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Plasmodium coatneyi produced ring-infected erythrocyte surface antigen (RESA) during infection of the rhesus monkey. This antigen was immunogenic and elicited an antibody response that was not persistent but was boosted by repeated infections in a manner similar to that seen in *P. falciparum* infections in humans. Preliminary data showed that the appearance and increasing titer of antibodies to *P. coatneyi* RESA-like antigen were associated with prolongation of intervals from inoculation to potency and with control of parasitemia. Studies using both immunofluorescence assay and western blot analysis showed that *P. coatneyi*-immune rhesus serum cross-reacted with *P. falciparum* antigens, but *P. falciparum* immune human serum did not recognize *P. Coatneyi* antigen in either assay. These results show that *P. Coatneyi* express RESA-like antigen that elicits an antibody response similar to that observed for human antibody to *P. falciparum* RESA. However, antibodies to *P. coatneyi* did not cross-react with *P. falciparum* RESA in erythrocyte membrane immunofluorescence assay and dot immunoblot analysis, suggesting that different immunogenic epitopes are present on the two molecules. Our observations support the use of this primate model in RESA-based vaccine development.

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PLASMODIUM COATNEYI RING-INFECTED ERYTHROCYTE SURFACE ANTIGENS

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Abstract. *Plasmodium coatneyi* produced ring-infected erythrocyte surface antigen (RESA) during infection of the rhesus monkey. This antigen was immunogenic and elicited an antibody response that was not persistent but was boosted by repeated infections in a manner similar to that seen in *P. falciparum* infections in humans. Preliminary data showed that the appearance and increasing titer of antibodies to *P. coatneyi* RESA-like antigen were associated with prolongation of intervals from inoculation to patency and with control of parasitemia. Studies using both immunofluorescence assay and Western blot analysis showed that *P. coatneyi*-immune rhesus serum cross-reacted with *P. falciparum* antigens, but *P. falciparum* immune human serum did not recognize *P. coatneyi* antigens in either assay. These results show that *P. coatneyi* expresses RESA-like antigen that elicits an antibody response similar to that observed for human antibody to *P. falciparum* RESA. However, antibodies to *P. coatneyi* did not cross-react with *P. falciparum* RESA in erythrocyte membrane immunofluorescence assay and dot immunoblot analysis, suggesting that different immunogenic epitopes are present on the two molecules. Our observations support the use of this primate model in RESA-based vaccine development.

Plasmodium coatneyi is a non-relapsing malaria parasite of macaque monkeys with a number of falciparum-like characteristics. Mature asexual blood-stage forms sequester in deep tissue vasculature^{1,2} in association with the formation of knob-like protrusions on the surface of parasitized red blood cells (PRBC)³ and cytoadhere to microvascular endothelium.⁴ Furthermore, *P. coatneyi* trophozoite/schizont (T/S)-stage PRBC have been shown to cytoadhere to uninfected red blood cells (RBC) to form rosettes.⁵

Little is known about the antigen characteristics of *P. coatneyi* or of the specificity of elicited immune responses. Using rhesus monkeys (*Macaca mulatta*) as the laboratory host, we investigated whether *P. coatneyi* produces ring-infected erythrocyte surface antigen (RESA) analogous to that of *P. falciparum*^{6,7} and whether there is immune cross-reactivity between these antigens. Since RESA is a candidate molecule for a blood-stage malaria vaccine,⁸⁻¹⁰ identifying a nonhuman primate malaria model with these characteristics may facilitate the process of vaccine development.

MATERIALS AND METHODS

Infection of rhesus monkeys with P. coatneyi

Four adult rhesus monkeys without prior exposure to *P. coatneyi* were selected from a primate colony for study using a protocol approved by the Committee of Laboratory Animal Care and Use at the Armed Forces Research Institute of Medical Sciences. Each animal was inoculated intravenously with blood-stage parasites of the type strain¹¹ of *P. coatneyi*. Three monkeys (AF-B298, AF-B464, and AF-G425) were infected using 1 ml of a previously frozen stablitate (1.6×10^5 PRBC/ μ l, 90% hematocrit). One monkey (AF-B425) was repeatedly infected with fresh heparinized blood (1-2 ml) from donor animals with low level parasitemias (140-520 PRBC/ μ l). The other three animals were followed through the course of infection, had sera collected, and were killed for tissue studies,⁴ while monkey AF-B425 was given curative treatment with chloroquine and twice rechallenged.

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Parasite and antigen preparations

Plasmodium coatneyi was taken from infected rhesus monkeys when the parasitemia (ring-stage) was 7–10%. Blood was collected in RPMI 1640 medium containing heparin, pH 7.4, and washed three times in a serum-free RPMI 1640 medium. After the buffy coat was removed, a 1% cell suspension in phosphate-buffered saline (PBS) was made and used to prepare glutaraldehyde (GA)-fixed, ring-stage PRBC monolayers for RESA as described by Perlmann and others.⁶ The remaining parasites were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated AB⁺ human serum, 25 mM HEPES, 2 mM glutamine, and 20 µg/ml of gentamicin, pH 7.4. Cultures were kept for at least 30 hr in an incubator at 37°C and in an atmosphere of 5% CO₂, plus air by continuous flow. Maturation of parasites to the T/S-stage in the first schizogonic cycle after isolation was confirmed by examination of Giemsa-stained thin blood films, after which RBC were harvested by washing twice with serum-free RPMI 1640 medium. A small aliquot of packed RBC was resuspended in an equal volume of 1% bovine serum albumin (BSA) in PBS and used for preparation of unfixed, air-dried thin blood films. The remaining T/S-stage parasites were resuspended in an equal volume of PBS and stored at –70°C for Western blot analysis.

P. falciparum Thai isolate, BR15, was maintained in vitro as previously described¹² for one month in human group O⁺ RBC using the same culture medium as above. The PRBC were harvested when the parasitemia reached 8–10%, either ring- or T/S-stage parasites, and handled as above. Uninfected RBC from group O⁺ human and rhesus monkey donors were prepared and frozen as above to provide control preparations for Western blot analyses.

Antibodies

Sera from *P. coatneyi*-infected rhesus monkeys during one or multiple infections, pooled sera from humans who had a *P. falciparum* infection, sera from uninfected rhesus monkeys, and sera from malaria-naïve human volunteers were collected and stored at –20°C.

Immunofluorescence assay

A conventional immunofluorescence assay (IFA) detecting intracellular parasite antigens and

an erythrocyte membrane immunofluorescence (EMIF) assay detecting RESA were performed with unfixed, air-dried, T/S-stage PRBC and GA-fixed, ring-stage PRBC, respectively, as described by Perlmann and others.⁶ Sera were serially diluted from 1:5 to 1:3,125 using five-fold dilutions, applied to both air-dried T/S-stage and GA-fixed ring-stage antigens for 30 min, washed, stained with anti-human immunoglobulin conjugated with fluorescein isothiocyanate, washed, mounted with 50% glycerol in PBS, and examined using ultraviolet light microscopy. Rhesus and human control or immune sera were also used for staining of *P. falciparum* and *P. coatneyi* antigens, respectively, to study antibody cross-reactivity elicited by the two parasites in their natural hosts.

Western and dot blot analyses

Frozen samples from infected (*P. coatneyi* and *P. falciparum*) and uninfected rhesus monkey and human RBC were diluted 1:10 in sample buffer (0.1 M Tris buffer, 10% sodium dodecyl sulfate [SDS], 9% glycerine, 0.1 M dithiothreitol, pH 6.8) and boiled for 3 min. After the samples were cooled in an ice bath, 20 µl of each sample was applied to an SDS-polyacrylamide gel and proteins were separated by electrophoresis. The separated proteins were transferred onto nitrocellulose paper and incubated with 1% BSA in PBS for 1 hr. Proteins on the nitrocellulose paper were probed with immune or control sera diluted at 1:100 in 1% BSA-PBS for 1 hr, washed five times in 0.5% Tween 20 in PBS, incubated with anti-human Ig conjugated with horseradish peroxidase for 1 hr, washed five times in Tween-PBS, incubated with peroxidase substrate (4-chloro-1-naphthol in 15% methanol in PBS containing 0.01% hydrogen peroxide) for 15 min, and rinsed with distilled water.

For dot blotting, 10 µl of a solution containing 0.2 mg/ml of synthetic peptide (EENVEHDA)₃, corresponding to the 3' repeat sequence of *P. falciparum* RESA (a gift from Dr. Troye-Blomberg, Department of Immunology, University of Stockholm, Stockholm, Sweden) was spotted onto a nitrocellulose paper and dried. As a control protein, BSA at a concentration of 0.5 mg/ml was also spotted onto the paper. The nitrocellulose was then incubated with 1% BSA and probed with immune human or rhesus monkey sera and stained as described above.

RESULTS

Infection of rhesus monkeys with P. coatneyi

Four rhesus monkeys were infected with blood-stage parasites of *P. coatneyi*. All four *P. coatneyi*-naïve animals developed patent parasitemias on days 2–4, which then increased to 3–10% in less than two weeks. On days 12–16, sera were collected from three animals prior to their being killed for pathologic study. Monkey AF-B425 was followed through three separate infections with *P. coatneyi* (Figure 1). After the first inoculation, a low level parasitemia (60 PRBC/ μ l of blood) was detected on day 2. The parasitemia became subpatent on day 6 and was again detectable on day 10. The highest parasitemia, 1.7×10^4 PRBC/ μ l of blood, was detected on day 16 and the monkey was bled for serum collection and given curative treatment with chloroquine every day for three days. The monkey was intravenously reinoculated with *P. coatneyi* 84 days later. On this occasion, parasitemia was detected on day 9 and the highest parasitemia, 8×10^4 PRBC/ μ l of blood, was observed on day 16. Serum was then collected from the monkey and the animal was given curative treatment as described above. After a third infectious inoculation 75 days after the second infection, parasitemia was detected on day 13 and reached a peak of 2.8×10^4 PRBC/ μ l of blood on day 18. The monkey was bled on the day of inoculation and again 22 days later when the parasitemia had decreased to 40 PRBC/ μ l of blood, three days before the animal became aparasitemic and prior to the initiation of chemotherapy.

Antibody responses to P. coatneyi in infected rhesus monkeys

Plasmodium coatneyi-naïve, rhesus monkey sera at a 1:25 dilution did not react with *P. coatneyi* antigens using the IFA and EMIF assay with air-dried T/S-stage and GA-fixed ring-stage *P. coatneyi* antigen preparations, respectively. Antibody titers to *P. coatneyi* of infected animals are shown in Table 1. Sera collected from three monkeys after their primary infections with *P. coatneyi*, were assayed; a low positive anti-*P. coatneyi* antibody titer, 1:25, was detected in one monkey with the air-dried T/S-stage but not with the GA-fixed ring-stage antigen preparation. Monkey AF-B425, which was challenged and

cured on three occasions, had serum collected after the second and before and after the third infections. Antibodies to air-dried T/S-stage antigens increased with the second and third infections reaching titers of 1:625 and 1:3,125, respectively. Positivity by the EMIF assay, which was not seen in three animals after one infection, was demonstrated to GA-fixed ring-stage antigens (Figure 2) at a titer of 1:125 after the second infection and increased to 1:625 after the third infection.

Cross-reactivity of antibodies to P. coatneyi with native P. falciparum proteins

Sera collected from *P. coatneyi*-naïve and -immune rhesus monkeys were assayed with *P. coatneyi* and *P. falciparum* antigens in the IFA and EMIF assay with air-dried T/S-stage and GA-fixed ring-stage preparations. The results are summarized in Table 2. Naïve monkey serum at a 1:50 dilution did not react with *P. coatneyi* or *P. falciparum* antigens. Rhesus immune serum (RIS) from the third *P. coatneyi* infection at a 1:250 dilution reacted with both air-dried T/S-stage and GA-fixed ring-stage *P. coatneyi* and also with air-dried T/S-stage *P. falciparum* antigens, but the RIS did not react with GA-fixed, ring-stage *P. falciparum* antigens. Human immune serum (HIS) at a 1:250 dilution reacted with both air-dried T/S-stage and GA-fixed ring-stage *P. falciparum* antigens, but did not react with either antigen preparation of *P. coatneyi*. In an EMIF assay using *P. falciparum*, the HIS had an antibody titer of 1:6,250. Control, nonimmune, human serum at a 1:50 dilution did not react with either *P. coatneyi* or *P. falciparum* antigens.

Cross-reactivity of antibodies to P. coatneyi with denatured P. falciparum proteins

Plasmodium coatneyi-immune rhesus monkey and *P. falciparum*-immune human sera (RIS and HIS) were analyzed using Western blotting under reducing conditions with crude antigens prepared from T/S-stage *P. coatneyi*- and *P. falciparum*-infected blood. Uninfected rhesus monkey and human blood were included as control antigens. By probing the proteins with RIS at a 1:100 dilution, *P. coatneyi* proteins with *M_r* values of 120, 100, 97, 80, and 45 kD were detected (Figure 3A, lane c). The RIS also detected

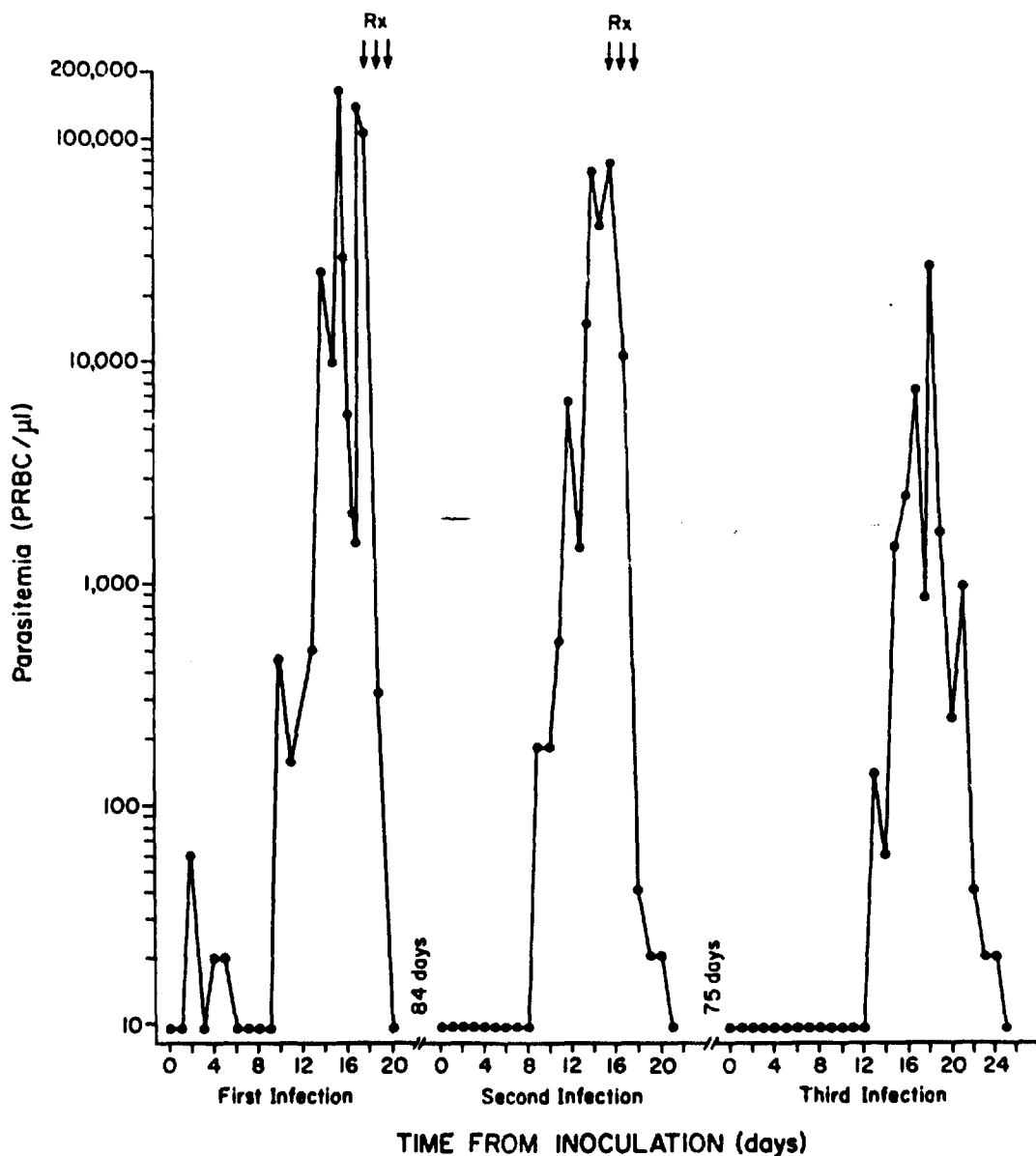


FIGURE 1. *Plasmodium coatneyi* parasitemia in rhesus monkey AF-B425 during the course of a primary and two subsequent infections. Intravenous inoculations of blood-stage parasites were done on day 0. Each dot represents parasitemia determined daily. The arrows indicate days of treatment (Rx) with chloroquine. PRBC = parasitized red blood cells.

a group of *P. falciparum* proteins with M_r values of 100, 90, 80, and 70 kD (Figure 3A, lane b), but it did not react with control antigens at the M_r values indicated above (Figure 3A, lanes a and d). Probing with HIS at a 1:100 dilution detected several *P. falciparum* proteins, including the polypeptide with an M_r of 155 kD (Figure 3B, lane g). In contrast to the RIS, the HIS did

not detect any proteins of *P. coatneyi* in the Western blot (Figure 3B, lane f). The HIS did not react with control antigens at the M_r values indicated above (Figure 3B, lanes e and h).

A dot immunoblot assay using the trimer of a synthetic peptide (EENVEHDA) that corresponded to the RESA of *P. falciparum* showed that HIS but not RIS reacted with the peptide.

TABLE 1

Antibody titers to *Plasmodium coatneyi* in rhesus monkeys during primary and repeated infections, as determined by immunofluorescence assays*

Monkeys	Infection			
	First	Second	Third	
	Day 18	Day 16	Day 0	Day 22
AF-B298†	0 (5)			
AF-G425†	0 (25)			
AF-B464†	0 (5)			
AF-B425	NT	125 (625)	25 (625)	625 (3,125)

* Values are reciprocal titers of antibodies in an erythrocyte membrane immunofluorescence assay and an immunofluorescence assay (values in parentheses). NT = not tested.

† These monkeys were killed after the first infection.

Neither HIS nor RIS reacted with spots of control protein.

DISCUSSION

Plasmodium coatneyi infection of macaque monkeys has attracted attention as an animal model for the study of falciparum malaria. The similarities previously identified between *P. coatneyi* infections of rhesus monkeys and *P. falciparum* infections of humans involve the pathophysiology of the infection. Serologic cross-reactivity between *P. falciparum* and *P. coatneyi* was assessed by Collins and others¹³ using sera from

syphilitic patients treated with induced malaria. These antisera to human malarias were tested against a panel of intra-erythrocytic antigens of simian malaria parasites. Occasional low-titer reactivity was observed against the *P. coatneyi* preparation. In this study, these immunologic relationships were further explored. Our preliminary results showed that rhesus monkeys develop antibody reactivity against the RESA-like antigen of *P. coatneyi* when GA-fixed ring-stage PRBC are used, and that some *P. falciparum* proteins other than RESA are recognized by *P. coatneyi*-immune rhesus sera.

During the course of *P. coatneyi* infections in

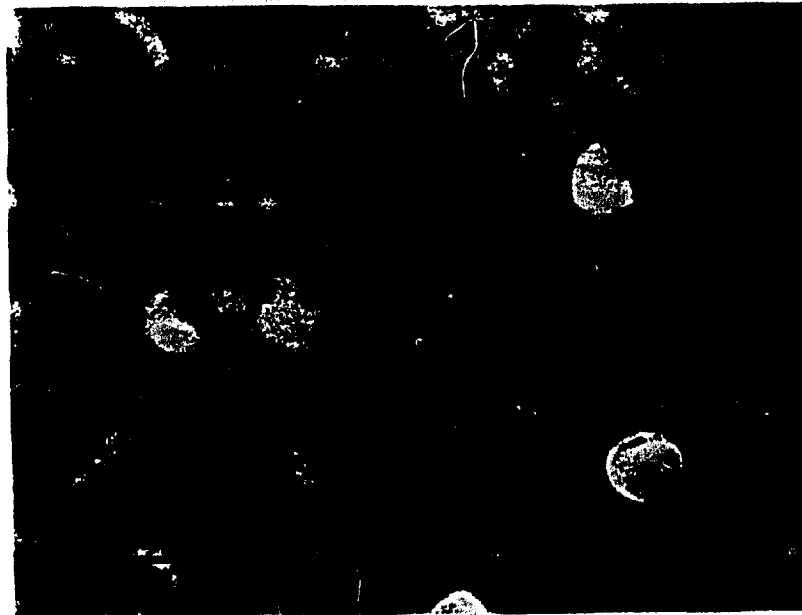


FIGURE 2. Ring-infected erythrocyte surface antigen of *Plasmodium coatneyi*-infected rhesus monkey red blood cells shown by an erythrocyte membrane immunofluorescence assay using immune serum from a *P. coatneyi*-infected rhesus monkey. Parasites were visualized by counterstaining with ethidium bromide (arrowheads).

TABLE 2

Serologic cross-reactivity between *Plasmodium coatneyi* and *P. falciparum* studied by immunofluorescence assays*

Anti-serum	Dilution	<i>P. coatneyi</i>		<i>P. falciparum</i>	
		IFA	EMIF	IFA	EMIF
RIS	1:250	+	-	+	-
HIS	1:250	-	-	+	+

* IFA = immunofluorescence assay of an unfixed, air-dried trophozoite-schizont-stage parasite preparation; EMIF = immunofluorescence assay of a glutaraldehyde-fixed ring-stage parasite preparation; RIS = rhesus immune serum from monkey AF-B425; HIS = human immune serum.

rhesus monkeys, the development of specific antibodies to RESA-like antigen was observed. Although the results were obtained from a small number of infected monkeys and kinetic studies have yet to be done, the data shown in Table 1 suggest that this antibody response required stimulation by repeated infections and was non-persistent, which is consistent with the properties of human antibodies to *P. falciparum* RESA.^{14, 15} In view of our preliminary results, the time to patency in the multiply infected rhesus monkey appeared progressively prolonged. Furthermore, the peak parasitemias in the second and third infections were two-fold and six-fold lower, respectively, than those observed during the primary infection. This is probably a reflection of the increasing levels of malaria-specific antibodies, including antibodies to RESA, as has been shown by the other investigators in *P. falciparum* infections.^{16, 17} Since antibodies to RESA are associated with falciparum malaria, but not other human malarias, these observations add an immunologic characteristic to the similarities between *P. coatneyi* and *P. falciparum*.

Plasmodium coatneyi-immune rhesus serum recognized native *P. falciparum* antigens as assayed by IFA of air-dried T/S-stage parasites. On the other hand, immune rhesus serum did not recognize the RESA of *P. falciparum* nor did the immune human serum recognize the RESA-like antigen of *P. coatneyi*. This suggests that the antigenic portion of the *P. coatneyi* RESA-like antigen exposed on the ring-infected rhesus RBC is different from that of the *P. falciparum* RESA on infected human RBC. The potent immunogenic B cell epitopes of the *P. falciparum* RESA have been shown to be localized predominantly in the repetitive amino acid sequences (EEN-VEHDA and EENV) in the 3' portion of the molecule.^{7, 18} Our preliminary results showed that

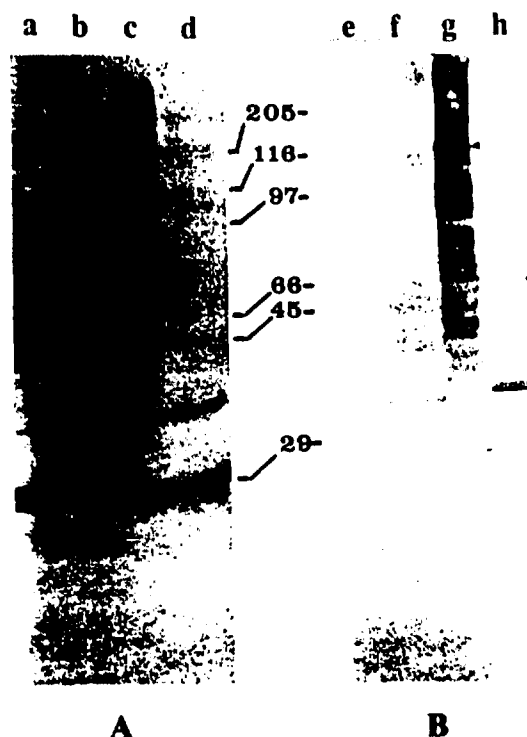


FIGURE 3. Cross-reactivity of antibodies to *Plasmodium coatneyi* with *P. falciparum* proteins studied by Western blot analysis. Protein extracts of uninfected human red blood cells (lanes a and h), trophozoite/schizont (T/S)-stage *P. falciparum*-infected human red blood cells (lanes b and g), T/S-stage *P. coatneyi*-infected rhesus red blood cells (lanes c and f), and uninfected rhesus red blood cells (lanes d and e) were electrophoresed and probed with rhesus immune serum from monkey AF-B425 (A) and human immune serum (B). Arrowheads indicate *P. coatneyi* polypeptides with M_r values of 120, 100, 97, 80 and 45 kD recognized by monkey serum AF-B425 (lane c) and *P. falciparum* RESA, M_r of 155 kD, recognized by human immune serum (lane g). The range of standard molecular masses are indicated by the numbers between the two blots.

the *P. coatneyi* RESA-like antigen is immunogenic in the rhesus monkey. Although the amino acid sequence of the *P. coatneyi* RESA-like antigen is not known and the existence of homology with the *P. falciparum* RESA has not been shown, it is unlikely that the RESA-like antigen of *P. coatneyi* will contain sequences similar to the repeat region of the *P. falciparum* RESA. In addition, it was shown in the EMIF assay and by dot immunoblotting that the immune rhesus serum did not recognize the RESA of *P. falciparum*.

We further investigated the serologic cross-reactivity of antibodies to *P. coatneyi* with denatured *P. falciparum* proteins by Western blotting under reducing conditions. The immune rhesus serum recognized several *P. coatneyi* polypeptides as well as a group of *P. falciparum* polypeptides and thus confirmed the cross-reactivity of antibodies to *P. coatneyi* with *P. falciparum* antigens detected by IFA of air-dried T/S-stage parasites. The polypeptide with an M_r value of 155 kD, the predominant antigen on the surface of *P. falciparum* ring-stage PRBC (Pf155/RESA),^{6,7} was not detected by the immune rhesus serum. This confirmed the negative result in EMIF assay with *P. falciparum* ring-stage PRBC and indicated that these two proteins, *P. coatneyi* RESA-like antigen and *P. falciparum* RESA, differ in their natural immunogenic epitopes.

Immune responses to the RESA have been associated with resistance to clinical falciparum malaria,^{14,15,16,19} leading to the proposal of RESA as a candidate vaccine. This report identifies a primate malaria model in which an antibody response to RESA occurs and may therefore be used in the development of RESA-based vaccines.

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