perceived by the parasite at the outer surface. Several RBC proteins bind to the membrane cytoskeleton, including glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphofructokinase and aldolase, calmodulin, and catalase, as well as hemoglobin; tropomyosin is also associated with the actin protofilaments.27 None of the cytosolic enzymes that we
examined was able to restore the capacity of the diluted cytosol to support invasion, however. In all these cases, dilution, as well as such factors as calcium and magnesium concentration, may be expected to change grossly the amount of the component bound to the membrane. Whether, and in what manner, different cytoplasmic proteins might affect the state of the membrane is not known at present. The enzymes already tested and hemoglobin can be excluded from consideration. Our results indicate that the factor is a monomeric, anionic protein of low mol wt. Its low stability in vitro may be the result of a tendency to aggregate or adsorb. A very low concentration (<1 μg/mL, as judged by the staining intensity of the fraction in gel electrophoresis) is required for promotion of invasion. It is thus most likely an enzyme or cofactor. Our earlier evidence suggests that phosphorly turnover on cytoskeletal proteins, in particular spectrin, may be closely associated with the invasion process, and cytoplasmic as well as membrane-bound kinases or phosphatases are thus likely to be involved.

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