Biotecnología Aplicada, Vol. 9, No. 2, 130-139 (1992)

Expression of a cDNA clone that recognizes an asexual blood stage antigen from the human malarial parasite *Plasmodium falciparum*.

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Received in December 1991
Approved in March 1992

SUMMARY

A cDNA clone, obtained from the human malarial parasite *P. falciparum*, was expressed in *E. coli* by fusion at the N-terminus of β-Galactosidase in a plasmid expression vector. The fusion protein was partially purified and injected into rabbits. The antibody response was evaluated by Indirect Immuno-fluorescence (IIF) on acetone fixed smears of parasite infected erythrocytes, by immunoprecipitation of polypeptides from cell lysates labeled by incorporation of ^{35}S-methionine *in vitro*, and western-blotting against different asexual blood stages of the cycle. As a result, these antibodies specifically recognized a high molecular weight precursor (120 kDa), which seems to be processed in at least two small fragments at the end of the schizogony. In addition, purified immunoglobulins G (IgG) from the whole serum, raised against the fusion protein, were tested for their capacity of inhibiting the growth of the parasite *in vitro*.

RESUMEN

Un clon ADNc, obtenido a partir del parásito de la malaria humana *P. falciparum* fue expresado en *E. coli*, fusionado al extremo N-terminal de la β-galactosidasa en un plasmidio de expresión. La proteína fusionada se purificó parcialmente y se inyectó en conejos. La respuesta de anticuerpos se evaluó por inmunofluorescencia indirecta sobre láminas que contenían eritrocitos infectados por el parásito, fijados con acetona; por inmuno-precipitación de polipéptidos provenientes de lisados celulares, marcados por incorporación de metionina-^{35}S *in vitro*; y por *Western-blotting* contra diferentes estadios del ciclo asexual de la sangre. Como resultado, estos anticuerpos reconocieron específicamente una proteína precursora de alto peso molecular (120 kDa), la cual parece ser procesada, al menos, en dos fragmentos de menor talla al final de la esquizogonia. Además, inmunoglobulinas G (IgG) purificadas a partir del suero total, elicitado contra la proteína fusionada, fueron evaluadas por su capacidad de inhibir el crecimiento del parásito *in vitro*.

INTRODUCTION

Malaria is a major cause of mortality and morbidity throughout the tropical regions. To investigate the possibility of vaccinating against this disease, work has focussed on identifying malarial proteins capable of stimulating a host protective immune-response. Target antigens identified in this way may be reproduced using recombinant DNA or synthetic peptide techniques.

The asexual blood stage, which is responsible for the clinical symptoms of malaria as parasites invade and destroy the host's erythrocytes, is also susceptible to immune-responses. In man, acquired

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Publicado por el Palacio de las Convenciones, La Habana, Cuba

immunity against this stage of infection is mediated at least partly by antibody (Cohen *et al.*, 1961). For vaccine development it is important, therefore, to identify and characterize those antigens synthesized by blood stage schizonts to which protective antibodies may be directed. A *P. falciparum* 195 kDa protein (P-195) has been identified (Holder and Freeman, 1982) and the biosynthesis of this precursor takes place in the mature intraerythrocytic forms, the schizonts; this protein is processed into discrete fragments (Holder and Freeman, 1982), and these fragments are major surface antigens of merozoites which are strongly recognized by human immune serum (Freeman and Holder, 1983; Holder and Freeman, 1984). For these reasons, the P-195 has been postulated as a possible candidate for a blood stage malaria vaccine. To achieve that, a cDNA library was made, and part of the whole P-195 gene was isolated (Odink *et al.*, 1984).

Here, it is reported the expression of a cDNA clone from the same library; obtained by hybridization to a probe enriched with mRNAs coding for high molecular weight proteins (Odink *et al.*, 1984). The clone expressed in *E. coli* as a fusion protein to the N-terminus of β-galactosidase was purified and injected into rabbits to raise antibodies. The antibody response was studied in the parasite using the techniques of *in vitro* culturing. As a result, a 120 kDa protein was recognized, which seems to be processed in late schizonts, and those antibodies directed either to the precursor or its processed fragments were also capable to inhibit the growth of the parasite *in vitro*.

MATERIALS AND METHODS

In vitro cultivation of *P. falciparum*

The continuous cultivation of the West African Wellcome strain of *P. falciparum* was performed as described previously (Holder and Freeman, 1982; Freeman and Holder, 1983).

Bacterial strains

E. coli JM-105: supE endA sbcB15 hsdR4 rpsL thi del(lac-proAB)

F'[traD36 proAB$^+$ lacI^q del(lacZ)M15] (Yanisch *et al.*, 1985)

Construction of the expression plasmid pX6-2

Expression of a specific region of a cDNA clone was performed using an open reading frame vector. After digestion with the suitable restriction endonucleases, a specific fragment was purified by gel electrophoresis and then treated sequentially with Bal 31 exonuclease and the Klenow fragment of DNA polymerase (Maniatis *et al.*, 1982). The DNA fragments were ligated into alkaline phosphatase treated SmaI cut pXY-460 plasmid (a derivative of pXY-410, Winther *et al.*, 1986) and used to transform *E. coli* JM-105 cells to ampicillin resistance.

Colonies that were blue on agar plates containing X-gal (BOEHRINGER), were picked and screened by restriction enzyme analysis of the plasmid DNA, coomassie blue staining and Western-blotting of cell lysates. Briefly, aliquots from overnight cultures were diluted into 5 mL of Luria broth (LB) (OXOID), supplemented with 50 μg ampicillin/mL and 60 μg IPTG/mL (BOEHRINGER). After 5 h of growth at 37°C, the cultures were harvested by centrifugation and the cell pellets were analyzed by 7.5% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) (LaemmLi, 1970). Clones containing a DNA insert and producing a hybrid protein reacting with IgG from adults living in endemic areas were picked.

Purification of the fusion protein pX6-2

After a partial solubilization of the fusion in 25 mM Tris pH 8.0 containing 0.2% (v/v) NP-40 (BDH), 1 mg lysozyme/mL (MERCK), 1 mM EDTA (BDH), and 1 mM PMSF (SIGMA), 1 h on ice; the insoluble material was harvested by centrifugation at 15 000 x g for 10 min.

The pellet was washed sequentially, first in 50 mM Tris pH 8.0 containing 1% (v/v) NP-40, 5 mM EDTA, 5 mM EGTA (BDH), and 1 mM PMSF; then in the same buffer containing 0.5 M KSCN (SIGMA), instead of NP-40. Upon centrifugation, the supernatant was dialyzed against the same buffer without KSCN, 3 h at 4°C; freeze-dried and stored at -20°C until required.

Antisera

Rabbits were immunized with the *E. coli*-derived protein on at least three occasions. The animals received about 500 μg of protein together with Freund's complete adjuvant (day 1) and were then boosted with the same antigen with Freund's incomplete adjuvant 21 and 49 days later. Sera were collected 63 days after the first immunization.

Indirect Immunofluorescence (IIF)

IIF tests of different blood stages were performed as described previously (Holder and Freeman, 1982). To do this, purified IgG from rabbits immunized with the fusion pX6-2 tenfold diluted (0.5 mg protein/mL) were used. As a second antibody, a goat antirabbit FITC-conjugate (MILES LABORATORIES) was used (1/250). IgG were purified by affinity chromatography on Protein A-Sepharose (Hjelm *et al.*, 1972).

^{35}S-Methionine biosynthetic labeling, cell solubilization, immunoprecipitation, electrophoretic and Western-blot analysis

Preparation of labeled extracts of intracellular blood stage, cell solubilization and immunoprecipitation, as well as Western-blotting analysis were performed as described by Holder and Freeman (1982) and by Freeman and Holder (1983). Molecular weight markers were human spectrin heterodimer (240 and 220 kDa respectively), β-Galactosidase (116 kDa), phosphorylase b (93 kDa), bovine serum albumine (68 kDa), aldolase (39 kDa), and lysozyme (14 kDa).

In vitro growth inhibition assay

For this assay, cultures were synchronized twice with 5% sorbitol (BDH). The mature parasites (late trophozoites and schizonts), obtained after culturing for 72 h, were diluted with washed human A^+ erythrocytes to a starting parasitaemia of 0.5% and adjusted to a 2.5% haematocrit with RPMI-1640 medium (FLOW LABORATORIES), supplemented with 22 mM glucose (BDH), 92 μM hypoxantine (BDH), 16 mM $NaHCO_3$ (BDH), 35 mM Hepes (FLOW), 20 μg gentamycin/mL (SCHERING CORP.), and 10% (v/v) human serum. Aliquots of these synchronized cultures (2 mL), were placed in 25 cm^2 culture flasks to which 0.5 mL of rabbit IgG anti pX6-2, human IgG, and pre-immune rabbit IgG were added to a final concentration of 0.5 mg protein/mL in saline buffer (PBS).

The cultures were incubated at 37°C in a 5% O_2, 2% CO_2, 93% N_2 gas mixture 30 h, to evaluate the serum effect during the next cycle of parasite growth. At the end of the incubation period, the red blood cells from each flask were centrifuged and thin blood smears stained with Giemsa were prepared. The percentage of parasitaemia was microscopically assessed reading 10 fields of erythrocytes.

Growth inhibition over a 30 h period was determined by comparing the percentage of parasitaemia in the culture containing rabbit IgG anti pX6-2 or human IgG, with that of the culture containing pre-immune rabbit IgG according to the formula described by Hui and Siddiqui (1986).

RESULTS AND DISCUSSION

Expression and purification of the fusion protein pX6-2

Previous reports about specific plasmid constructs leading to expression of part of other malaria genes in *E. coli* have been described (Holder *et al.*, 1985). Here, the expression of a specific region of a cDNA clone was achieved using the open reading frame vector pXY-460, to give an N-terminal fusion with β-Galactosidase (figure 1). Upon the induction with IPTG, the fusion protein showed an apparent molecular weight size of 130 kDa as estimated by SDS-PAGE (figure 2); and on western-blotting the protein reacted with purified IgG from adults living in endemic areas (data not shown). The product of this fusion was insoluble, hence, it was necessary to purify it by solubility fractionation from *E. coli* cell lysates as described in *Materials and Methods*.

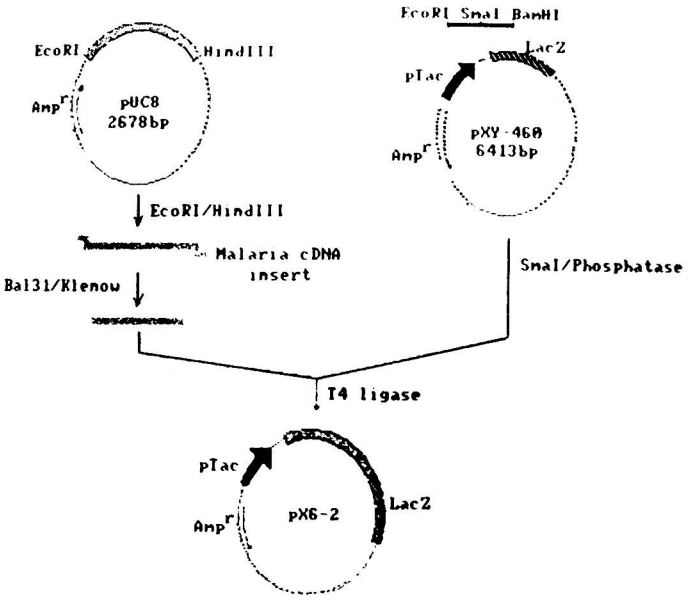

FIG. 1. Cloning strategy for the construction of a recombinant plasmid expressing a malaria sequence in *E. coli*. An EcoRI-HindIII restriction fragment from a cDNA clone in pUC-8 was treated with Bal31 (Boehringer), repaired with Klenow (Biolabs), and ligated into the SmaI site of pXY-460 to produce the inframe expression plasmid pX6-2 fusioned to the N-terminus of β-galactosidase.

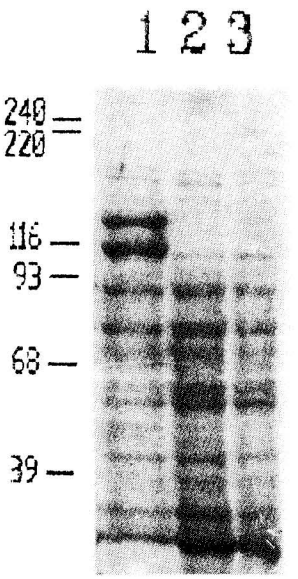

FIG. 2. SDS-PAGE analysis of the LacZ-fusion protein pX6-2 upon induction with IPTG. Proteins synthesized on induced culture (lane 1). Proteins synthesized in repressed culture (lane 2). Proteins from culture containing bacteria with the control plasmid pXY-460 without an insert (lane 3). Positions of the molecular weight markers (kDa) are indicated.

As shown in figure 3, most of the fusion went to the supernatant after KSCN treatment and, thereby, a quite good purified soluble preparation was obtained to immunize rabbits. Nevertheless, the recovery of the fusion was not quantitative, due probably to some loss during the washing steps; though, it is also worth to point out that the total amount of protein loaded on tracks 2 and 5 was different.

On IIF, IgG anti pX6-2 purified from the whole rabbit serum were specific for a parasite antigen associated to late schizonts (figure 4).

A maximum peak of incorporation after metabolic labeling of synchronous cultures of parasite was found at 36 h life cycle (late trophozoites - early schizonts), as described by Holder and Freeman (1982), and the morphology was followed

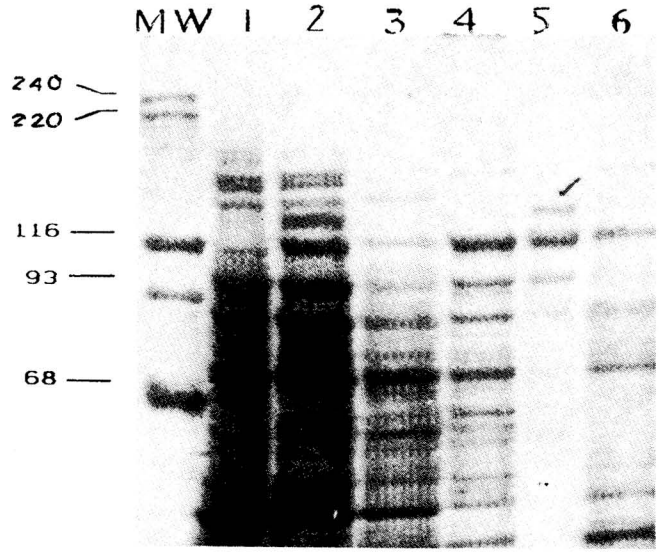

FIG. 3. SDS-PAGE analysis of different steps of the partially purified fusion pX6-2 by solubility fractionation from *E. coli* cell lysates. Non-induced pX6-2 cell lysates (lane 1). Induced pX6-2 cell lysates (lane 2). Supernatant after treatment of cell pellet with 0.2% (v/v) NP-40 (lane 3). As in lane 3 but 1% (v/v) NP-40 (lane 4). Supernatant after treatment of cell pellet with the chaotropic agent KSCN (lane 5); the fusion protein is pointed by an arrow. Pellet after KSCN treatment (lane 6). Positions of the molecular weight markers (kDa) are indicated.

Analysis of the antibody response

The antibodies in the sera from immunized animals were assayed by IIF on acetone fixed smears of parasite infected red blood cells, by immunoprecipitation of ^{35}S-methionine labeled proteins, and immunoblotting against different asexual blood stages.

by examination of Giemsa stained smears at 4 h intervals throughout the cycle (figure 5).

Cell lysates from these synchronous cultures were immunoprecipitated with rabbit serum raised against the fusion pX6-2. This serum specifically recognized a 120 kDa soluble parasite protein in late trophozoites - early schizonts, which was

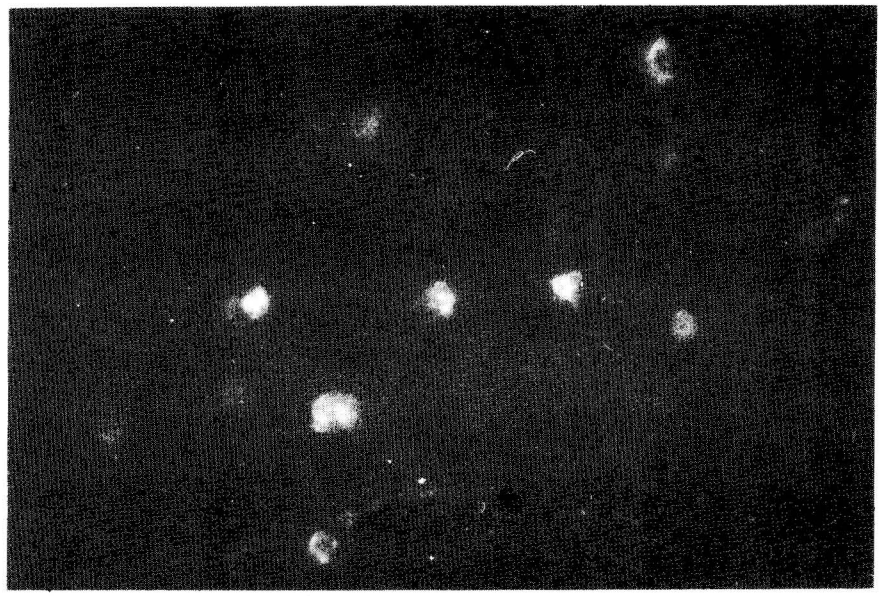

FIG. 4. IIF staining pattern of *P. falciparum* blood stages in acetone-fixed smears (see text for details). Rabbit IgG anti pX6-2 reacting with schizonts. Note localization of staining on mature segmented schizonts. Magnification was 100 x.

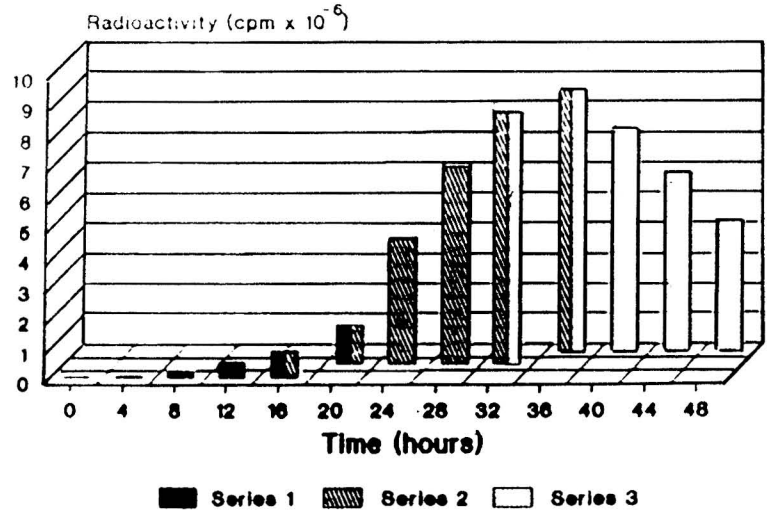

FIG. 5. Morphology of *P. falciparum* and ^{35}S-methionine incorporation during synchronous culturing *in vitro*. Series 1: ring forms (0-16 h); Series 2: Trophozoite forms (20-36 h); Series 3: Schizont forms (36-48 h). Stage overlappings between rings/trophozoites (16-20 h), and trophozoites/schizonts (32-36 h) were obtained. Total TCA precipitable radioactivity incorporated into parasite proteins during labeling with ^{35}S-methionine is shown. A maximum peak of incorporation was obtained at 36 h (late trophoz. - early schiz.).

not present in late schizonts (figure 6, lanes 3 and 4). In addition, this serum recognized strongly, at least, two low molecular weight proteins between 30-40 kDa in late schizonts (figure 6, lane 4) that appear to be present in parasites immunoprecipitated by IgG from adults living in endemic areas (figure 6, lane 1).

IgG anti pX6-2 was evaluated for its ability to inhibit the growth of parasites *in vitro*. As shown in figure 7, IgG anti pX6-2 caused 50.6% reduction of ring forms after 30 h of growth (6-22 h-stages of next cycle) when compared with pre-immune rabbit IgG. IgG from adults living in endemic areas (55.3% inhibition) were used

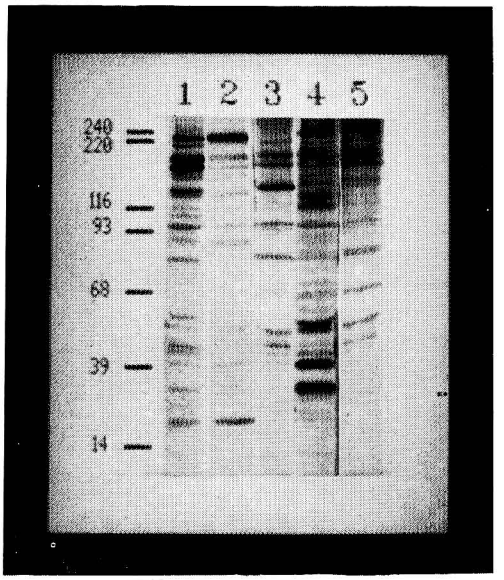

FIG. 6. SDS-PAGE and fluorography analysis of ^{35}S-methionine-labeled proteins from different blood stages. Late trophozoites - early schizonts (lanes 1-3, and 5); late schizonts (lane 4). Rabbit serum against pX6-2 immunoprecipitated a 120 kDa protein (lane 3); not seen by the same serum in late schizonts (lane 4). This serum also immunoprecipitated two low molecular weight fragments (lane 4) which appear to be present, as well as the 120 kDa precursor, in parasites immunoprecipitated by IgG from adults living in endemic areas (lane 1). Rabbit IgG anti P-195 kDa (lane 2) and pre-immune rabbit serum (lane 5) were used as controls. Positions of the molecular weight markers (kDa) are indicated.

For Western-blotting, solubilized parasite cells from different blood stages were run on 7.5% SDS-PAGE, transferred to nitrocellulose filters, and probed with either pre-immune rabbit serum or rabbit anti pX6-2. A series of discrete low molecular weight bands ranging from 20 to 40 kDa were detected by the anti pX6-2 in late schizonts (data not shown).

as a positive control since these antibodies inhibit the parasite growth *in vitro* (Brown *et al.*, 1982). Total percentage of parasitaemia from infected RPMI culture medium was measured and compared with that of pre-immune rabbit IgG, resulting in no differences between them; thus it was concluded that the inhibitory effect seen in this experiment was due only to the immunoglobulins themselves.

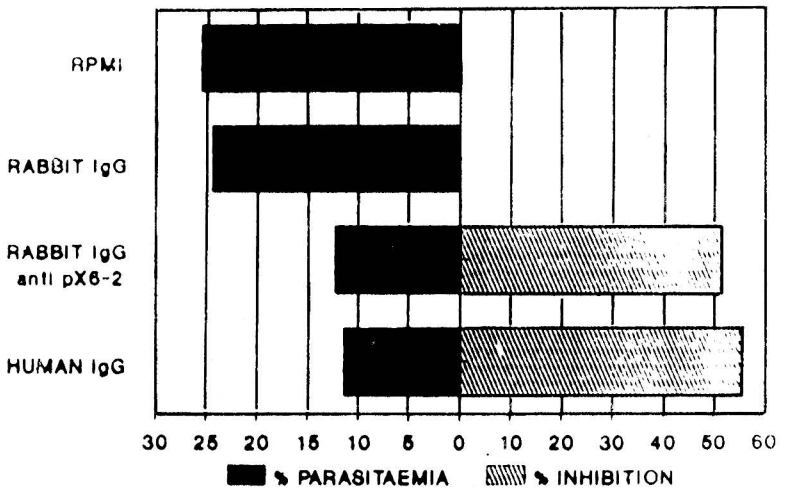

FIG. 7. *In vitro* growth inhibition assay. The ability of the rabbit IgG anti pX6-2 to inhibit the growth of the parasite *in vitro* over a 30 h period was determined. A 50.6% inhibition of the parasitaemia compared to that of the pre-immune rabbit (Hui and Siddiqui, 1986) was obtained. IgG from adults of endemic areas were used as a control (55.3% inhibition). Percentages of parasitaemia were: RPMI-medium, 25.3; pre-immune rabbit IgG, 24.4; IgG anti pX6-2, 12.3; and human IgG, 11.1. The experiment was duplicated and the average value taken.

The aim of this work was to express a malaria cDNA clone in *E. coli* in order to obtain a protein for antibodies generation and characterize those antigens associated to this protein, within the blood stage of *P. falciparum*, against which the host may effect a protective immune-response. Using a rabbit serum against the purified pX6-2, a 120 kDa parasite protein was immunoprecipitated from synchronous cultures (late trophozoites - early schizonts), which seems to be also present in parasites immunoprecipitated by IgG from adults living in endemic areas.

This protein did not appear in late schizonts as a high molecular weight form; accordingly, two possible explanations arise from this result. First, the protein could be processed, or second, it is degraded. The latter should not be the case, since IgG anti pX6-2 identified a parasite antigen associated to late schizonts on IIF; and this result is consistent with those obtained by immunoprecipitation and Western-blotting of late schizonts in which a set of low molecular weight fragments (20-40 kDa) were identified.

Thus, it can be concluded that the antibodies raised against the fusion protein studied in this work recognized a 120 kDa protein in the parasite that behaves as a precursor form subsequently processed in at least two small fragments in late schizogony. This agrees with the fact that the expressed cDNA clone was obtained by hybridization to a probe enriched with mRNAs coding for high molecular weight proteins synthesized mostly at around 36-42 h life cycle (Odink *et al.*, 1984). Moreover, purified IgG from rabbit serum against the fusion pX6-2 inhibited the growth of the parasite *in vitro* to an extent similar to that of IgG from individuals living in endemic areas. This is in fact, the most important result obtained in this work, concerning this malarial protein. So far,

what should only be said is that the immune-response directed against this protein is at least antibody-mediated, suggesting that the inhibition occurred at a point before the release of merozoites and attachment or entry of them to erythrocytes, because of the finding that the IgG anti pX6-2 did not recognize ring forms on IIF (data not shown). Moreover, it is known that schizonts containing red blood cells have an increased permeability, therefore, one can not exclude the possibility that some antibody molecules reach intracellular parasites at the last stage of development (Perrin *et al.*, 1981).

These results are compatible with those observed by others (Freeman and Holder, 1983; Holder *et al.*, 1987).

The sequence of this cDNA clone, as well as its location within the *P. falciparum* genome, have been determined (data pending publication). Further studies would elucidate the function of that schizont protein recognized by antibodies raised against the fusion polypeptide pX6-2, the mechanisms of its biosynthesis and processing, and its role in protective immunity against *P. falciparum* malaria.

ACKNOWLEDGEMENTS

The author thanks all members of the Department of Molecular Biology within Wellcome Biotechnology, specially Drs. V.R. Moreno, M. Smith, and A.A. Holder, for their kind technical assistance, and S.C. Nicholls for helping him initiate the culturing procedure. The author also thanks Drs. A. Suárez, J. Roca, M. Jesus, and G. Lemos for their help in the preparation of graphs.

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