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STUDY OF HUMORAL IMMUNE RESPONSE IN MAMMALS IMMUNIZED WITH *PLASMODIUM FALCIPARUM* ANTIGENIC PREPARATIONS

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Six Plasmodium falciparum *protein fractions, isolated under reducing conditions, were used to immunize mice, rabbits and the squirrel monkey* Saimiri sciureus. *Five or seven subcutaneous injections of each antigenic preparation, in conjunction with Freund's complete or incomplete adjuvants, were administered. This led to the development of specific antibodies detected by IFAT, ELISA or immunobloting which inhibited merozoite reinvasion in* in vitro P. falciparum *cultures. This activity seems to be associated with rhoptry proteins contained in fractions Pf F2 and Pf F4.*

Key words: *Plasmodium falciparum* – protein fractions – *in vitro* parasite inhibition – rhoptry proteins

Plasmodium falciparum-type malaria is a major health problem in tropical and sub-tropical regions. With the spread of chemoresistance and the difficulties in vector control, the search for effective immunoprophylatic methods becomes more indispensable.

Several asexual blood stage *P. falciparum* antigens have been shown capable of inducing a partially protective immune responses in susceptible monkeys and also in humans (Patarroyo et al., 1987). Earlier attempts focused on critical malaria antigens present on the surface of merozoites or deposited on the erythrocyte membrane (Perrin et al., 1984).

However, the great diversity in parasite proteins and their extensive polymorphism complicates the identification and molecular characterization of components which may have a protective function. Moreover, the host's immunological status, including MHC linked immune restriction, leads to the development of varied effector mechanisms not yet fully understood.

In this study, we analyzed the patterns and *in vitro* efficiency of the humoral response in mammals immunized with six partially purified protein fractions isolated from *P. falciparum* grown *in vitro*.

MATERIALS AND METHODS

Animals – Female BALB/c mice were obtained from an inbred colony maintained at the Institut de Recherches sur le Cancer, Centre National de la Recherche Scientifique, Villejuif, France. They were used between 8 and 14 weeks of age. Female New Zealand rabbits were purchased from Charles River, France. Animals weighing 3 kg were used, they were between 9 and 12 weeks of age. Squirrel monkeys *Saimiri sciureus* captured in the amazonic jungle (Brazil) were maintained at the Center of Research, Aggeu Magalhães-FIOCRUZ (Recife, Brazil). Adult males and females previously treated for helminthic parasitoses and weighing 465 +/– 11.5 g were selected for the present work. Phenotype-matched animals were spleen-intact, and had hematocrits 40%, haemoglobin 10g%, and negative parasitological tests for plasmodia.

Parasite – *P. falciparum*-parasitized erythrocytes (isolate "H") were obtained from a patient living in Niger (West Africa), and were adapted in our laboratory to *in vitro* culture

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according to the method of Trager & Jensen (1976). Non-synchronized cultures grown in human O Rh$^-$ red blood cells were used for antigen production.

Preparation of antigen fractions – Parasitized erythrocytes were collected when parasitaemia reached 10% and the schizont stage was observed. After two washes in sterile phosphate-buffered saline (PBS, pH 7.2), erythrocytes were lysed by 0.2% saponin (30 min at 4 °C), and the suspension was centrifuged and washed several times with sterile PBS. The pellet was homogenized in a buffer containing 0.5% (v/v) Nonidet P-40, 1% (w/v) sodium dodecyl sulphate (SDS) and protease inhibitors. The extract was sonicated, insoluble material was removed by centrifugation and the supernatant made up to a final concentration of 0.06M Tris hydrochloride (pH 6.8), 2% (v/v) glycerol and 5% <(v/v) 2-mercaptoethanol, and was then electrophoresed on 10% SDS polyacrylamide gels (Laemmli, 1970). After electrophoretic migration, gels were sliced into six strips by referring to molecular weight markers run in parallel, and the proteins were electroeluted from each fragmented gel strip, dialyzed and lyophilized. The six fractions thus obtained were defined by their apparent molecular mass ranges: Pf F1 > 94KDa; Pf F2 = 94-67KDa: Pf F3 = 67-40KDa; Pf F4 = 40-30KDa; Pf F5 = 30-20KDa and Pf F6 < 20KDa.

Immunization schedule – Mice, rabbits and monkeys were immunized subcutaneously 3, 5 or 7 times respectively, at 21 day intervals, with 50µg of each fraction. This was reconstituted just before use in 250µl physiological saline with an equal volume of Freund's complete adjuvant for the first injection and Freund's incomplete adjuvant for the others. Multiple inoculations (8-10) were performed in monkeys for each immunization.

Control animals received the adjuvant in PBS or PBS alone on the same days. Sera were collected on days 0 and 49 for all animals, 101 for rabbits, 108 and 136 for monkeys, then kept at -20 °C.

Antibody assays – IFAT was performed on acetone-fixed smears of a schizont-enriched preparation of *P. falciparum*. Specific antibodies contained in serial dilutions of serum were revealed with species-specific fluorescein isothiocyanate-conjugate (Nordic, The Netherlands). For ELISA, serial dilutions of sera (200µl/well) were incubated for 2 h at 37 °C in EIA plates (Costar) coated with 5µg/ml of an ultrasonicated *P. falciparum* lysate. After several washes with PBS-Tween 20 (0.1%), bound antibodies were detected using species-specific antiglobulin-HPO (Nordic, The Netherlands) for 2 h at 37 °C with O-phenylenediamine as substrate. Isotype-specific reagents were used to assay for IgM or IgG antibody production separately. The absorbance was read at 492 nm. Ultrasonicated red blood cell lysates were used as control antigen. Western blot analysis was performed with *P. falciparum* blood stage lysates resolved in 10% SDS-PAGE and electroblotted onto nitrocellulose as described by Towbin et al. (1979). Blocked nitrocellulose strips were incubated overnight with immune sera from mice, rabbits or monkeys. Blots were probed with species-specific antiglobulin-HPO. Isotype-specific HPO conjugates were used for detection of IgM or IgG antibodies. Invasion and growth inhibition as measured by radioisotope incorporation. To assess the inhibitory activity of each simian antiserum, a *P. falciparum* inhibition assay was performed. Briefly, H isolate-infected RBC were diluted with normal O Rh$^-$ RBC to a parasitaemia of 0.5% and hematocrit adjusted with complete medium (25mM HEPES-buffered RPMI 1640 medium (Gibco) containing 10% heat-inactivated normal serum (A+), 1% Gentamycin and 2mM L-glutamine). Non heat-inactivated monkey sera were tested in duplicate at various concentrations.

Experiments were conducted in 96-well flat-bottom microdilution plates (Nunc), comparing six test sera with two control simian sera. All samples were sterilized on 22µm pore size filters (Millipore) before use. For the assay, 9µl aliquots of non-synchronized cultures were added to 121µl of complete medium and 50µl of diluted monkey serum. The wells were pulsed with 0.4 uCI (3H) Hypoxanthine (Amersham Corp.) and the cultures incubated for 72 hours at 35.7 °C according to the method of Trager & Jensen (1976). Individual well contents were then collected onto glass filtre-paper with a semi-automated sample harvester (Skatron, Norway) and radioisotope incorporation was measured with a liquid scintillation counter (Beckman LS1701, UK).

Percentage inhibition of parasite growth was calculated using the following formula:

$$I = \frac{\text{(1 - cpm of parasite culture with serum) x 100}}{\text{cpm of parasite culture without serum}}$$

(where cpm = counts per minute).

Monoclonal antibodies – The monoclonal antibodies used in this study were 2.13 (against rhoptry proteins); 12.3, 12.5 and 13.4 against Merozoite Surface Antigen (MSA-2). They were gifts of Dr R. Ridley and Dr J. Mc Bride of Edinburgh University.

RESULTS

Composition of P. falciparum *antigenic preparations* – Electroeluted *P. falciparum* fractions were reanalyzed on 10% SDS-PAGE and the gels stained with silver nitrate (See Figs 1, 2). mAb 2.13 recognized by immunoblotting 5 proteins with Mr 69, 63 (in Pf F2) 40, 36, 34 kDa in Pf F4.

Pooled mAbs 12.3, 12.5 and 13.4 identified two bands with Mr 49-50 and 55 kDa in Pf F3 (Fig. 3).

TABLE I

IgG reactivity in Western-blotting of simian antisera to *Plasmodium falciparum* polypeptides (sample on D49)

Polypeptide	Antiserum		
Mr (kD)	Pf F1	Pf F2	Pf F3
> 150	+		
85		++	
81-78			
69-64	+	+++	+
44-40		+	+++

TABLE II

IgG reactivity in Western-blotting of simian antisera to *Plasmodium falciparum* polypeptides (sample on D49)

Polypeptide	Antiserum	
Mr	Pf F4	Pf F5
37 kD	++	
30 kD		+++

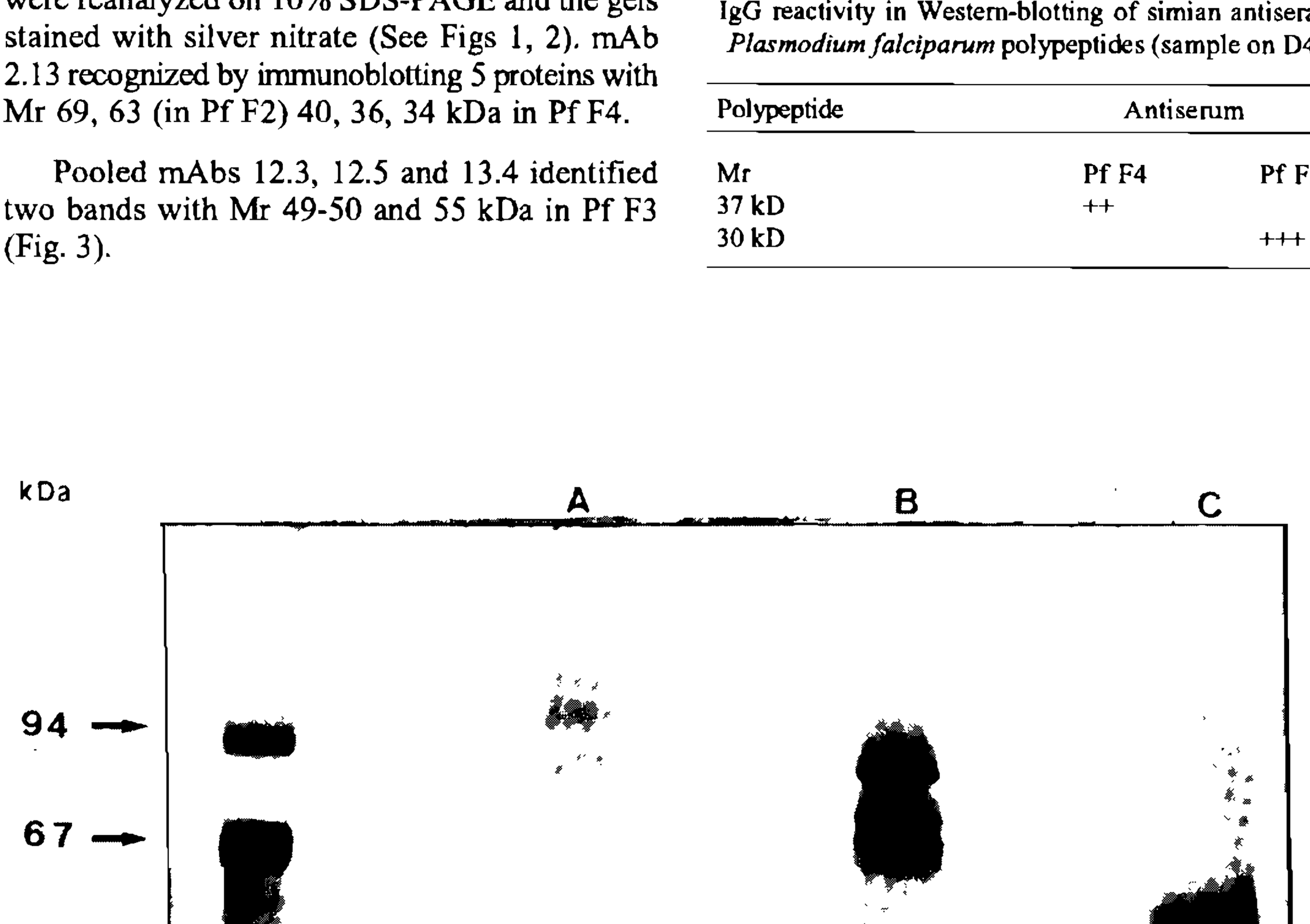
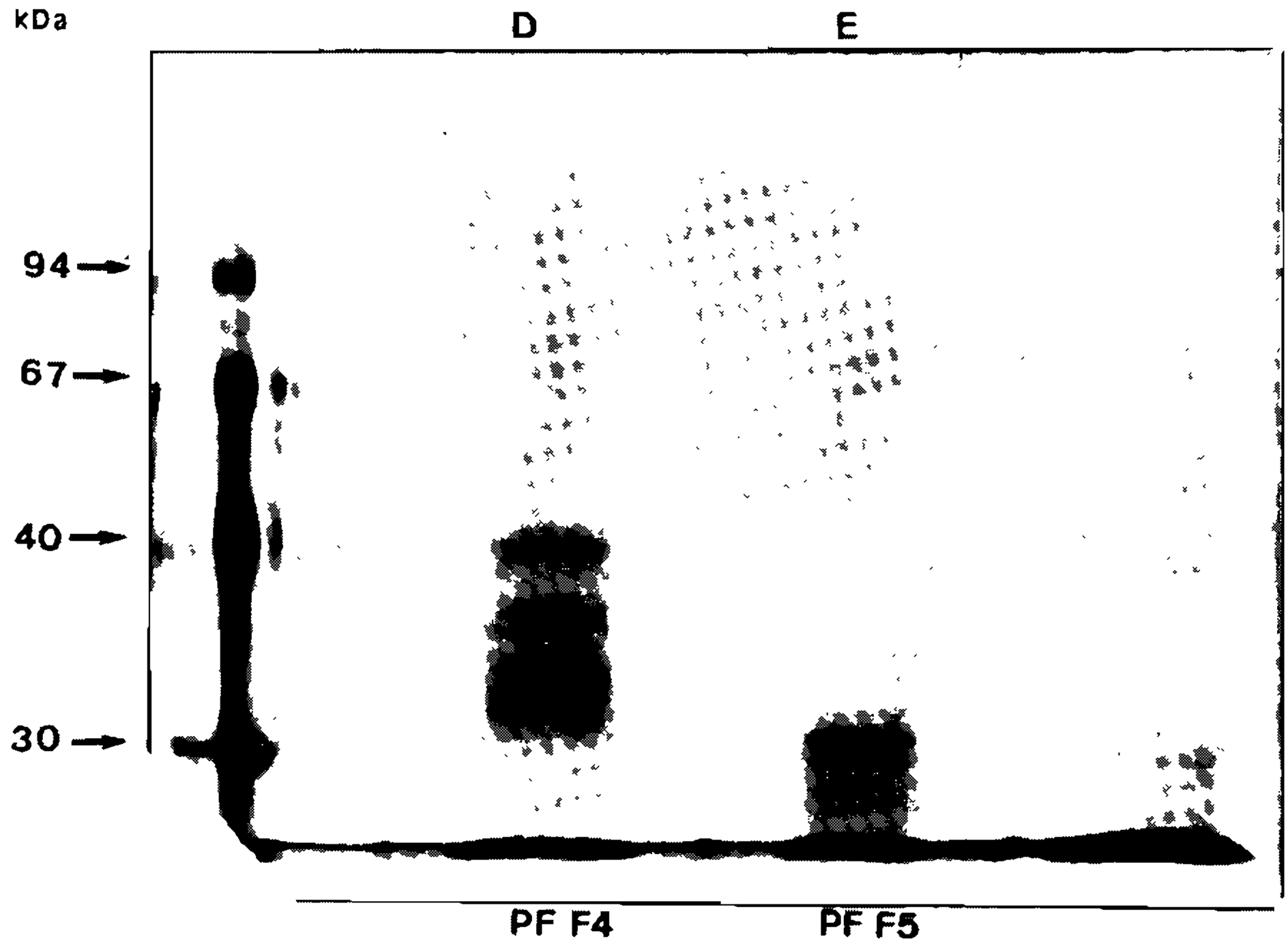


Fig. 1: silver nitrate stained gel 10% SDS-PAGE. Analytical electrophoresis of Pf F1, Pf F2 and Pf F3 fractions.



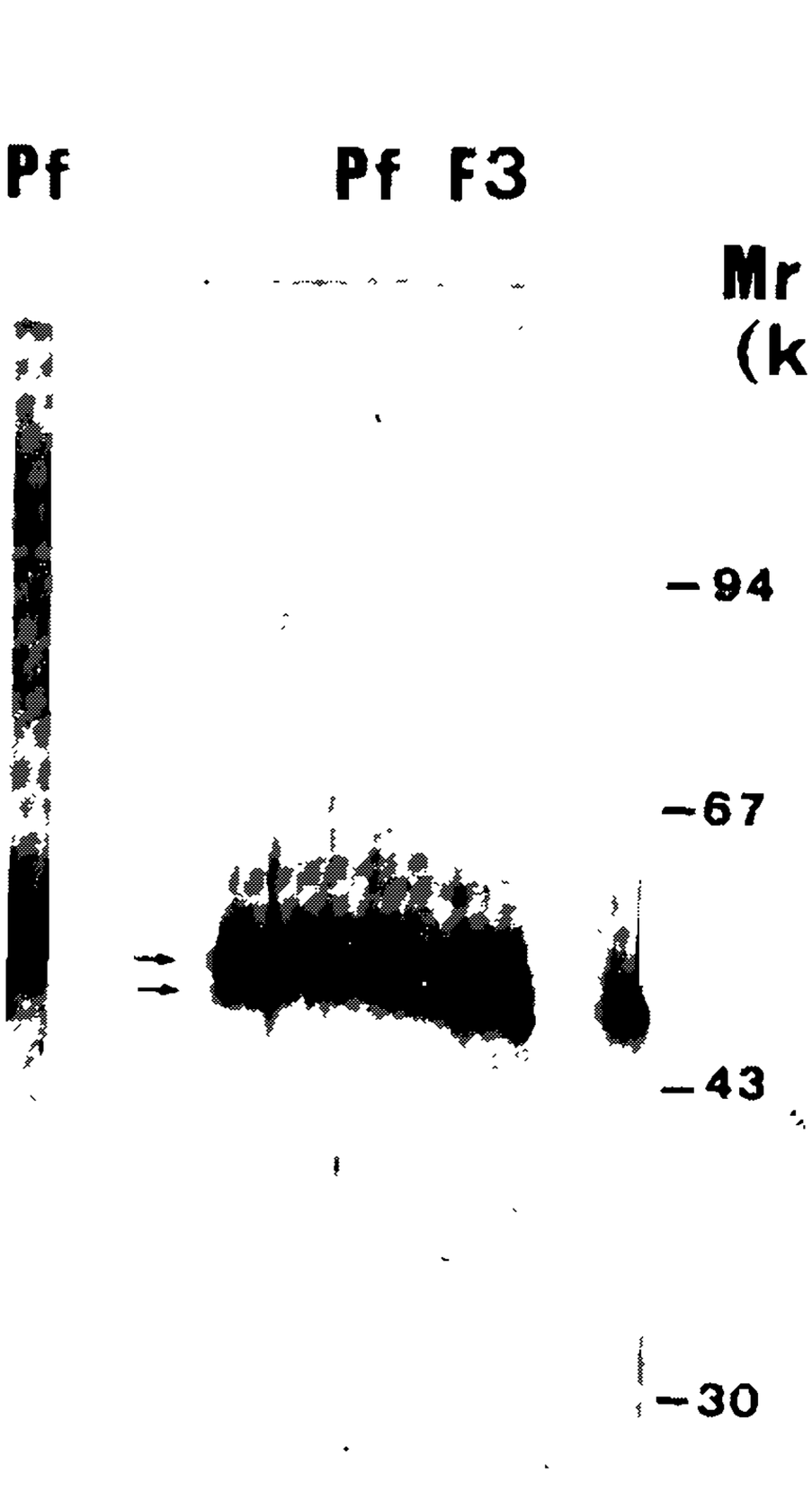


Fig. 3: immunoblotting with a *Plasmodium falciparum* extract separated in 10% SDS-PAGE and electroblotted onto nitrocellulose. Pf F3: separated in 10% SDS-PAGE. Pooled mAbs anti-MSA 2 recognized two polypeptides (49-50 and 55 kDa).

TABLE III

IgG reactivity in Western-blotting of simian antisera to *Plasmodium falciparum* polypeptides (D 136)

Polypeptide	Antiserum		
Mr(kD)	Pf F1	Pf F2	Pf F3
> 150	++		+
138-135	++	+	+
89-85		+++	
70-68	++	++++	
64		+++	
44-40	+		+/–

TABLE IV

IgG reactivity in Western-blotting of simian antisera to *Plasmodium falciparum* polypeptides (D 136)

Polypeptide	Antiserum	
Mr (kD)	Pf F4	Pf F5
> 150		++
138-135		++
102-93	+	++
85		++
77	+	
49-44		++
40-36-34	+/++	
30-26		++

Isotypes of malaria antibodies as measured by ELISA – Varied levels of anti-Pf F antibody titres were observed in all immunized animals. When isotypes of antibodies were analyzed, *Saimiri*

monkeys had decreasing titres of IgG but increasing levels of IgM between the 3rd and 6th immunizations.

IgG and IgM reactivity against P. falciparum *polypeptides* – The number of protein components linked to IgG antibodies, but not to IgM, increased with the number of immunizations. Tables I-IV summarized these results.

In vitro growth inhibition – No inhibition of parasite proliferation was observed with anti-Pf F1, Pf F3, Pf F6 and control sera. In contrast, anti-Pf F2, Pf F4 and Pf F5 immune sera at several concentrations induced partial inhibition (Tables V, VI).

TABLE V

Inhibitory effects of immune monkey sera on *Plasmodium falciparum* cultures

Concentration[a]	% Growth inhibition by antiserum		
%	Pf F2	Pf F4	Pf F5
4	NA	28.8	28.1
2	59.3	17.7	17.6
1	37.8	0	10.6
0.5	0	0	0

a: final concentration of antiserum. NA: non available

DISCUSSION

The purpose of the present study was to determine the immunogenicity of six denaturated *P. falciparum* protein fractions and, eventually, the *in vitro* efficiency of the humoral immune response induced in mammals injected with these antigenic preparations.

Using three different serological techniques, specific malaria antibodies were detected and analyzed in mice, rabbits and monkeys. The immunofluorescent staining pattern obtained with murine, rabbit or simian antisera did not differ widely (data no shown). All fraction-induced antibodies elicited almost similar diffuse cytoplasmic reactivity with acetone-fixed smears of *P. falciparum* parasitized erythrocytes ("H" isolate), regardless of animal species involved. The humoral response in squirrel monkeys was much more promising than in rabbits and mice, so additional analyses were carried out on the former.

By Western blotting of simian sera we found that samples collected after the 6th vaccination recognized a larger number of polypeptides than sera after the third vaccination. However, this increased reactivity was observed for IgG but not for IgM antibodies and was more marked with antisera to Pf F2, Pf F4 and to a lesser extent Pf F5.

These observations and the *in vitro* partial antiplasmodial inhibitory activity deployed by the above mentioned three-fraction simian antisera (as measured by radioisotope incorporation assay), suggest the presence in the corresponding fractions (Pf F2, Pf F4 and Pf F5) of critical antigens capable of inducing the production of neutralizing antibodies.

It seems that there are rhoptry antigens among the polypeptides in Pf F2 and Pf F4 which were detectable by mAb 2.13. Based on this information, it is conceivable that these fractions could exert their action by inducing antibody formation in squirrel monkeys, which can bind to rhoptry antigens, thereby inhibiting merozoite invasion of erythrocytes *in vitro*.

Experiments are under way in our laboratory to further characterize these fractions.

TABLE VI

[^{3}H] Hypoxanthine incorportation by *Plasmodium falciparum* cultures with immune monkey sera (IMS) or control sera (CS)

Concentrations[a]	Mean CPM of culture with IMS or CS			
%	Pf F2	Pf F4	Pf F5	CS
4	NA	9070.1[b]	9461.6[b]	12122.5
2	4468.0[b]	10491.6	10939.1	12610.3
1	8345.0[b]	12409.1	11773.2	13037.3
0.5	12981.1	12342.6	133.46.7	13203.5

a: final concentration of serum NA: non available
b: statisticaly significant difference

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