IDENTIFICATION OF A STRAIN-SPECIFIC MALARIAL ANTIGEN EXPOSED ON THE SURFACE OF *PLASMODIUM FALCIPARUM*–INFECTED ERYTHROCYTES

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New antigens appear on the surface of erythrocytes containing the trophozoite and schizont stages of the asexual malaria parasite (1–5). These antigens may theoretically be altered host antigens or of parasite origin and are potential targets of immune responses leading to the destruction of intracellular parasites. One such new antigen is involved in cytoadherence between *Plasmodium falciparum*–infected erythrocytes (IE)[1] and endothelium. IE develop knobs on the erythrocyte surface by which they adhere to venular endothelium in vivo (6) and to human endothelial cells (7) and amelanotic melanoma cells (8) in vitro. Immune serum inhibits cytoadherence of IE to these cells (4). Inhibition is mediated by strain-specific antibody (4), indicating antigenic diversity of the molecules involved in cytoadherence.

In this study we have attempted to identify strain-specific malarial antigens exposed on the surface of *P. falciparum* IE. We have used two strains of *P. falciparum* isolated in different parts of the world (Malaysia and El Salvador) together with sera shown to be strain specific by their capacity to inhibit cytoadherence to melanoma cells (4). Our biochemical approach was similar to the one that led to identification of the variant antigen on *P. knowlesi* IE as a protein of parasite origin (9). By comparing the radioiodinated and metabolically labeled proteins which were immunoprecipitated from these strains by strain-specific sera, we have identified a strain-specific malarial protein with the properties of a new surface antigen on IE.

Materials and Methods

Parasites. The Camp (Malaysia) and the Santa (St.) Lucia (El Salvador) strains (4) of *P. falciparum* were maintained by passage in *Aotus trivirgatus griseimembra* monkeys. Both strains induced knobs (K^+) on IE during development to trophozoites. IE in *Aotus* blood

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[1] *Abbreviations used in this paper:* DFP, diisopropyl fluorophosphate; IE, infected erythrocytes; K^+, Knob inducing; PBS, phosphate-buffered saline, pH 7.3; SDS, sodium dodecyl sulfate; TLCK, *N*-α-*p*-tosyllysine chloromethyl ketone; TPCK, *N*-*p*-tosylphenylalanine chloromethyl ketone.

were cryopreserved at the ring stage (18–40% parasitemia). Parasites were thawed and cultured by a modification of the Trager and Jensen method (4, 10). Cytoadherence of IE containing trophozoites to melanoma cells was performed as described previously (8).

Sera. Strain-specific immune sera were produced in two *Aotus* monkeys. Each monkey was infected three times with either Camp or St. Lucia strains before the sera were obtained. After each infection, the monkey was drug cured. Each animal was immune to the homologous strain, as shown by the failure to develop a patent parasitemia after the last challenge. Each immune serum was tested for blocking and reversal of cytoadherence to melanoma cells by IE as previously described (4).

Metabolic Labeling. After thawing the blood, parasites were cultured for 20–27 h to allow development from rings to trophozoites in the presence of 100 μCi/ml L-[4,5-^{3}H]-isoleucine (80–120 Ci/mmol), L-[2,5-^{3}H]histidine (40–60 Ci/mmol), L-[4,5-^{3}H]leucine (130 Ci/mmol), or L-[^{35}S]methionine (>600 Ci/mmol) (Amersham Corp., Arlington Heights, IL). Culture media containing RPMI 1640 90% deficient in the amino acid being incorporated was prepared using the Select-Amine kit (Gibco Laboratories, Grand Island, NY).

Lactoperoxidase-catalyzed Radioiodination. Suspensions of IE containing trophozoites were radioiodinated as described previously (11).

Detergent Extraction of Radioiodinated IE. Radioiodinated IE were washed once in phosphate-buffered saline, pH 7.3 (PBS) and resuspended at 2.5×10^8/ml in PBS. This suspension was layered over 5 vol of 55% Percoll (Pharmacia, Uppsala, Sweden) in RPMI 1640. The sample was centrifuged at 300 *g* for 20 min and the interface containing erythrocyte ghosts and IE ghosts was removed by aspiration. The pellet containing radioiodinated intact erythrocytes and IE was extracted either directly with electrophoresis sample buffer or sequentially with 1% Triton X-100 followed by electrophoresis sample buffer. Electrophoresis sample buffer (12) contained 5% sodium dodecyl sulfate (SDS) (Calbiochem-Behring Corp., San Diego, CA), 5% 2-mercaptoethanol, 0.001% bromphenol blue, and 62.5 mM Tris, pH 6.8; extractions were at a ratio of 5×10^7 cells per 100 μl of sample buffer. For sequential extractions, 2.5×10^8 cells were incubated for 30 min on ice in 1 ml of PBS containing 1% Triton X-100 (Sigma Chemical Co., St. Louis, MO), 5 mM diisopropyl fluorophosphate (DFP) (Sigma Chemical Co.), 10 μg/ml leupeptin (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 10 μg/ml chymostatin (Sigma Chemical Co.). The extract was separated into a soluble extract and an insoluble residue by centrifugation (13,000 *g* for 5 min). The soluble extract was added to an equal volume of double strength sample buffer containing 10% SDS, 10% 2-mercaptoethanol, 0.002% bromphenol blue, and 125 mM Tris, pH 6.8. The insoluble residue was extracted in sample buffer (5% SDS) and the extract was used for gel electrophoresis.

Trypsin Treatment of Intact Radioiodinated IE. One ml of 2×10^8 radioiodinated IE in PBS was incubated with various concentrations of trypsin-TPCK (Worthington Biochemical Corp., Freehold, NJ) for 5 min at room temperature followed by the addition of soybean trypsin inhibitor (Worthington, Biochemical Corp.) at a concentration 10 times that of the trypsin. One sample contained no enzyme and one contained a mixture of 100 μg/ml trypsin and 1 mg/ml soybean trypsin inhibitor. The cells were then washed twice in PBS, layered over 55% Percoll, and centrifuged as described above. The pelleted intact IE were extracted in Triton X-100 and separated into a soluble extract and insoluble residue as described above.

Reaction of Antibody with IE. Intact radioiodinated and metabolically labeled IE were incubated with immune sera as described previously (9) with the following modifications. A suspension of 5×10^8 erythrocytes in 50 μl RPMI 1640, 30 mM Hepes (Sigma Chemical Co.), pH 7.3, and 50 μl immune serum were incubated for 60–90 min at room temperature. The cells were washed three times in RPMI/Hepes containing 10% bovine serum albumin. The cells were suspended in 1 ml of a cocktail of protease inhibitors in PBS which included 1 mM EDTA (Fisher Scientific Co., Pittsburgh, PA), 1 mM EGTA, 1 mM *p*-chloromercuribenzoate, 0.2 mM *N*-α-*p*-tosyllysine chloromethyl ketone (TLCK), 0.1 mM *N*-*p*-tosylphenylalanine chloromethyl ketone (TPCK), and 2 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.) for radioiodinated cells and 1 mM EDTA, 1 mM

EDTA, 0.2 mM TLCK, 5 mM DFP, 10 μg/ml leupeptin, and 10 μg/ml chymostatin for metabolically labeled cells. An equal volume of 2% Triton X-100 in PBS was added and a soluble extract was prepared as described above. The soluble immune complexes were bound to protein A Sepharose and were washed as described previously (6). Antigen-antibody complexes were eluted in sample buffer and used for gel electrophoresis.

Gel Electrophoresis. The methods were as described previously (9).

Results

Cytoadherence of IE to Melanoma Cells and Inhibition by Immune Serum. Both K^+ Camp and K^+ St. Lucia IE bound to melanoma cells as previously reported (4). Anti-Camp immune serum completely blocked and reversed cytoadherence by K^+ Camp IE but not by K^+ St. Lucia IE. Anti–St. Lucia immune serum completely blocked and reversed cytoadherence by St. Lucia IE but not by Camp IE. This strain specificity of the sera was consistently observed when K^+ Camp and K^+ St. Lucia IE from three different monkeys were used.

Pattern of Radioiodinated Surface Proteins of IE. Intact erythrocytes containing predominantly mid to late trophozoites were radioiodinated by the lactoperoxidase method and extracted sequentially with 1% Triton X-100 (to obtain Triton X-100-soluble proteins) and 5% SDS (to obtain Triton X-100-insoluble proteins soluble in 5% SDS). The Triton X-100-soluble ^{125}I-proteins of Camp and St. Lucia IE were identical and indistinguishable from the pattern for uninfected erythrocytes. ^{125}I-labeled band 3 was a major component of this fraction, together with a broad band of M_r 200,000–220,000 (data not shown).

Examination of the Triton X-100-insoluble ^{125}I-proteins (extracted in 5% SDS) of Camp and St. Lucia IE revealed a difference in radiolabeling patterns. A new ^{125}I-protein absent from comparable extracts of radiolabeled uninfected erythrocytes was identified with IE from both strains in the M_r range 255,000–290,000 (Fig. 1). The M_r of this ^{125}I-protein was different for St. Lucia and Camp IE: M_r 280,000–290,000 (mean 287,000) in three experiments with Camp IE; M_r 255,000 and 265,000 (mean 260,000) in two experiments with St. Lucia IE. The Triton X-100-insoluble fraction also contained some radiolabeled band 3, a broad band of M_r 200,000–220,000 that is probably aggregated band 3 (13), and weakly labeled ^{125}I-proteins that co-migrated with spectrin. Broad ^{125}I-bands of M_r 300,000 were also seen in this fraction. There were several other ^{125}I-proteins of lower M_r with both Camp and St. Lucia IE that were not seen with uninfected erythrocytes.

Immunoprecipitation of Radioiodinated and Metabolically Labeled Proteins of Camp and St. Lucia IE. Intact, radioiodinated Camp IE and intact, metabolically labeled Camp and St. Lucia IE were incubated with anti-Camp or anti–St. Lucia immune serum. After unbound antibody was removed by washing, IE with antibody bound to the surface were solubilized with 1% Triton X-100. Immune complexes were bound to protein A-Sepharose and eluted with 5% SDS electrophoresis sample buffer for SDS–gel electrophoresis.

Anti-Camp, but not anti–St. Lucia, immune serum specifically immunoprecipitated a high M_r radioiodinated protein from Camp IE in three independent experiments (Fig. 2, lanes 1 and 2). In one experiment, this protein was not immunoprecipitated by either anti-Camp or anti–St. Lucia immune serum. This molecule therefore appears to be a strain-specific antigen. This antigen migrated

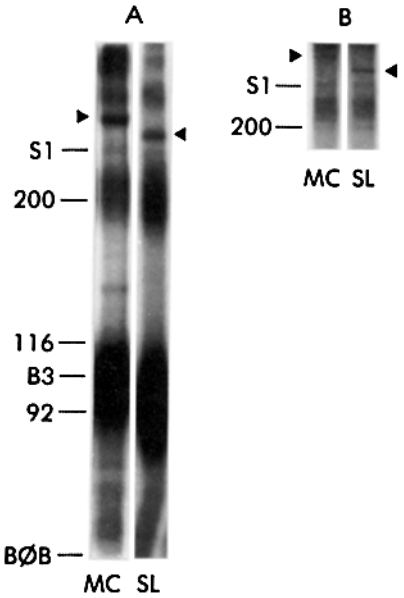

FIGURE 1. Autoradiograph of radioiodinated proteins of Malayan Camp (MC) and St. Lucia (SL) strains of *P. falciparum* electrophoresed on 5–7.5% polyacrylamide gels. Intact IE were solubilized in 1% Triton X-100 and centrifuged to obtain a Triton X-100-insoluble pellet. The pellet was solubilized in sample buffer and electrophoresed. Results shown for MC and SL run on separate gels (*A*) and on the same gel (*B*). In *A* the gels lanes were aligned by the Coomassie Blue–stained spectrin bands. To the left of the gel lanes are molecular weight standards, the bromphenol blue dye front (BϕB), band 3 (B3) and spectrin (S1). The strain-specific antigens are marked with a solid arrowhead (▶).

above spectrin, as judged by comparison with an adjacent gel lane, and had an M_r of 290,000 in each experiment. This protein was not immunoprecipitated from radioiodinated normal erythrocytes (data not shown).

With metabolically labeled Camp IE, anti-Camp immune serum specifically immunoprecipitated a high M_r protein (Fig. 2, lanes 3 and 4). Normal *Aotus* serum failed to immunoprecipitate this protein (data not shown). This metabolically labeled protein was immunoprecipitated in a strain-specific fashion in four independent experiments using four different metabolic labels ([^{3}H]isoleucine, [^{35}S]methionine, [^{3}H]leucine, and [^{3}H]histidine; results with [^{3}H]isoleucine are shown). The M_r of this strain-specific protein ranged from 280,000 to 295,000 (mean 289,000). Overexposed fluorographs showed that immunoprecipitation with anti–St. Lucia immune serum resulted in immunoprecipitation of a small amount of radioactive protein in this region (Fig. 2, lane 4).

With metabolically labeled St. Lucia IE, both anti–St. Lucia immune serum and anti-Camp immune serum immunoprecipitated a high molecular weight protein (Fig. 2, lanes 5 and 6). However, with anti–St. Lucia immune serum, the amount of radioactivity that accumulated in this band was much greater than with anti-Camp immune serum. This result was obtained in three independent experiments using three different metabolic labels (results for [^{3}H]isoleucine are shown). The M_r of the protein in three experiments ranged from 255,000 to 265,000 (mean 258,000).

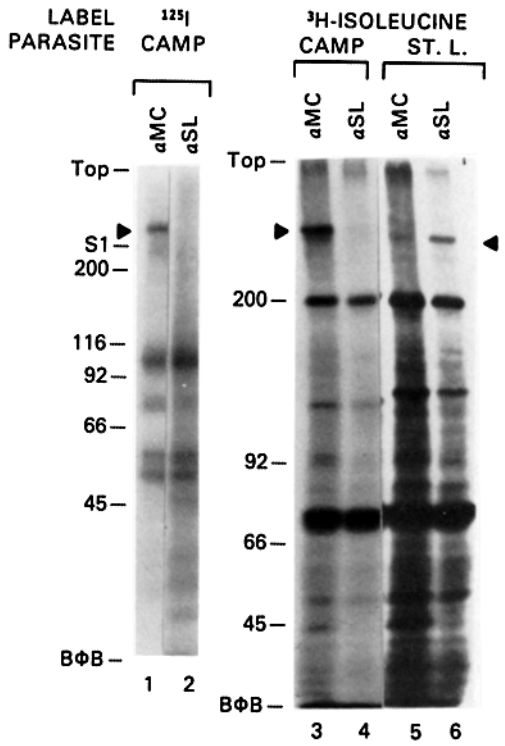

FIGURE 2. Autoradiograph (^{125}I) and fluorograph ([^{3}H]isoleucine) of Malayan Camp (Camp) and St. Lucia (St. L.) strains of *P. falciparum*. The intact IE were incubated with anti-Malayan Camp serum (aMC) or anti-St. Lucia serum (aSL). The protein-antibody complexes were solubilized in Triton X-100, bound to protein A Sepharose and electrophoresed in sample buffer on a 5–15% polyacrylamide gradient gel (lanes 1 and 2) and on a 5–10% polyacrylamide gradient gel (lanes 3–6). Lanes 1 and 2: radioiodinated Malayan Camp IE. Lanes 3 and 4: metabolically labeled ([^{3}H]isoleucine) Malayan Camp IE. Lanes 5 and 6: metabolically labeled ([^{3}H]isoleucine) St. Lucia IE. The notations to the left of the figures and the solid arrowheads are the same as in Fig. 1*A*. Lanes 3–6 are from the same gel.

Other metabolically labeled proteins of M_r 200,000 and less were immunoprecipitated by both immune sera from both parasite strains.

Effect of Trypsin Treatment on Cytoadherence to Melanoma Cells and on Radioiodinated Surface Proteins. Cytoadherence of Camp and St. Lucia IE to melanoma cells was partially reduced by treatment of IE with 0.1 μg/ml of trypsin for 5 min. Treatment of IE with 1 μg/ml for 5 min completely inhibited cytoadherence.

Intact, radioiodinated IE were treated with trypsin and the Triton X-100-insoluble ^{125}I-proteins were compared (Fig. 3). The M_r 280,000 ^{125}I-protein of Camp parasites and the M_r 255,000 ^{125}I-protein of St. Lucia parasites both showed loss of radioactivity after treatment with 0.1 μg/ml of trypsin (Fig. 3, lane 3). After treatment with 1.0 μg/ml trypsin, no strain-specific ^{125}I-proteins were recovered (Fig. 3, lane 4). Thus, there is a direct correlation between the trypsin sensitivity of cytoadherence to melanoma cells and tryptic cleavage of the ^{125}I-labeled strain-specific proteins.

After treatment of intact radioiodinated IE with levels of trypsin that cleaved

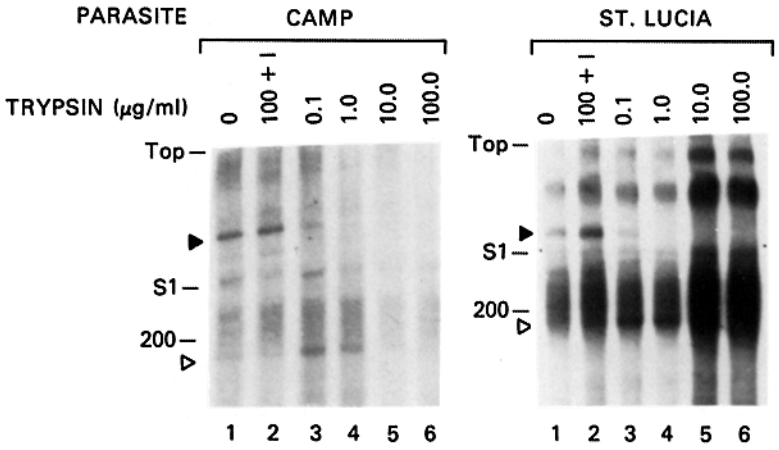

FIGURE 3. Autoradiograph of the upper part of a 5% polyacrylamide gel showing trypsin-sensitive radioiodinated proteins on Malayan Camp and St. Lucia IE. The Triton X-100-insoluble residues were solubilized in sample buffer for electrophoresis. The trypsin concentrations in μg/ml are shown at the top of the gels. The first two lanes contained no enzyme (0) and 100 μg/ml trypsin with 1 mg/ml soybean trypsin inhibitor (100 + I), respectively. The strain-specific trypsin-sensitive proteins of Malayan Camp and St. Lucia are marked with a solid arrowhead (▶). The apparent cleavage products in lanes 3 and 4 are marked with an open arrowhead (▷). The notations to the left of the figures are as in Fig. 1.

the strain-specific ^{125}I-proteins, new ^{125}I-proteins could be identified with the washed IE (Fig. 3, lanes 3 and 4). These ^{125}I-proteins appeared to be cleavage fragments of the M_r 280,000 and 255,000 strain-specific molecules that remained on the IE membrane after cleavage. The Camp strain fragment had M_r of 180,000 and that of St. Lucia strain had M_r of 185,000. Treatment of IE with ≥10 μg/ml trypsin removed these ^{125}I-labeled cleavage products from the washed cells (Fig. 3, lanes 5 and 6).

Discussion

This study has led to the identification of high M_r protein antigens of Camp and St. Lucia strains of *P. falciparum* that are specifically immunoprecipitated from each strain by immune serum from *Aotus* monkeys infected with the homologous strain. Immune serum from animals infected with the heterologous strain either did not immunoprecipitate the protein or immunoprecipitated much less of the protein than immune serum from animals infected with the homologous strain. Taken together, the results with two parasite strains and two immune sera indicate that these antigens are strain specific. In addition to a difference in antigenicity, these antigens displayed a difference in electrophoretic mobility on SDS-polyacrylamide gels. The strain-specific antigen of Camp IE had an M_r of 280,000–295,000 while that of St. Lucia IE had an M_r of 255,000–265,000. Since these strain-specific antigens could not be identified by metabolic labeling methods on uninfected *Aotus* erythrocytes, and because they were metabolically labeled by radioactive amino acids, they are malarial proteins rather than host proteins. These high molecular weight parasite proteins may be in the same molecular weight region as the molecule described by Gruenberg and Sherman

(14). In their study, intact erythrocytes infected with *P. falciparum* were bound to polyacrylamide beads coated with polyethyleneimine. The membranes that remained on the beads after lysis were extracted in sample buffer. A protein of M_r 230,000 was differentially concentrated in this preparation.

Although the antigens in the present study were strain-specific and differed in M_r, they shared certain properties which suggest that these molecules are homologous and are exposed on the surface of *P. falciparum* IE. The properties that indicate surface membrane orientation were as follows. First, the strain-specific antigens were radiolabeled by lactoperoxidase-catalyzed radioiodination under conditions that predominantly label erythrocyte membrane proteins. Radioiodination of intact synchronous populations of late trophozoite/early schizont–infected cells could be achieved with little or no detectable labeling of hemoglobin. Second, treatment of intact IE with low concentrations of trypsin cleaved the strain-specific antigens. Third, we used antibody as a membrane-impermeant probe added to intact IE to demonstrate reactivity of the strain-specific antigens. Although none of these techniques are absolutely surface specific, the fact that the strain-specific antigens were consistently identified by them is compatible with location in the erythrocyte membrane. It should be noted that we have combined the data for ^{125}I-labeled proteins and for metabolically labeled proteins because they had similar mobilities on SDS-gels, because they fit the criteria for location in the erythrocyte surface and because they were strain specific. Proof of identity must await the availability of cross-reactive monoclonal antibodies to these proteins.

These strain-specific antigens have certain characteristics in common with the molecules on the surface of IE involved in cytoadherence to melanoma cells. First, both cytoadherence (15) and the molecules described in this paper were sensitive to low concentrations of trypsin (1 μg/ml). Second, antibody blocked binding to melanoma cells (4) and immunoprecipitated antigens in a strain-specific fashion when serum was added to intact IE. Although we speculate on the basis of these results that the strain-specific antigen may serve as the ligand for binding to endothelial cells, specific receptor studies will be required to answer this question.

The strain-specific antigens of *P. falciparum* have many biochemical properties in common with the variant antigen of *P. knowlesi* that is also on the surface of IE (9). In addition to accessibility to surface radioiodination, sensitivity to trypsin, and specific immunoprecipitation by antibody added to intact cells (6 and R. J. Howard, unpublished data), both the *P. knowlesi* variant antigen (16 and R. J. Howard, unpublished data) and *P. falciparum* strain antigen are insoluble in Triton X-100. These detergent solubility properties suggest that both antigens may be anchored to the erythrocyte skeleton, which has similar detergent solubility properties (17). This characteristic detergent solubility is further evidence for the homology between the two strain-specific antigens of *P. falciparum* and may indicate an homology with the variant antigen of *P. knowlesi.* When antibody was added to intact IE, however, both the *P. knowlesi* variant antigen (6) and the *P. falciparum* strain-specific antigen became soluble in 1% Triton X-100. Whether this effect is due to the addition of protease inhibitors with

perturbant properties or to the incorporation of the antigen into an immune complex is unknown.

Thus we have identified a strain-specific antigen of *P. falciparum* that has characteristics of a molecule localized on the surface of IE. Whether it is the binding ligand to endothelial cells (4), the variant antigen on the surface of IE (3), or both, remains to be determined. It will also be important to determine whether this strain-specific antigen has common epitopes that could serve as antigenic targets for strain-transcending antimalarial immunity.

Summary

We have identified strain-specific antigens with Camp and St. Lucia strains of *P. falciparum* of M_r ~285,000 and ~260,000, respectively. These strain-specific antigens were metabolically labeled with radioactive amino acids, indicating that they were of parasite origin rather than altered host components. These proteins had the properties of a molecule exposed on the surface of infected erythrocytes (IE). First, the proteins are accessible to lactoperoxidase-catalyzed radioiodination of intact IE. Second, the radioiodinated proteins were cleaved by low concentrations of trypsin (0.1 μg/ml). Third, these antigens were immunoprecipitated after addition of immune sera to intact IE. Fourth, the strain-specific immunoprecipitation of these proteins correlated with the capacity of immune sera to block cytoadherence of IE in a strain-specific fashion. Fifth, the strain-specific antigen had detergent solubility properties (i.e., insolubility in 1% Triton X-100, solubility in 5% sodium dodecyl sulfate) similar to the variant antigen of *P. knowlesi*, which has been proven to be a malarial protein exposed on the erythrocyte surface.

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