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Plasmodium falciparum polypeptides released during *in vitro* cultivation*

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Synchronous cultures of Plasmodium falciparum *were successively labelled with (^{35}S)-methionine and both the supernatants and the pellets of infected red blood cells were collected. The release of TCA-precipitable material in the culture supernatants was low during the development of ring forms and trophozoites, increased during schizogony, and was maximum at the time of schizont rupture and merozoite reinvasion. Analysis of the supernatants by SDS–PAGE and autoradiography showed that both polypeptides common to the various developmental stages of the parasite and schizont/merozoite-specific polypeptides were released. Polypeptides of relative molecular mass 140000, 82000 and, to a lower degree, 41000 were present in high amounts in the culture supernatants. These polypeptides have been shown to be the target of monoclonal antibodies that are able to inhibit the growth of* P. falciparum *cultures, and may be involved in protective immunity. The released polypeptides may also be used as target antigens in immunodiagnostic tests aiming at the detection of malaria infection.*

The sera of individuals with an acute *Plasmodium falciparum* infection are known to contain soluble malaria antigens (*1*, *2*). These antigens have been detected by double diffusion in gels using, as antisera, the sera of adult individuals who had repeatedly been exposed to *P. falciparum* infection (*1*, *2*). Several malaria antigens have been recognized and they have been classified on the basis of their thermostability (*3*). Some of these antigens have also been characterized by their relative molecular mass and susceptibility to proteolytic enzymes (*4*). Other studies, using short-term *in vitro* cultures of *P. knowlesi* and *P. falciparum*, showed that malaria antigens are released during the schizont developmental stage and/or at the time of schizont rupture and merozoite invasion of new erythrocytes (*5*, *6*). Some of the polypeptides released by the parasite in the supernatant of short-term *P. falciparum* cultures *in vitro*, have the same immunological reactivity as antigens detected in the sera of infected individuals (*5*). The precise origin of the released antigens has not been elucidated.

The aim of the present study was to determine and quantify the sequential release of *P. falciparum* antigens during the asexual development of the parasite and to identify at the molecular level the antigens released in the culture medium.

MATERIALS AND METHODS

Culture conditions and synchronization of the culture

P. falciparum (SGE1 strain) was adapted to *in vitro* culture by using blood taken from a European patient who had a first acute *P. falciparum* infection after returning from Senegal. The adapted isolate was propagated in group A red blood cells (RBC) by the candle jar method (*7*). This isolate did not produce gametocytes under the culture conditions used.

Synchronized cultures were initiated and the multinucleated forms of the parasite were separated from ring forms and normal RBCs by flotation in Physiogel (*8*). Over 75% of the cells floating in Physiogel were parasitized RBCs. These cells were mixed in a ratio of 1 to 3 with fresh uninfected RBCs. The reinvasion period was limited to 5 h, after which the remaining mature forms were lysed with 5% mannitol (*9*). The procedure was repeated

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over a second replication cycle to increase synchrony and parasitaemia. In the experiment reported here (the experimental protocol was repeated three times with similar results), a pellet of 0.7 ml RBC with a parasitaemia of 23% (93.7% ring forms) was diluted in 28 ml of culture medium and divided in seven Petri dishes (60 mm diameter). The culture medium was changed every 6 h and the course of the erythrocytic development was assessed microscopically by using Giemsa-stained thin smears.

Labelling conditions

The synchronized cultures were labelled successively every 6 hours as follows: 0-6, 6-12, 12-18, 18-24, 24-30, 30-36, and 36-42 hours. Successive cultures were washed once in methionine-free MEM (minimum essential medium), supplemented with 10% human serum, 2 mmol of glutamine and 25 mmol of HEPES buffer, and resuspended in 3.5 ml of the same medium containing 1.48×10^4 Bq (40 μCi) per ml of L-(^{35}S)-methionine (specific activity 3.84×10^2 Bq (10.39 mCi)/ml). After 6 h of labelling, the parasitized RBC were pelleted by centrifugation at 1000 *g* for 10 min and then the pellets and the supernatants were treated separately. The pelleted cells were washed twice in cold phosphate-buffered saline (PBS), lysed by the addition (to the last pellet) of 0.6 ml of TNE (Tris-NaCl-EDTA buffer, pH 8) containing 0.5% of Nonidet P40 (NP40) and kept in aliquots at -75 °C. The supernatants were centrifuged at 20 000 *g* for 30 min at 4 °C and dialysed overnight at 4 °C against PBS and stored at -75 °C.

Analysis of the labelled material

Measurement of radioactivity associated with infected RBCs and released in culture medium. Duplicate 10 μl aliquots of the various infected RBC lysates and 100 μl aliquots of the supernatants were incubated for 15 min at 37 °C in 0.5 ml of 1 mol/l NaOH containing 25 μl of hydrogen peroxide (30 volumes) and precipitated with 3 ml of cold 25% trichloroacetic acid (TCA). The precipitates were filtered on 0.2 μ millipore filters and, after several washings with 5% TCA and alcohol, dissolved in Readysol scintillation fluid and counted in a Beckman LS20 β-counter. The radioactivity associated with macromolecules in the infected RBCs and culture supernatants was expressed as a percentage of the total radioactivity (counts per minute (cpm) of (^{35}S)-methionine added to individual cultures at the beginning of each labelling).

Immunoprecipitation assay. Immunoprecipitation was carried out as described by Kessler (*10*). Aliquots of culture supernatants containing TCA-precipitated polypeptides (0.5×10^6 cpm) were brought to a final concentration of 0.5% NP40 and a final volume of 400 μl by the addition of TNE buffer. These samples were were incubated for 1 h at room temperature with 10 μl of each individual serum. A volume of 50 μl of a 10% suspension of formalin-treated *Staphylococcus aureus* ("Pansorbin" from Behring) was added to the antigen-antibody mixture. After 15 min of incubation, the mixture was centrifuged (2000 *g* for 10 min), and the precipitates washed five times. The bound antigen-antibody complexes were eluted with 50 μl of sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) sample buffer at 60 °C for 15 min. The pellets of labelled infected RBCs were solubilized in TNE buffer, pH 7.2, containing 0.5% NP40 and then centrifuged at 20 000 *g* for 30 min at 4 °C. The supernatants containing soluble polypeptides (accounting for 90% of the measured cpm) were used for immunoprecipitation, following the same procedure as for the culture supernatants. Three sera from adults living in a malaria endemic area (in Kenaba, the Gambia) and a serum from a European who had never suffered from malaria were used for the immunoprecipitation assay.

SDS-PAGE analysis and autoradiography. Electrophoresis was carried out in 10% slab gel, as described by Laemmli (*11*). All samples were equilibrated in Tris-HCl-buffer, pH 6.8, containing 1% SDS and 2% mercaptoethanol. The gels were calibrated with standard protein markers (Pharmacia Fine Chemicals), fixed, stained with 0.1% Coomassie blue, dried, and autoradiographed on X-ray films (Kodak) for 5-10 days at -75 °C.

RESULTS

A good synchrony of *P. falciparum* cultures was achieved by using the methodology described (Fig. 1). The incorporation of (^{35}S)-methionine into parasitized RBCs varied during the course of the asexual cycle. As ring forms matured into trophozoites, the incorporation of (^{35}S)-methionine increased, reaching its highest level in mature trophozoites. A decline of incorporation was later observed in schizonts. The measure (cpm) of TCA-precipitable polypeptides in the supernatants of cultures was used to quantify the amount of parasite polypeptides released into the supernatants. Low counts were obtained from supernatants of cultures containing ring forms and trophozoites. The highest counts were obtained with the supernatants of cultures containing mature schizont segmenters and new ring forms after reinvasion, at which time the TCA-precipitable polypeptides in the supernatant represented 7% of the

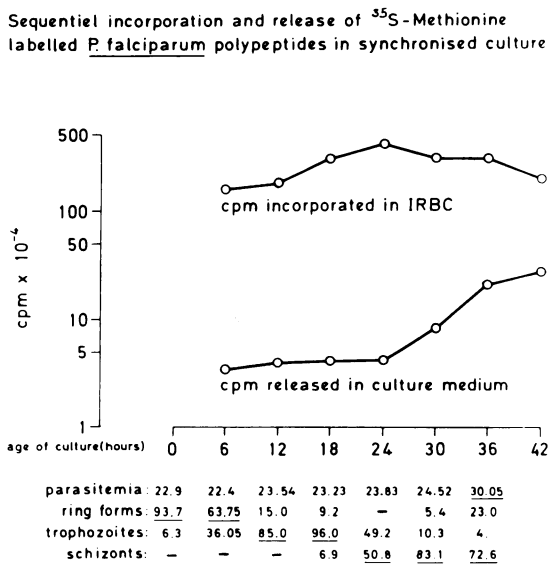

Fig. 1. Incorporation of (^{35}S)-methionine in infected RBCs (IRBC) and release of radiolabelled material in culture supernatants during the asexual erythrocytic cycle. Each of the successive individual cultures was incubated for 6 hours with a constant amount of (^{35}S)-methionine. At the bottom of the figure, the percentage of each erythrocytic stage of the parasite is indicated at various time points during the experiment. Non-infected RBCs were also used as a control under identical conditions: there was less than 0.5×10^4 cpm incorporated in normal RBCs and less than 0.2×10^4 incorporated in the supernatant.

Table 1. Percentage of (^{35}S)-methionine counts incorporated in infected RBCs and released in culture supernatants

Labelling intervals (hours)	Developmental stages	% counts[a] incorporated in infected RBCs	% counts[a] released in culture medium
0–6	Early rings	5.1	0.06
6–12	Rings	5.8	0.07
12–18	Rings + trophozoites	11.5	0.07
18–24	Rings + trophozoites	15.1	0.07
24–30	Trophozoites + schizonts	11.4	0.15
30–36	Schizonts	10.2	0.36
36–42	Schizonts + rings	3.4	0.48

[a] 100% represents the total cpm added to each culture at the start of the labelling.

radioactivity (as cpm) incorporated in the infected RBCs (Fig. 1, Table 1).

Both the parasitized RBCs (collected successively during the asexual cycle) and the corresponding supernatants were analysed by SDS-PAGE. Fig. 2A presents autoradiographs of successive samples of parasitized RBCs applied on SDS-PAGE. All the labelled polypeptides were parasitized gene products since mature RBCs are depleted of their gene pool. Most of the *P. falciparum* polypeptides are synthesized throughout the asexual cycle, as reported previously (*12*). However, some polypeptides are synthesized or processed only, or mainly, at the time of schizogony. Their localization on the gel is indicated by arrows (Fig. 2A) and their relative molecular masses (M_r) are respectively > 200 000, 160 000, 140 000, 105 000, 82 000, 55 000 and 41 000. Supernatants recovered from each of the successive cultures have been analysed under identical conditions (Fig. 2B). As would be expected from the results shown in Table 1, parasite-specific polypeptides are released in small amounts in the supernatants before schizogony and the number of detectable polypeptides increases at the time of schizont rupture, which is when the supernatant contains most of the schizont-specific polypeptides. In addition, four polypeptides of M_r 180 000, 76 000, 60 000 and 45 000 that are common to the three developmental stages are clearly detectable. Based on the intensity of the radiolabelled bands, polypeptides of M_r > 200 000, 180 000, 140 000, 82 000, 60 000 and 45 000 were identified as the major components released (Fig. 2B and Fig. 3, second band).

To further identify the labelled polypeptides as parasite-specific products and assess their immunogenicity, the supernatants were used as a source of malaria antigens and immunoprecipitated by sera of individuals living in endemic areas, as shown in Fig. 3 (bands 1s, 2s, 3s). Lysates of unsynchronized, labelled infected cells were also used as a source of antigens and immunoprecipitated by the same sera (Fig. 3, bands 1c, 2c, 3c). The results demonstrate, on the basis of the intensity of the polypeptide bands detected on the autoradiographs, that some malaria antigens are present in higher concentration in the culture supernatants than in the lysate of infected RBCs. This difference is particularly evident for the schizont- and merozoite-specific polypeptides of M_r 140 000, 82 000 and 41 000. Another schizont-specific polypeptide of M_r 160 000 is precipitated similarly from both antigenic sources, whereas the M_r 200 000 polypeptide is detected in higher concentration in the immunoprecipitates obtained by using the infected cells as a source of malaria antigens.

DISCUSSION

This study shows that, during the *in vitro* culture of asexual erythrocytic stages of *P. falciparum*, parasite-specific polypeptides are released in the culture medium. The results also confirm previous data demonstrating that the release of parasitic material is very low during the maturation of ring forms and trophozoites, increases at the end of schizont maturation, and is maximum at the time of schizont rupture and reinvasion of RBCs by merozoites (*4*, *5*). All the radiolabelled polypeptides are parasite-specific since mature human RBCs do not contain nucleic acids. In addition, the radiolabelled polypeptides released are precipitated by sera from immune individuals living in malaria endemic areas. In this study, the relative molecular masses of the released polypeptides were determined, two groups being identified: polypeptides common to the various developmental stages of the parasites (with M_r 180 000, 76 000 and 45 000, for example) and polypeptides that are synthesized or processed during the schizont developmental stage. The polypeptides with low M_r migrate with the front of the gels and probably account for most of the TCA-precipitable material detected in the supernatants of cultures containing ring forms and early trophozoites. This material probably consists of small polypeptide chains that leak from infected RBCs.

The exact origin of the released polypeptides is difficult to specify. They probably do not arise from lysis of schizonts that fail to mature or from lysis of merozoites that fail to invade the erythrocytes, since some of the major polypeptides of schizonts are not represented in the supernatants, and since the culture conditions have been optimized. One possibility is that the released antigens represent residual parasitic material that is not incorporated into schizonts. Alternatively, they may be parasite antigens associated with the knobs present at the surface of erythrocytes containing schizonts, or antigens associated with the coat of merozoites. The parasite polypeptides common to the various developmental stages probably represent parasite material that is not incorporated into the merozoites. The schizont-specific polypeptides have previously been identified and are shown in Fig. 2A (*12*, *13*). They are good candidates for expression at the surface of schizonts and/or merozoites since polypeptides expressed late in sporogony and schizogony have been shown in other plasmodia species to be expressed at the surface of sporozoites and merozoites and to be involved in protective immune responses (*14*, *15*). The apparent M_r of the more intensively labelled schizont-specific polypeptides are respectively about 200 000, 160 000, 140 000, 105 000, 82 000 and 41 000. Some of these polypeptides are present in high concentration in the supernatant of *P. falciparum* cultures at the time of reinvasion, notably polypeptides of M_r 140 000, 82 000 and, to a lesser extent, 41 000. Interestingly, these polypeptides have been shown to be the target of monoclonal antibodies that are able to inhibit the *in vitro* growth of *P. falciparum* cultures (*16*). These monoclonal antibodies are known to react with schizonts and merozoites, as demonstrated by the indirect immunofluorescence technique (*13*, *16*). It has been shown by electron microscopy that merozoites release their coat during the invasion of RBCs (*17*). There is evidence here to suggest that the released polypeptides of M_r 140 000, 82 000 and 41 000 are part of the merozoite coat and may be components of the merozoites. In addition, the M_r 82 000 polypeptides may be identical with the knob-associated protein described by Kilejian (*18*). This is on the basis of its relative molecular mass and stage specificity. It has not been elucidated whether or not these polypeptides are intact polypeptides or fragments of a polypeptide of higher M_r, as shown in other malaria species (*15*). Comparison of the polypeptide bands precipitated, using either infected RBC lysate or culture supernatants, indicates that there is no (or only limited) additional degradation of malaria antigens in the culture supernatants, with the possible exception of the polypeptide with $M_r > 200\,000$.

The importance of soluble antigens released by haemosporidia during *in vitro* culture has been emphasized by the recent demonstration that vaccination with soluble *B. bovis* antigens derived from cultures can induce protective immunity in cattle (*19*). These experiments and other vaccination experiments, using soluble antigens of various plasmodia (*20*, *21*), suggest that the supernatants of *P. falciparum* cultures may be a good source of malaria antigens that are suitable for inducing protection. Soluble parasite antigens may also be of importance for immunodiagnosis, since they may be used as target antigens in serodiagnostic assays that are designed to detect current or recent malaria infection.

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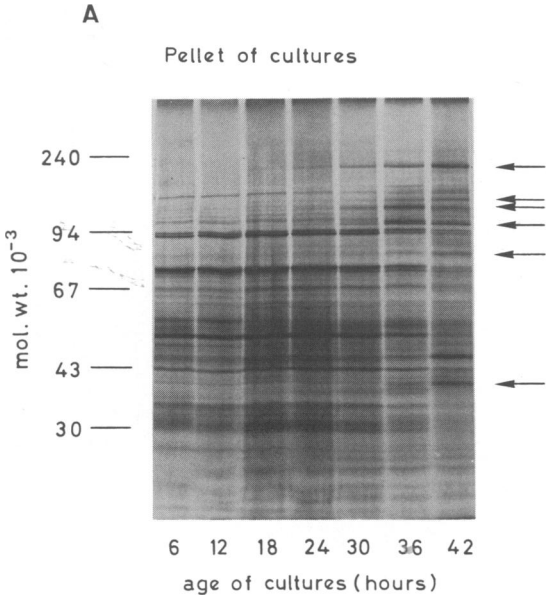

Fig. 2A. Autoradiographs (by SDS-PAGE analysis) of infected RBCs collected from successive cultures of *P. falciparum*, metabolically labelled with (^{35}S)-methionine at 6-hour intervals. The arrows point to the schizont-specific polypeptides with M_r > 200 000, 160 000, 140 000, 105 000, 82 000, 55 000 and 41 000.

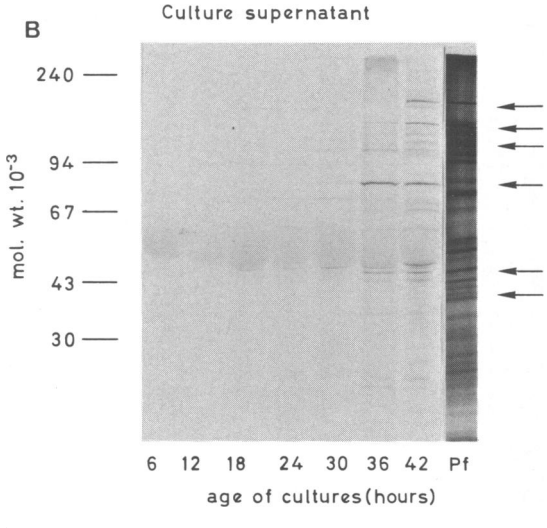

Fig. 2B. Autoradiographs (by SDS-PAGE analysis) of successive culture supernatants of *P. falciparum*, metabolically labelled with (^{35}S)-methionine. The culture supernatant corresponds to the infected cells analysed in Fig. 2A. Identical volume of culture supernatant (8 μl) was applied on each lane of the gel. Pf: unsynchronized *P. falciparum*-infected RBC preparation labelled with (^{35}S)-methionine. The arrows point to polypeptides of M_r > 200 000, 140 000, 82 000 and 45 000.

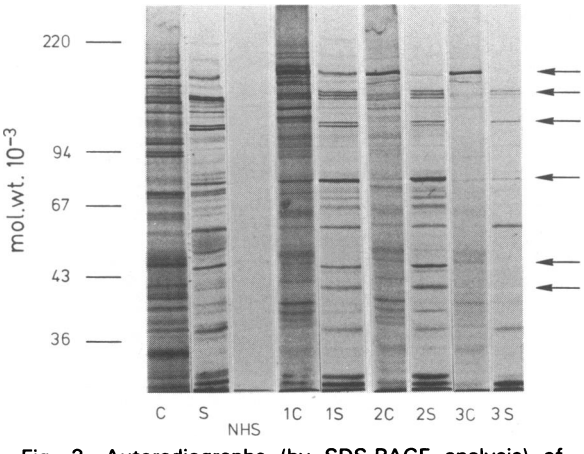

Fig. 3. Autoradiographs (by SDS-PAGE analysis) of immunoprecipitates obtained by using immune sera and either *P. falciparum*-infected RBCs (bands 1c, 2c, 3c) or culture supernatants (bands 1s, 2s, 3s). Abbreviations: C = *P. falciparum*-infected RBCs; S = culture supernatant; NHS = normal human serum.

RÉSUMÉ

CARACTÉRISATION DES POLYPEPTIDES LIBÉRÉS PAR *PLASMODIUM FALCIPARUM* LORS DE LA CULTURE *IN VITRO*

Des antigènes paludiques ont été mis en évidence dans le sérum de malades souffrant de paludisme à *P. falciparum*. Certains de ces antigènes ont été retrouvés dans le milieu employé pour les cultures continues *in vitro* des formes érythrocytaires asexuées de *P. falciparum*.

Pour les expériences présentées ici, des cultures synchrones successives de *P. falciparum* ont été marquées pendant 6 heures à la (^{35}S)-méthionine. On a constaté que l'incorporation de cet acide aminé était maximale à la fin du stade trophozoïte. L'étude des surnageants des cultures a montré que très peu de polypeptides marqués étaient libérés durant la maturation des formes annulaires et des trophozoïtes. Assez marquée à la fin de la maturation des schizontes, cette libération devient importante au moment de la rupture des schizontes et de l'invasion de nouveaux globules rouges. On déduit l'origine parasitaire des polypeptides marqués trouvés dans le milieu de culture du fait qu'il n'y a pas d'incorporation de (^{35}S)-méthionine en présence de globules rouges normaux non parasités et que les polypeptides marqués libérés dans le milieu de culture sont précipités sélectivement par des sérums humains d'individus partiellement immuns.

Les surnageants de culture et les globules rouges parasités ont été analysés par SDS-PAGE, suivi d'autoradiographie. Il apparaît que les polypeptides libérés appartiennent à deux groupes principaux: le premier comprend des polypeptides qui sont synthétisés et exprimés à tous les stades de développement érythrocytaire asexué du parasite; le second comprend des polypeptides synthétisés ou exprimés à la fin de la schizogonie; ces derniers sont donc schizonto- ou mérozoïto-spécifiques. On peut envisager que les polypeptides libérés communs aux divers stades de développement érythrocytaire (principalement polypeptides de M_r égale à 180 000, 76 000, 60 000, et 45 000) représentent du matériel parasitaire non incorporé dans les mérozoïtes; ce matériel serait libéré par l'éclatement des schizontes. Les polypeptides schizonto/mérozoïto-spécifiques dont la masse molaire relative (M_r) est de 140 000 ou 82 000 et, dans une moindre mesure, ceux dont la M_r est de 41 000, sont particulièrement abondants dans le surnageant des cultures. Il est intéressant de relever que ces polypeptides sont reconnus par des anticorps monoclonaux qui ont la capacité d'inhiber la croissance de *P. falciparum in vitro*. Ces anticorps réagissent avec les mérozoïtes en immunofluorescence indirecte. Il apparaît donc que le surnageant des cultures de *P. falciparum* peut représenter une source de matériel intéressante pour la purification d'antigènes paludiques susceptibles de jouer un rôle dans la réponse immune de l'hôte.

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