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PROTECTION OF AOTUS MONKEYS AFTER IMMUNIZATION WITH RECOMBINANT ANTIGENS OF *PLASMODIUM FALCIPARUM*

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The genus Aotus spp. *(owl monkey) is one of the WHO recommended experimental models for* Plasmodium falciparum *blood stage infection, especially relevant for vaccination studies with asexual blood stage antigens of this parasite. For several immunization trials with purified recombinant merozoite/schizont antigens, the susceptible Aotus karyotypes II, III, IV and VI were immunized with* Escherichia coli *derived fusion proteins containing partial sequences of the proteins MSAI (merozoite surface antigen I), SERP (serine-stretch protein) and HRPII (histidine alanine rich protein II) as well as with a group of recombinant antigens obtained by an antiserum raised against a protective 41 kD protein band. The subcutaneous application (3x) of the antigen preparations was carried out in intact animals followed by splenectomy prior to challenge, in order to increase the susceptibility of the experimental hosts to the parasite. A partial sequence of HRPII, the combination of three different fusion proteins of the 41 kD group and a mixture of two sequences of SERP in the presence of a modified Al(OH)3 adjuvant conferred significant protection against a challenge infection with* P. falciparum *blood stages (2-5 x 10^6) i. RBC). Monkeys immunized with the MS2-fusion protein carrying the N-terminal part of the 195 kD precursor of the major merozoite surface antigens induced only marginal protection showing some correlation between antibody titer and degree of parasitaemia. Based on the protective capacity of these recombinant antigens we have expressed two hybrid proteins (MS2/SERP/HRPII and SERP/MSAI/HRPII) in* E. coli *containing selected partial sequences of SERP, HRPII and MSAI. Antibodies raised against both hybrid proteins in rabbits and Aotus monkeys recognize the corresponding schizont polypeptides. In two independent immunization trials using 13 animals (age 7 months to 3 years) we could show that immunization of Aotus monkeys with either of the two hybrid proteins administered in an oil-based well tolerated formulation protected the animals from a severe experimental* P. falciparum *(strain Palo Alto) infection.*

Key words: *Plasmodium falciparum* – Aotus monkeys – blood stage antigens – recombinant polypeptides – hybrid proteins – protection

The increasing spread of multidrug-resistant malaria demands the development of an effective vaccine against the major pathogen *Plasmodium falciparum.*

It is obvious that the enormous amount of protective malarial antigens required for a worldwide vaccinational programme can only be produced by recombinant DNA technology as a basis for large-scale vaccine production.

Recombinant vaccines need to include several of the antigenic components of the parasite capable of eliciting a protective immune response in the recipient, based on appropriate B-cell as well as T-cell epitopes within these antigens.

A malaria vaccine has to protect individuals, especially children living in endemic areas, therefore the primary immunization must be able to establish T-cell memory that responds to boosting from subsequent natural infection.

Necessary prerequisites for vaccine development are the identification and characterization of particular antigens from the complex array of hundreds of malarial proteins that characterize each life cycle stage.

It is well accepted that the merozoites and schizont infected erythrocytes appear to be the

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most promising targets for the development of a vaccine, because they are the only asexual stages which actually expose antigens to the immune system of the host and the erythrocytes represent the only cells for the merozoite to invade.

In early studies (Mitchell et al., 1977) it was shown that vaccination of owl monkeys with crude preparations containing merozoites and Freund's complete adjuvant protected against a *P. falciparum* challenge. This protection was long lasting and raised hopes that an antimerozoite vaccine might be feasible, if it is composed of defined antigens that could be administered with a clinically acceptable adjuvant in humans.

The location of an immunorelevant antigen is of major consideration in determining the vaccine candidacy of an asexual blood-stage antigen. High priority has been considered to structures of the merozoite and schizont infected erythrocyte surface and to the merozoite secretory organelles (rhoptries) because they are obviously directly accessible to immune attack.

Many merozoite and schizont antigens have been identified and their genes are cloned and sequenced. Among others, protein bands eluted from SDS-gels of 200, 140 and 41 kD conferred partial protection to Saimiri monkeys (Perrin et al., 1984a, b, 1985).

Our objective was to isolate the coding sequences for these protective antigens, to express these sequences in *E. coli,* and to test the expression products as single proteins or either fused to hybrid proteins for their protective potential against an experimental *P. falciparum* infection in Aotus monkeys.

In vivo – immunization/challenge studies in Aotus – or Saimiri-monkeys are an obligatory criterion for selection of protective antigens to be included in antimalarial vaccines.

The best model for assessing asexual *P. falciparum* blood stage vaccine candidates is *Aotus lemurinus griseimembra* from Columbia (WHO, 1988). Since this species/ karyotype is available only in very limited numbers, the Bolivian Aotus monkey (*A. azarae boliviensis*) is an alternative model with adequate susceptibility to *P. falciparum* infection if the animals are splenectomized.

MATERIAL AND METHODS

Monkeys – The monkeys (*A. lemurinus griseimembra* and *A. azarae boliviensis*) were bred in the animal facilities of Behringwerke AG. Each experimental group was matched for weight, sex and age. No previous exposure to *P. falciparum* of *P. brasilianum* was evident according to serology and clinical history. All monkeys were splenectomized 6-8 days after the last immunization and 7 days prior to challenge.

Adjuvants – (a) 10% modified alhydrogel: consisting of 50% Al(OH)3 (2% conc.), 45% lecithin (20% conc.) and 5% aescin (SCHWABE, Karlsruhe; FRG); (b) 10% Polyalphaolefine (PAO): a highly branched isoparaffinic synthetic white oil (ESSO; MW ~ 500), water in oil emulsion.

Both adjuvant formulations were developed in our laboratories and were proven to be well tolerated and effective in different animal models (Enders et al., 1990).

Antigens – From previous vaccination experiments it was shown that the native purified protein bands (SDS-PAGE) with molecular weights of 200, 140 and 41 kD induced protective immunity in Saimiri monkeys. (Perrin, et al., 1984a, b, 1985). In our laboratories we have isolated the coding sequences of these proteins as well as from HRP II, expressed parts of these sequences in E. coli and purified the expression products for immunization purposes.

Malarial antigens characterized by Behringwerke AG (Table).

Single recombinant antigens

MSA I (PMMSA, gp 195) – This antigen is a predominant glycoprotein of 195 kD in schizonts recognized by patient sera (Holder et al., 1985). This protein exhibits antigenic polymorphism, however it contains conserved regions (Peterson et al., 1988). Using an antiserum raised against the native 195 kD protein band we have isolated a DNA fragment coding for part of the conserved N-terminal sequence from which the repeat region was deleted, and expressed the sequence in *E. coli.*

SERP (113 kD) – By screening of a λgt11 library from *P. falciparum* schizont cDNA with an antiserum raised against the protective 140

TABLE

Malaria antigens (*Plasmodium falciparum*) characterized by Behringwerke AG

Antigens	Protective (native protein)	Localization	Function	MW (kD)	Cloned gene fragment	Cloned gene complete	Protective (recombinant fragment)
gp 195	+	merozoite-surface		195	+	–	–
SERP	+	parasitophorous	protease	113	+	+	(+)
SERP H	n.d.	vacuole		130	+	+	n.d.
HRPII	n.d.	erythr. surface secreted	?	72	+	+	+
41-2	n.d.	schizont-membrane; erythr.-surface; transport-membrane	?	29	+	+	–
41-3	+	n.d.	?	41	+	+	(+)
41-7		cytoplasma (membrane assoc.)	aldolase	41	+	+	–
GBPH	(+)	n.d.	?	40	+	+	n.d.

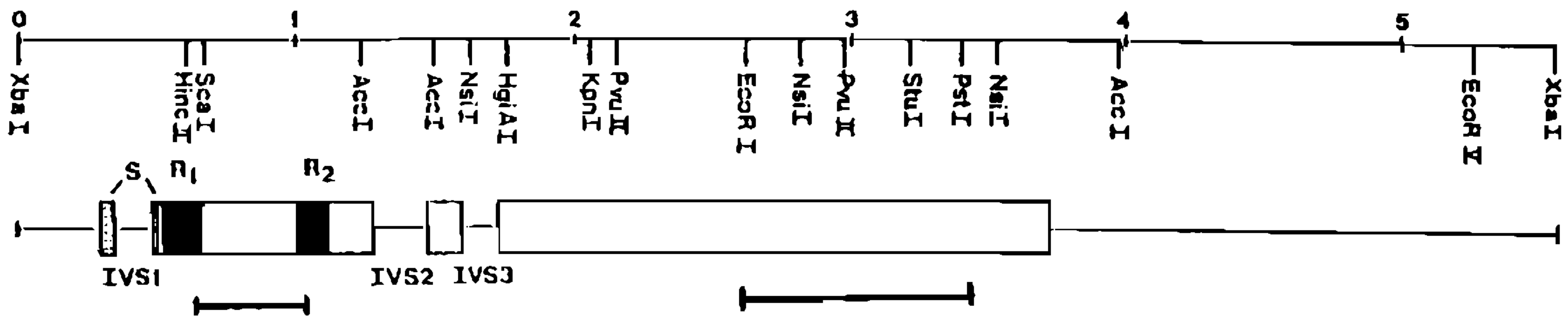
kD protein band two corresponding phage clones were isolated. Their insert DNAs were used to identify and clone a genomic fragment which carries the entire sequence coding for a protein of 113 kD characterized by a stretch of 37 serine residues (SERP) (Knapp et al., 1988). Different protein regions of SERP were expressed in *E. coli* and used to determine the capacity of human lymphocytes to recognize these subfragments. We defined one region of SERP which was recognized by human lymphocytes, allowing the identification of two T-cell epitopes on a narrow region of SERP (Roussilhon et al., 1990). This region of the SERP antigen, which was shown to be well conserved between different isolates, was selected as a vaccine component.

Figure 1 shows the restriction map and structure of the SERP gene. The regions which were used for vaccination of Aotus monkeys are indicated.

41-3 Protein – Using an antiserum raised against the protective 41 kD protein band for screening of λgt11 libraries, we have isolated 16 phage clones coding for different gene products. Three of these clones – 41-1, 41-2, and 41-3 – were analysed in more detail. The insert DNA of the phage clone 41-3 was used to isolate the entire 41-3 gene. The genomic structure is very complex: Nine exons are interrupted by eight intervening sequences. The 41-3 protein carries a hydrophobic N-terminal region typical for a signal sequence. Assuming that this sequence is cleaved off, the molecular weight of the protein is 41.2 kD and therefore the 41-3 protein could well be a component of the protective 41 kD protein band.

HRP II – This antigen also found by crossreaction with the antiserum raised against the 41 kD protein band was identified as the histidine-alanine rich protein HRP II (Knapp et al., 1988). This protein is characterized by an extended repeat region consisting of the tripeptide Ala-His-His and the tripeptide Ala-Ala-Asp (Wellems & Howard, 1986; Knapp et al., 1988). HRPII antigens of different *P. falciparum* strains show only slight variability in number and arrangement of the Ala-His-His tripeptide repeats (Knapp et al., 1988). The HRP II antigen was shown to be released from the infected red blood cell (Howard et al., 1986). However, data from cell surface radioiodination have shown that HRP II stays also associated with the outer surface of the erythrocyte membrane (Rock et al., 1987). Parts of the repeat region were expressed in *E. coli.*

P. falciparum-*Hybrid proteins* – Based on investigations on the blood stage antigens



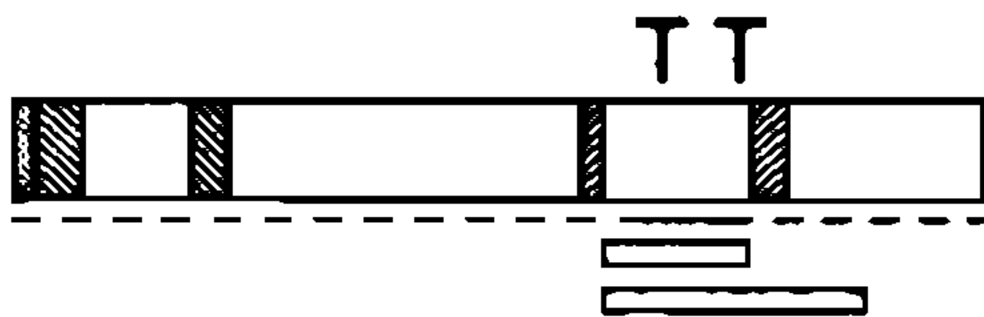
R1 = repeat 1 = 6 x Thr – Gly – Gly – Ser/Gly – Gln – Ala/X – Gly – Ser/Asn

R2 = repeat 2 = 19 x Ser – Ser

IVS = intervening sequences 1, 2, 3

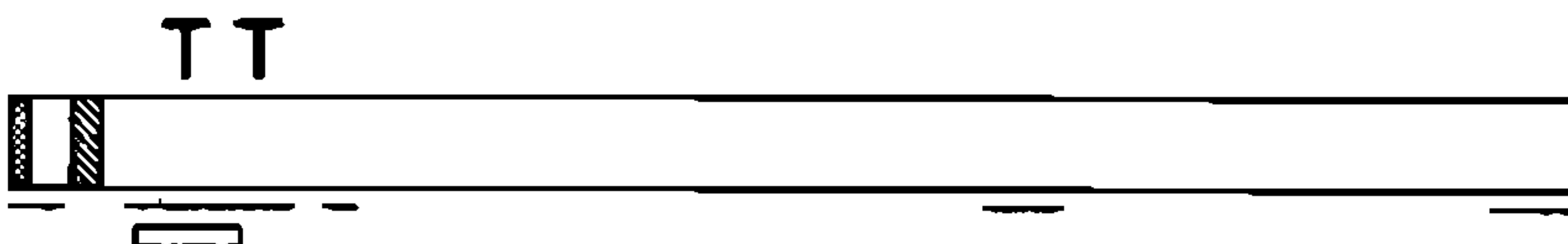
Fig. 1: restriction map and structure of the SERP I gene. Corresponding to the restriction map of the upper lane the gene structure is shown schematically. The coding regions (boxes) which carry the sequences coding for the putative signal sequences (S) and for both repeat sequences (R1, R2) are separated by three intervening sequences (IVS1, IVS2, IVS3).

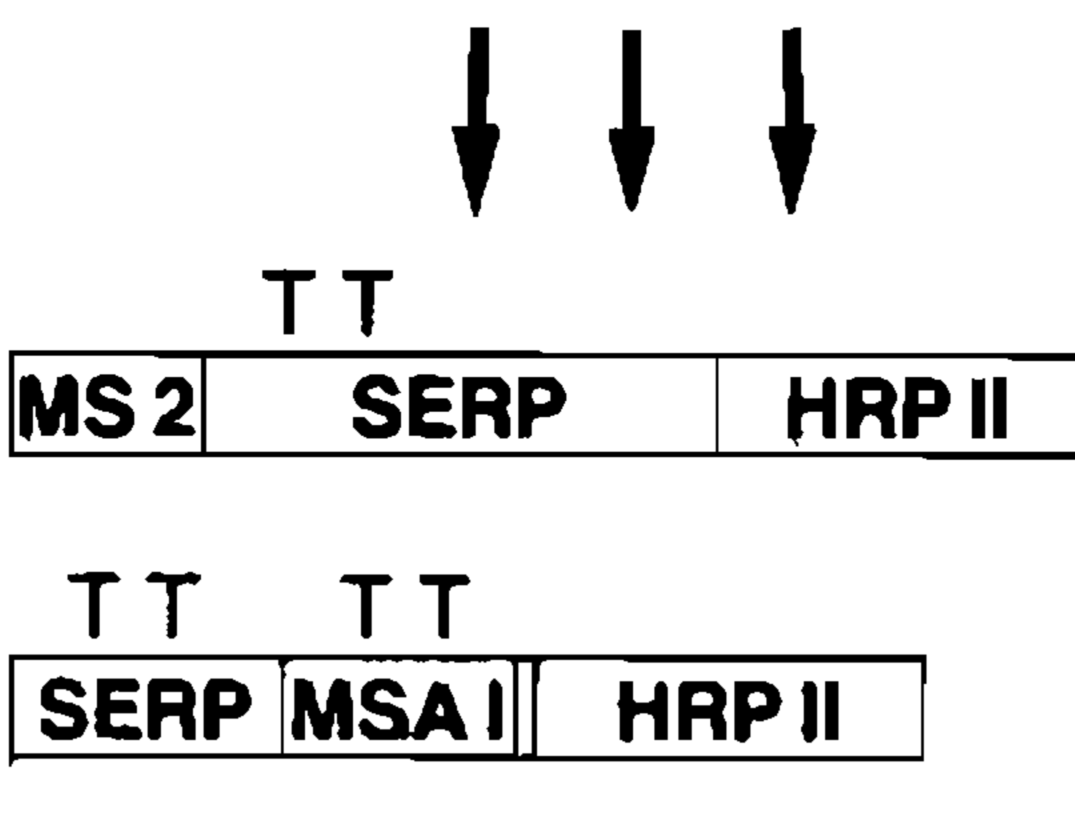
SERP (113 kD; 984 aa), parasitophorous vacuole; protease?



HRP II (72 kD; 327 aa), secreted; surface of parasitized erythrocyte

MSA I (195 kD; 1720 aa), merozoite surface





leader sequence

repetitive region

sequence homologous to cysteine proteinases

conserved region

sequence selected for vaccine construction

Fig. 2: selection of partial protein sequences for the construction of hybrid vaccine antigens.

SERP, HRP II and MSA I we have expressed two hybrid proteins in E. coli containing selected partial sequences of these antigens. Antibodies raised against both hybrid proteins in rabbits and Aotus monkeys recognize the corresponding schizont polypeptides.

Figure 2 shows a selection of partial protein sequences for the construction of hybrid vaccine antigens.

MS2/SERP/HRP II – The construction of the SERP/HRP II hybrid gene and its expression as MS2-polymerase fusion protein in *E. coli* using the pEX31b vector was described by Knapp et al. (1991). This fusion protein comprises amino acids 631-892 of the SERP antigen (Knapp et al., 1989), a region which includes two T-cell epitopes between amino acid positions 640 and 700 (Roussilhon et al., 1990) and a sequence being homologous to cysteine proteinases from amino acid positions 745 to 786 (Higgins et al., 1989). Additionally, the hybrid gene encodes the 189 C-terminal amino acid residues of HRP II (Knapp et al., 1988).

SERP/MSA I/HRP II – The hybrid antigen SERP/MSA I/HRP II (Knapp et al., 1992) carries aa 630-764 of SERP (Knapp et al., 1989) initiating translation at met-630, followed by amino acid residues 146 to 260 corresponding to MSA I (Holder et al., 1985), a conserved region additional including two T-cell epitopes (Crisanti, A. et al., 1988). The resulting hybrid antigen carries at least four T-cell epitopes and was combined with the 189 C-terminal amino acid residued of HRP II (Knapp et al., 1988). The antigen is expressed in *E. coli* using the vector p TRC.

The hybrid antigens MS2/SERP/HRP II and SERP/MSA I/HRP II migrate as 75 kD and 62 kD proteins in SDS-polyacrylamide gels, respectively, and were detected by Western blot analysis using antisera raised against the corresponding partial sequences of SERP, HRP II and MSA I. Both hybrid proteins induce antibodies in rabbits which recognize the corresponding malarial proteins SERP, HRP II and MSA I from *P. falciparum* schizonts to a similar extent, determined by Western blot analysis. This result shows that none of the fusion partners of the hybrid protein dominates the immune response.

Figure 3 shows the schematic representation of two hybrid proteins expressed by *E. coli*.

Production and purification of expression products – The heat induction of the bacterial cultures were performed as described by Küpper et al., (1982) and the analysis of the expression products was carried out by SDS-polyacrylamide-gel-electrophoresis (SDS-PAGE). For large scale antigen production 5 liter cultures were used. After heat induction, cells were centrifuged and disruptured by french press treatment. The soluble components were removed by centrifugation and the pellet containing the expression products were washed twice with PBS and subsequently treated with increasing concentrations of urea until the fusion protein was solubilized. The protein solution was dialyzed stepwise against decreasing urea concentrations down to the minimal concentration sufficient to keep the protein soluble. During these steps most of the *E. coli* contaminants were removed. The purification was monitored by SDS-PAGE.

Immunization – The antigens (1,0 ml) were

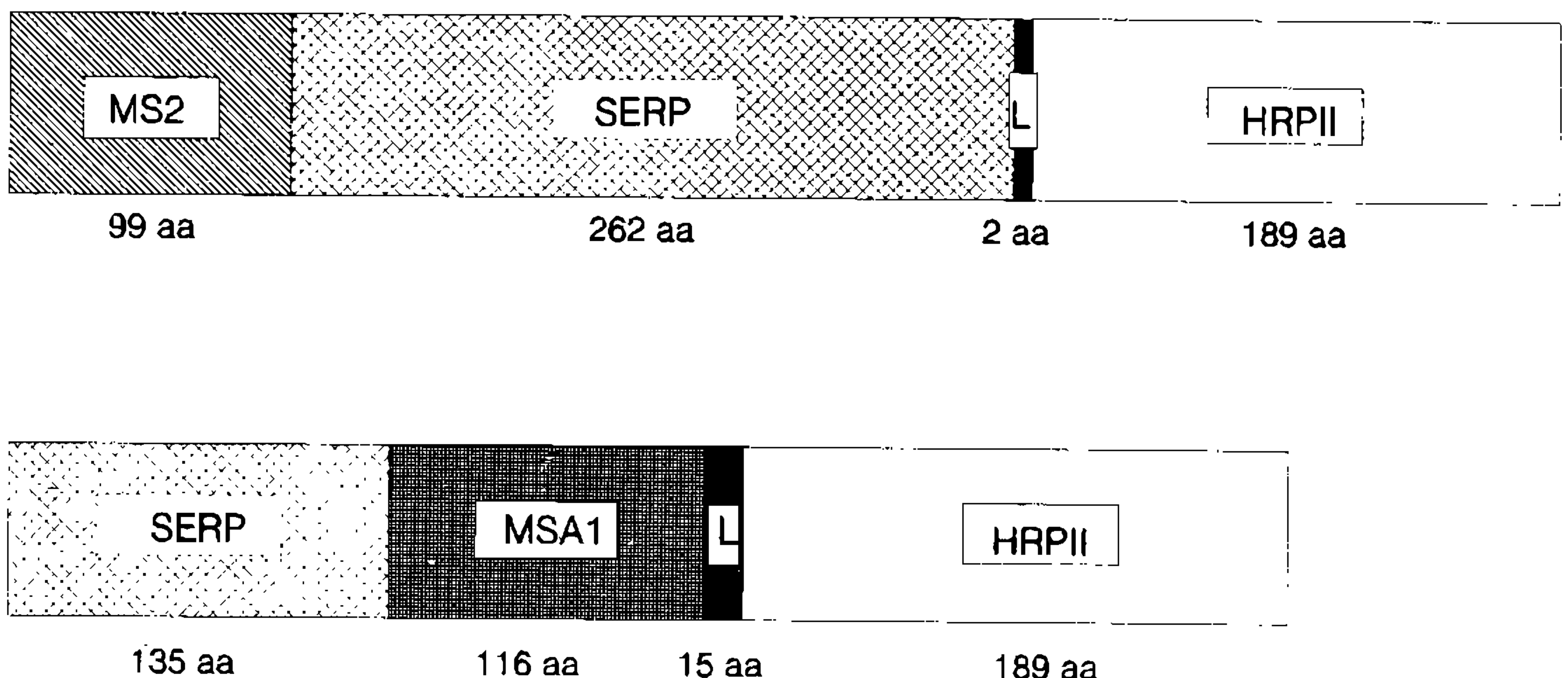


Fig. 3: combination-expression of *Plasmodium falciparum* antigens in *Escherichia coli*.

injected subcutaneously in intact monkeys on days 1, 22, 43. The vaccine preparations contained always of 100 μg fusion protein/ml in PBS 3 m urea or either the solvent alone, respectively, mixed with 10% of the modified $Al(OH)_3$-or PAO adjuvant.

Parasites – Two *P. falciparum* isolates, FUP Palo Alto (Uganda) and FCB-1 (Columbia) previously adapted to Aotus monkeys were used for challenge. Parasite infected monkey blood from liquid nitrogen stocks was thawed and used directly to infect the donor monkeys.

Parasite challenge – After splenectomy of the monkeys on day 48-53, they were challenged on day 58-60 with 5 x 10^6 parasitized erythrocytes (2 x 10^6 in younger animals) injected i.v. obtained from a donor monkey.

Parameters – Blood samples for serological analysis were taken at weekly intervals. Serumconversion was determined by indirect immunofluorescence (IFT) on acetone-fixed schizont-infected erythrocytes; specific binding was visualized with FITC-labelled anti human-Ig. ELISA was performed in microtiter plates coated with the respective recombinant proteins. Binding of specific antibodies was detected with peroxidase-labelled anti human-IgG. Parasitemia was determined daily on Giemsa stained blood smears and expressed as the percentage of parasitized erythrocytes. Monkeys with parasitemias exceeding 15% were treated with Mefloquine (Lariam^R) which was shown to be effective against the Chloroquine resistant challenge strains used.

Blood (serum and plasma) was collected weekly and monitored for clinico-chemical parameters (erythrocytes, hematocrit, blood sedimentation rate, serumenzymes GOT, GPT, and LDH).

RESULTS

Trial 1 – We used 9 Aotus monkeys which were divided into 3 groups. Group 1 received the C-terminal half of HRP II; Group 2 was immunized with the N-terminal sequence of MSA I expressed and purified in an analogous manner. Group 3 served as a control and was treated with PBS 3M urea and adjuvant.

The course of *P. falciparum* infection in control and immunized monkeys is presented in Fig. 4. All three monkeys immunized with the HRP II fusion protein were protected against the challenge infection as demonstrated by delayed and low parasitemias (< 2%). One monkey in this group showed total protection – only a few parasites per microscopic slide could be detected on day 10, whereas the following examinations were parasitologically negative.

In contrast the control group as well as the animals immunized with the MSA I fusion protein showed parasitemias higher than 4%. One monkey had to be treated with Mefloquine after the parasitemia had reached 15%. Following this treatment the monkey survived. In the other non protected animals the challenge infection was controlled without treatment or supportive care and parasites were no longer detectable in the peripheral blood after days 25 to 30.

All vaccinated monkeys showed high antibody responses against the respective recombinant antigens with maxima at the time of challenge.

Immunofluorescence titers were low but significant during the immunization period, increased after challenge and persisted after clearance of parasitemia.

Figure 4 shows the parasitemia after *P. falciparum* challenge infection of Aotus monkeys immunized with HRP II fusion protein (A) and with the MSA I partial sequence (B) or with adjuvant alone as a control (C).

Trial 2 – In this vaccination experiment we immunized 3 Aotus monkeys with a combination of the MS2 fusion proteins 41-1, 41-2 and 41-3. Vaccination, splenectomy and challenge were identical to trial 1. Modified Al(OH)3 was used as adjuvant. As demonstrated by parasitological examination infection was completely controlled by the immunized monkeys in contrast to the nonimmune animals which reached much higher peak parasitemias and two of which had to be treated with Mefloquine.

Figure 5 shows parasitemias in Aotus monkeys vaccinated with a combination of MS2 fusion proteins 41-1, 41-2 and 41-3.

Trial 3 – From a subsequent experiment (identical design as for trials 1 and 2) with 3 Aotus monkeys per group, immunized with the single MS 2 fusion proteins 41-1, 41-2 and 41-3 it could be concluded that antigen 41-3 was

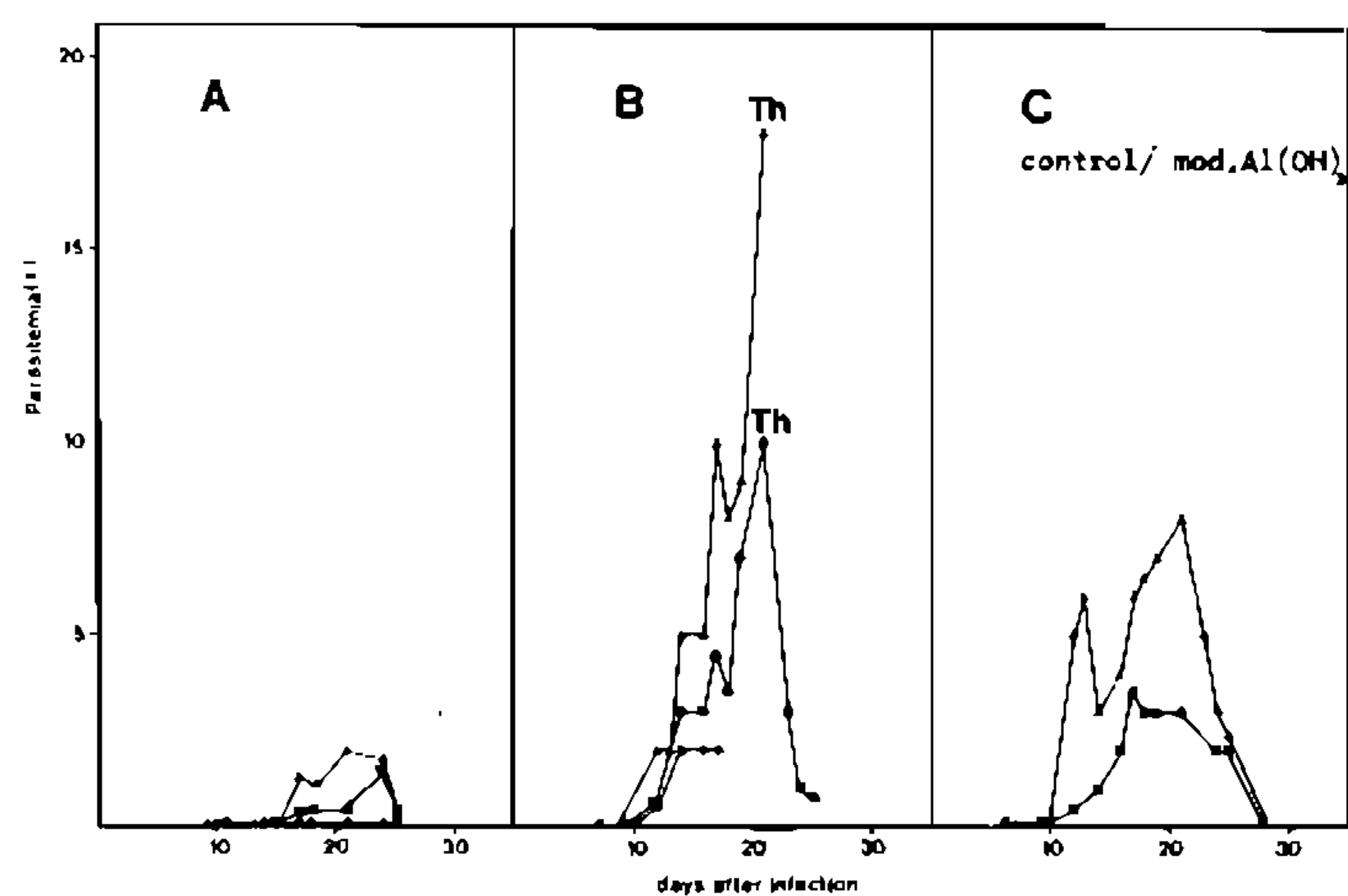


Fig. 4: parasitemia after *Plasmodium falciparum* challenge infection of Aotus monkeys immunized with HRP II fusion protein (A) and with rec. MSA I antigen (B).

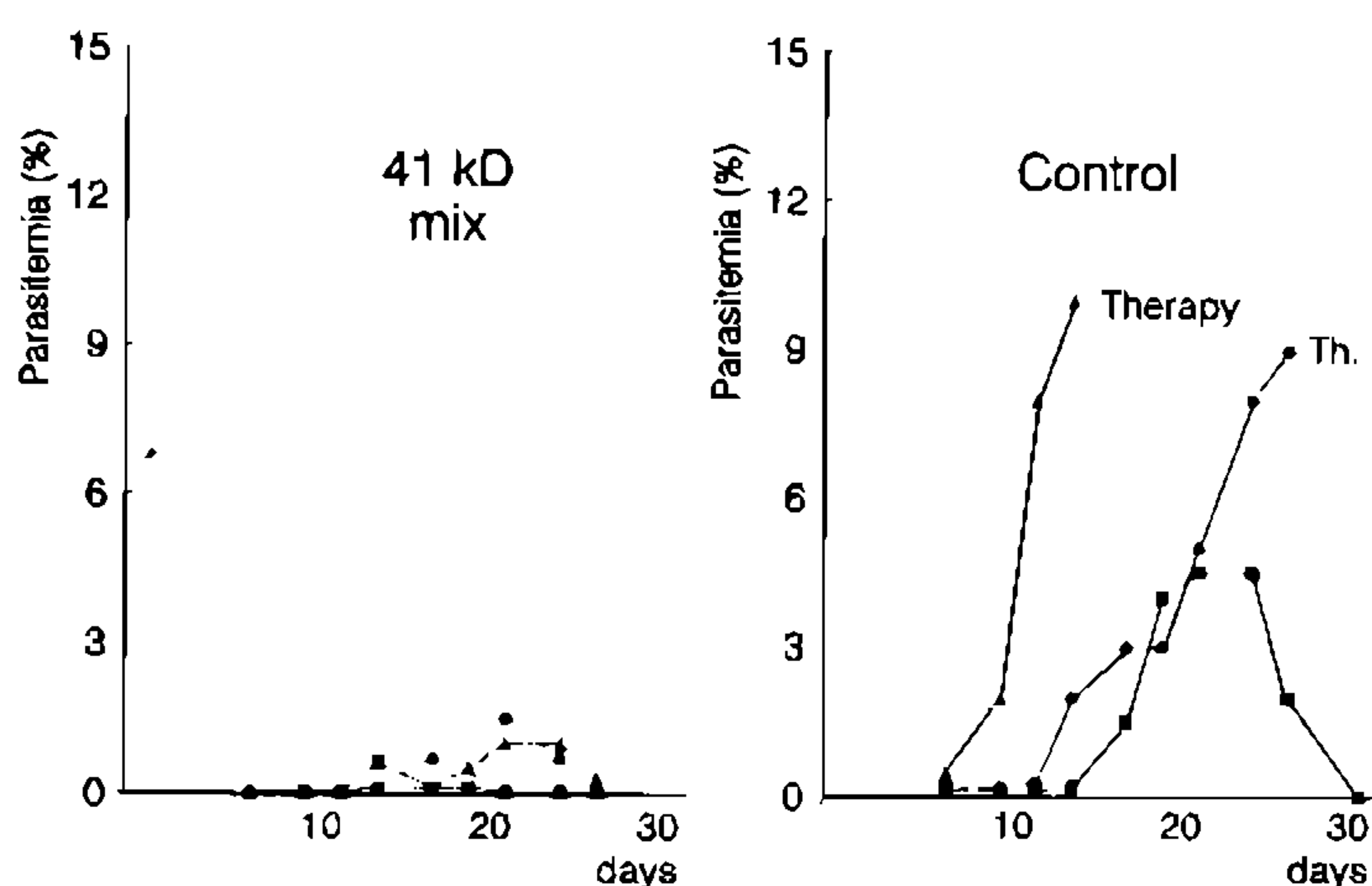


Fig. 5: parasitemias of Aotus monkeys vaccinated with a combination of MS2 fusion proteins 41-1, 41-2, 41-3 and control.

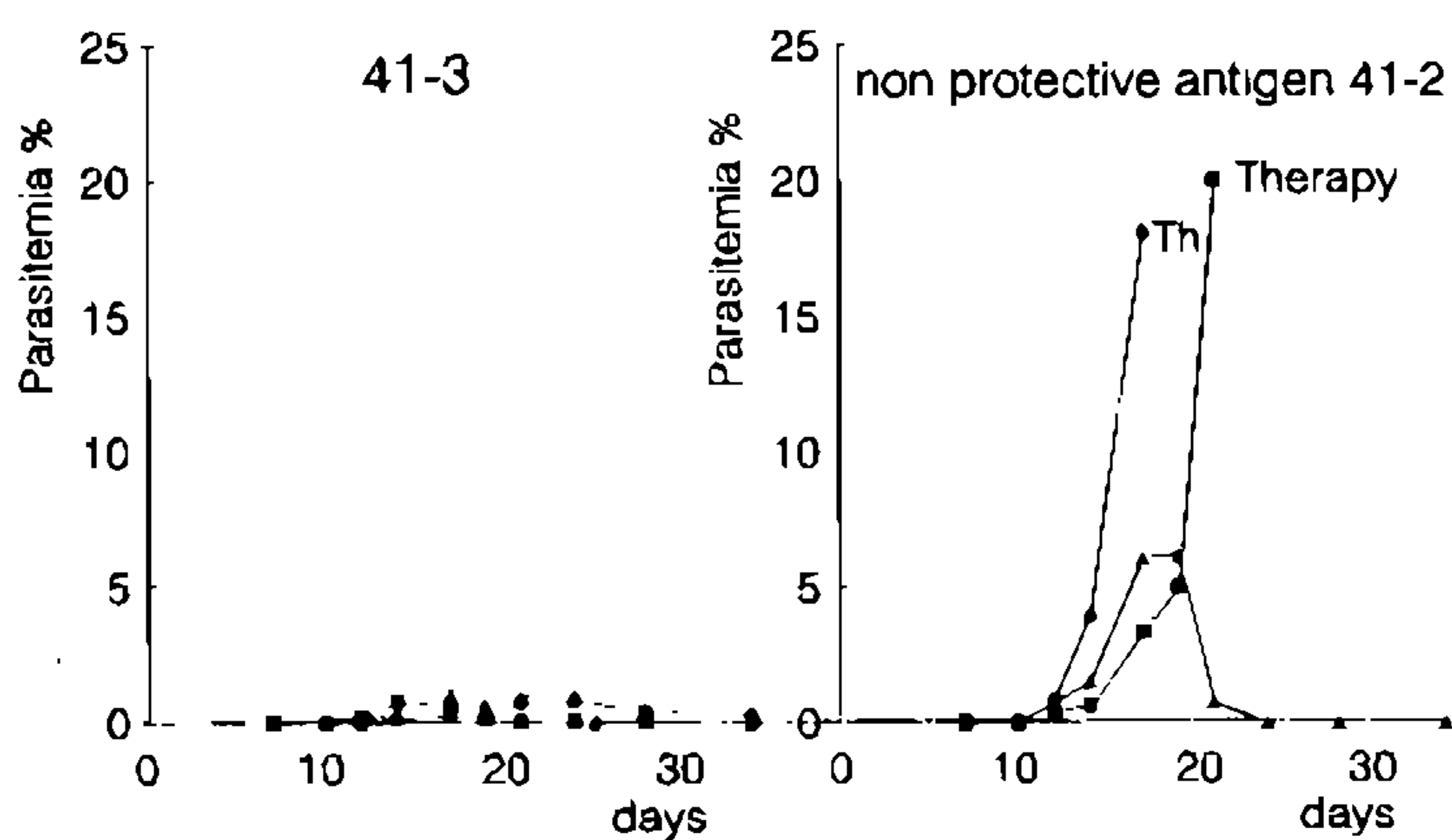


Fig. 6: parasitemias of Aotus monkeys vaccinated with MS2 fusion protein 41-3 and 41-2.

the constituent responsible for the protective effect obtained with the antigen mixture in trial 2.

Figure 6 shows parasitemias in Aotus monkeys vaccinated with MS 2 fusion proteins 41-2 and 41-3.

Trial 4 – Four Aotus monkeys were immunized by a schedule identical as in the a.m. trials with a combination of aa 68-184 and aa 631-892 of the SERP antigen, both fused to MS 2 polymerase. All treated monkeys developed partial protection in comparison to the non-vaccinated group. Protection of Aotus with partial sequences of SERP was also recently described by Inselburg et al. (1991).

Figure 7 shows parasitemias of Aotus monkeys vaccinated with a combination of recombinant sequences of SERP.

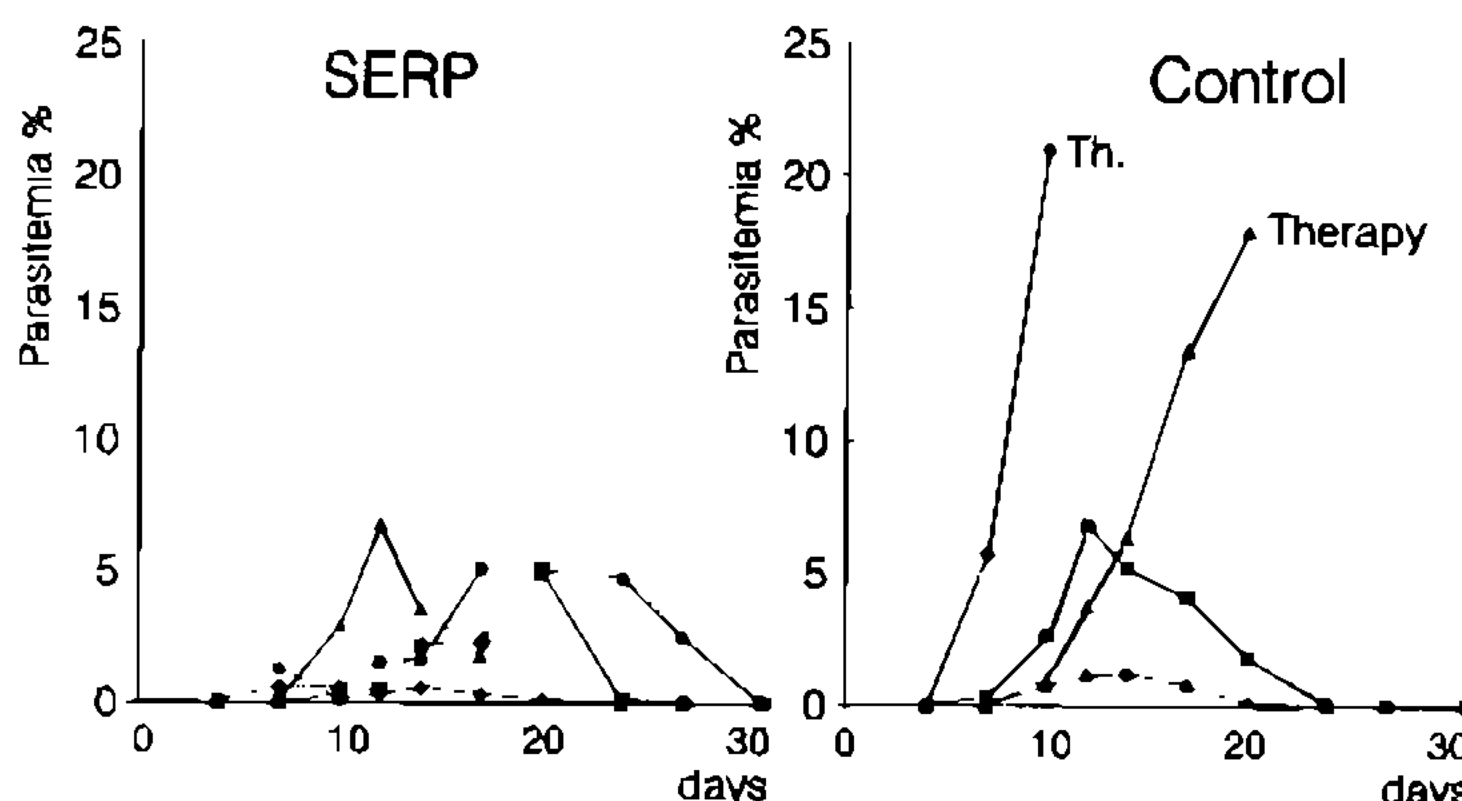


Fig. 7: parasitemia of Aotus monkeys vaccinated with a combination of aa 68-184 and aa 631-892 of SERP, both expressed in *Escherichia coli* as MS2 fusion proteins.

Based on the results of the vaccination trials with the recombinant P. falciparum blood stage antigens SERP, HRP II and MSA I we constructed two hybrid proteins containing selected partial sequences of these antigens and expressed these constructs in *E. coli* (see Figs 2 and 3). In order to assess the ability of both hybrid proteins to induce a protective immune response we performed vaccination experiments using Aotus monkeys.

Trial 5 – In trial 5 two animals (ca-2 years old) were immunized with the fusion protein MS2/SERP/HRP II while two other animals served as controls. After challenge with infected erythrocytes (5 x 10^6 i.RBC), the two control animals showed a rapid increase in parasitemia up to 28% and 14%, respectively. The animal with the higher parasitemia was treated with Mefloquine to prevent death whereas the animal with the lower parasitemia was able to control the infection without treatment. In contrast parasitemias of only up to 2% could be detected in the two monkeys immunized with the hybrid protein MS2/SERP/HRP II.

Figure 8 shows the parasitemia after *P. falciparum* infection of Aotus monkeys immunized with MS2/SERP/HRP II hybrid protein.

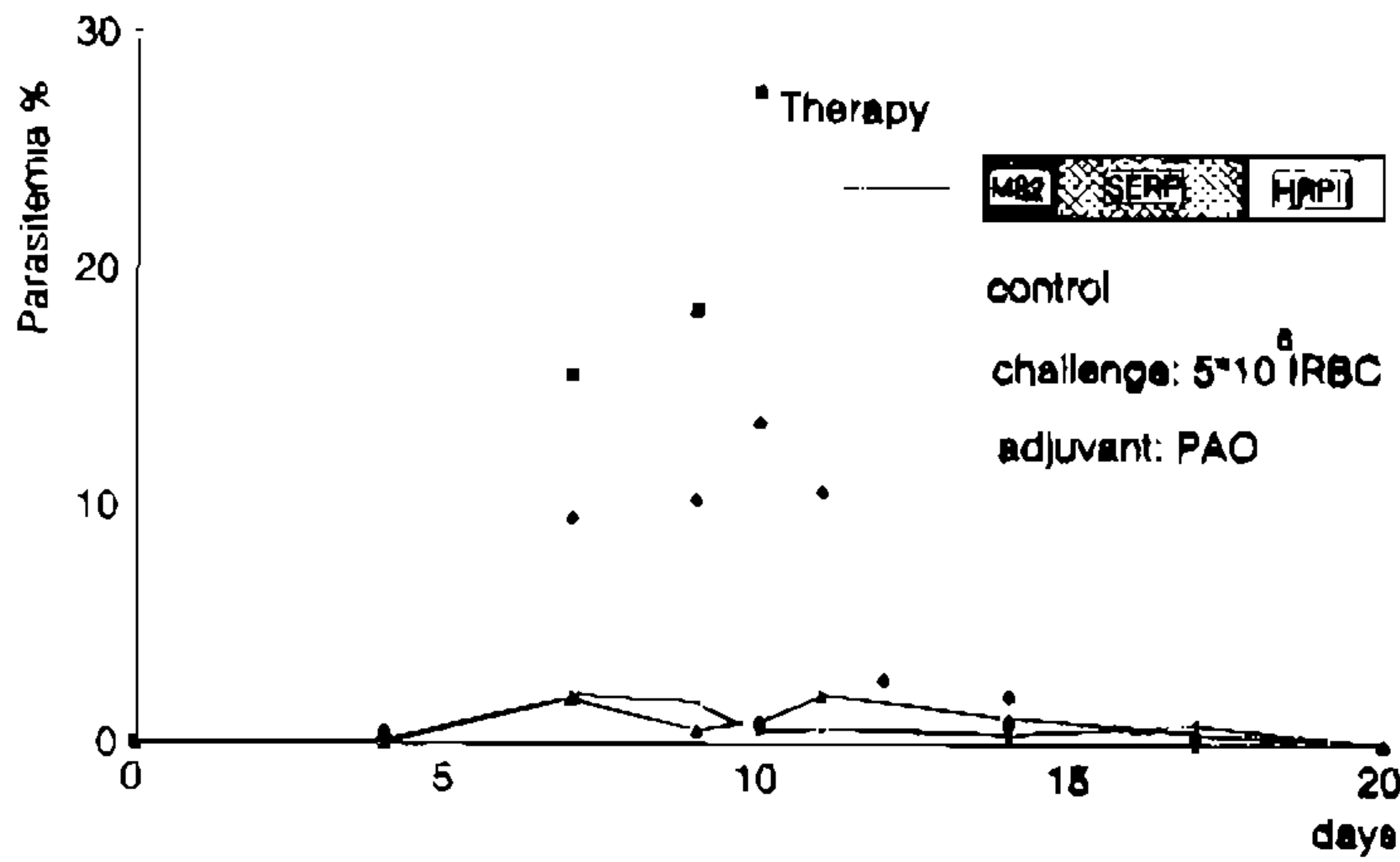


Fig. 8: parasitemia in Aotus monkeys after immunization with fusioprotein MS2/SERP/HRPII and infection with *Plasmodium falciparum*.

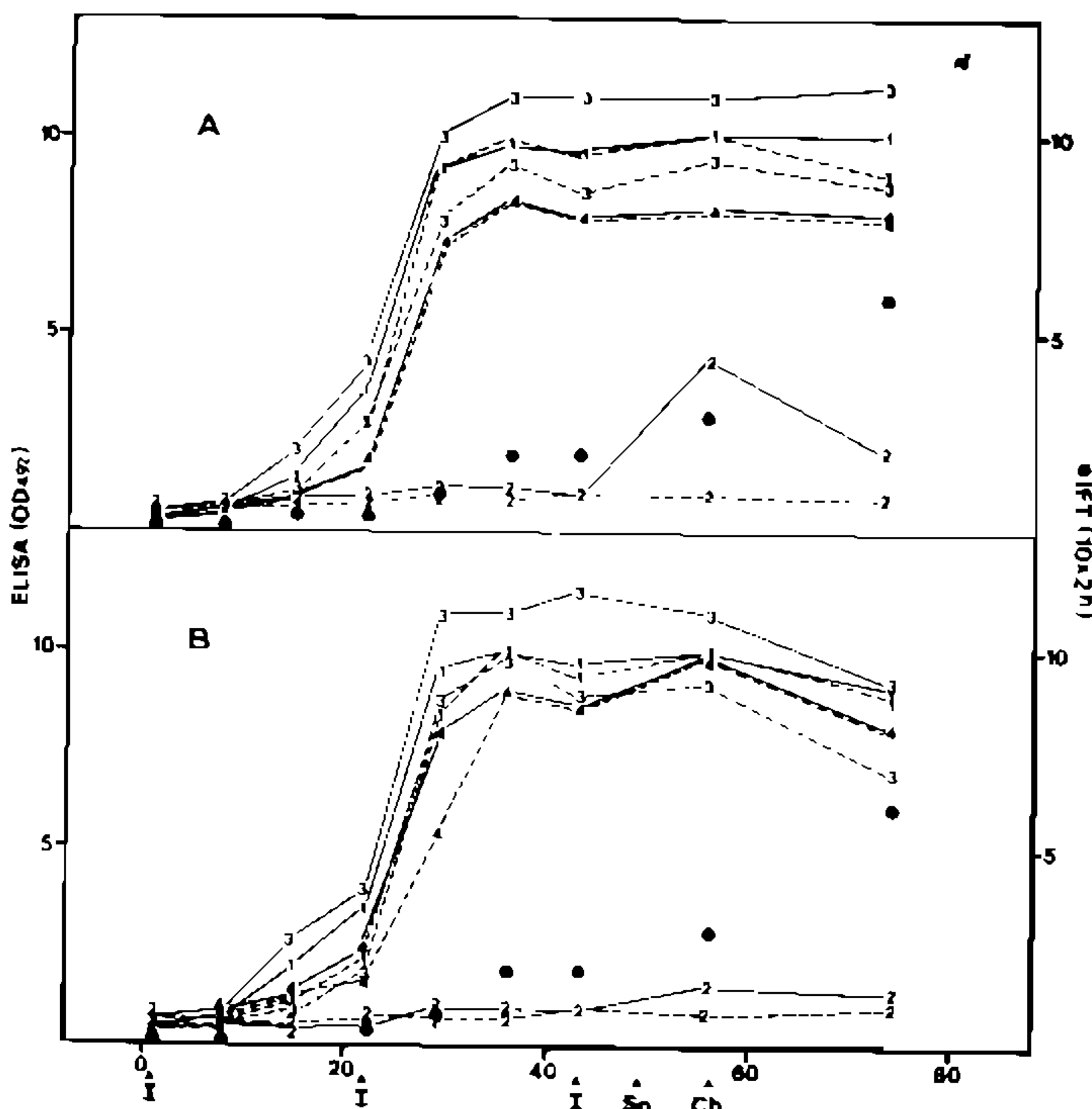


Fig. 9: serological responses of Aotus monkeys A and B immunized with the SERP/HRPII hybrid protein. ELISA on immunization antigen (1), MS2 (2), SERP-MS2 fusion protein (3) and HRPII-MS2 fusion protein (4) before (——) and after (----) absorption with MS2 polymerase. Days of immunization, splenectomy and challenge are indicated by arrowheads. ● IFA.

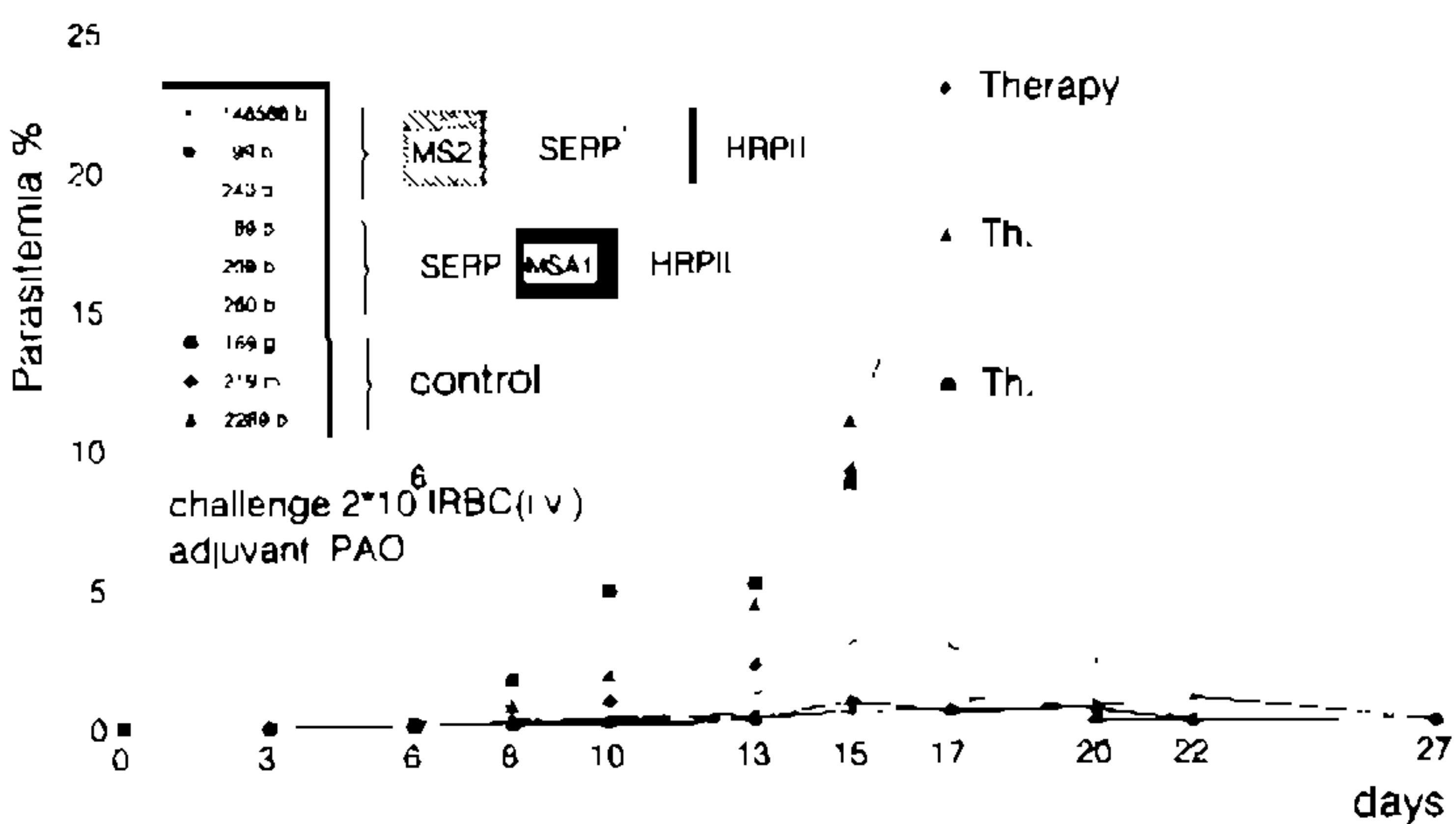


Fig. 10: parasitemia in young Aotus monkeys after immunization with hybrid fusion proteins and infection with *Plasmodium falciparum*.

Figure 9 shows the serological responses of Aotus monkeys A and B immunized with the MS2/SERP/HRP II hybrid protein (ELISA).

Trial 6 – In trial 6 three groups of three animals (7-9 months old) each were either immunized with MS2/SERP/HRP II or SERP/MASI/HRP II or served as control. Upon challenge the three control animals developed parasitemias of 12%, 18% and 23%, respectively, and were treated with Mefloquine. One animal each of the immunized groups showed a parasitemia of 2% and 2,8%, respectively, whereas the residual two monkeys of each group developed parasitemias of less than 1%.

Figure 10 shows the parasitemia after *P. falciparum* challenge (2 x 10^6 i.RBC) of young Aotus monkeys immunized with MS2/SERP/HRP II; SERP/MSA I/HRP II or PAO adjuvant as control.

Serum samples of the monkeys immunized with MS 2/SERP/HRP II and SERP/MSA I/HRP II, respectively, showed high antibody levels (ELISA) against the corresponding malarial antigens, arriving at a plateau after the second immunization; immunofluorescence titers were low but significant during the immunization period. Prechallenge sera analyzed by Western blot analysis on *P. falciparum* schizonts detect the corresponding malarial antigens SERP, HRP II and MSA I.

DISCUSSION

In search of a vaccine against the blood stage of *P. falciparum* we have identified several protein protective in Aotus monkeys (K + IV, VI) sequences. In order to combine the protective effects of the immuno-relevant antigenic fragments, we designed two hybrid proteins combining partial sequences of the blood stage antigens SERP (Knapp et al., 1989; Roussilhon et al., 1990) HRP II (Knapp et al., 1988) and MSAI (Holder et al., 1985; Crisanti et al., 1988).

SERP, a protein characterized by a serine stretch (Bzik et al., 1988; Knapp et al., 1989), is the major component of a protein band of 140 kD which was shown to induce protective antibodies in Saimiri monkeys (Perrin et al., 1984). The antigen is localized within the parasitophorous vacuole where it may function as a cysteine proteinase during merozoite release (Higgins et al., 1989; Knapp et al.,

1991). We were able to obtain partial protection of Aotus monkeys immunized with a recombinant protein containing a region of SERP which carries two T-cell epitopes (Roussilhon et al., 1990) and seems to be conserved among different *P. falciparum* strains (Knapp, same issue).

The histidine-alanine rich protein HRP II is characterized by an extended repeat region consisting of the tripeptide units Ala-His-His and Ala-Ala-Asp (Wellems et al., 1986; Knapp et al., 1988) which show only slight variability in number and arrangement among different *P. falciparum* strains (Knapp et al., 1988). HRP II was shown to be released from the infected red blood cell (Howard et al., 1986); however, part of the antigen stays associated with the outer surface of the erythrocyte membrane (Rock et al., 1987). A recombinant protein containing the C-terminal half of HRP II was shown to partially protect Aotus monkeys against a parasite challenge (Knapp et al., 1988).

The merozoite surface antigen MSAI is a polymorphic glycoprotein of 195 kD (Lyon et al., 1986; Peterson et al., 1988). The antigen purified by a monoclonal antibody could protect Aotus monkeys from malaria (Siddiqui et al., 1987); however, so far three recombinant proteins carrying conserved regions of MSAI showed only very low or no protection (Knapp et al., 1988; Herrera et al., 1990). For the construction of the hybrid protein (SERP/MSA I/HRP II) we have selected a conserved region from the N-terminus which was shown to contain two T-cell epitopes (Crisanti et al., 1988).

Our results show that recombinant hybrid proteins comprising of combinations of different candidate vaccine antigens are capable to induce protective immunity in Aotus monkeys. A combination of different malarial antigens would reduce the risk of the parasite escape by mutation under immunological pressure. Hybrid antigens have the additional advantage of expression and purification of just a single polypeptide versus a cocktail of antigens. Therefore such combined antigens should be considered as potential vaccines against malaria.

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