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Synthesis of *Plasmodium knowlesi* polypeptides in a cell-free system*

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Preliminary results are presented concerning the characterization of Plasmodium knowlesi *antigens. RNA with messenger activity has been extracted from* P. knowlesi *and translated in a rabbit reticulocyte cell-free system. The products of this system have been analysed both before and after precipitation with specific* P. knowlesi *antiserum and many polypeptides ranging in molecular weight from over 100 000 to approximately 20 000 have been found. A good correlation was found between these results and those obtained by McColm with* in vitro *labelling of* P. knowlesi *with (^{3}H)-isoleucine. The major polypeptide in both systems had a molecular weight of about 50 000–55 000. These results indicate the parasite origin of the antigens.*

As the malaria parasite progresses through its life cycle it undergoes a number of changes (*1*). These changes involve not only different morphological forms but also changes in antigenicity (*2*).

Several different classes of antigen have been identified from *Plasmodium falciparum* and have been partially characterized (*3–5*), but it is not clear whether all these antigens are of parasitic or erythrocyte origin. As with trypanosome infections, malaria parasites are capable of undergoing antigenic variation (*2*). This variation has been demonstrated by the schizont-infected cell agglutination (SICA) test (*6*), but the variable antigen has not yet been characterized.

It is the purpose of our work to investigate the occurrence of malarial antigens both common and variable. For this we have examined *P. knowlesi* proteins indirectly through *P. knowlesi* RNA, and directly by extraction, fractionation, and analysis of parasite extracts. This report is solely concerned with the work using *P. knowlesi* RNA.

MATERIALS AND METHODS

L-(^{35}S)-methionine (specific activity: 18.5–22.2 x 10^8 Bq (500–600 μCi) per mmol) was obtained from the Radiochemical Centre, Amersham, Bucks, England, biochemicals from Sigma, London, England, and chemicals from BDH, Poole, Dorset, England.

Preparation of cells

P. knowlesi: *Macaca mulatta* approximately 3 kg in body weight were infected with a reference stabilate of cryopreserved parasitized blood of the Nuri strain (*6*). Parasitized erythrocytes, predominantly large trophozoites and early schizonts, were isolated according to the method of Brown et al. (*6*), except that the temperature was maintained at +2°C throughout.

RNA preparation

The parasitized cells were resuspended in a buffer (pH 7.4) containing 0.3 mol of sucrose, 1 mmol of EDTA, and 0.01 mol of Tris-HCl per litre, pelleted by centrifugation at 1500 *g* for 5 min, resuspended in an equal volume of the same buffer, and disrupted by freezing (with solid carbon dioxide) and thawing several times. The unbroken cells and cell debris were removed by centrifugation at 30 000 *g* for 20 min, and RNA was extracted from the supernatant using a phenol/chloroform method described by Berridge et al. (*7*). RNA was precipitated overnight at –20°C by the addition of 2.5 volumes of ethanol, then pelleted by centrifugation, washed twice with ethanol and once with an ethanol–ether mixture (1:1); it was then ready for assaying for messenger RNA activity.

Rabbit reticulocyte in vitro *assay*

The rabbit reticulocyte cell-free protein synthesizing system was prepared and set up as described by Eggitt et al. (*8*), based on the methods of Clemens et al. (*9*), Pelham & Jackson (*10*), and Hunt et al. (*11*). 18.5 x 10^4 Bq (5 μCi) of (^{35}S)-methionine and 26 μg of *P. knowlesi* RNA were added to 100 μl of cell-free assay preparation.

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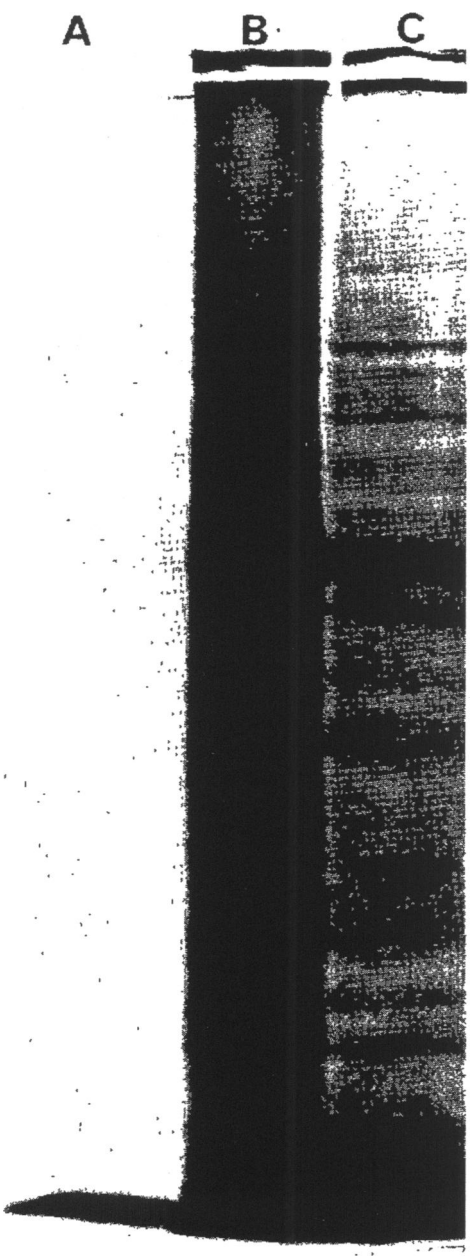

Fig. 1. Autoradiograph of an SDS–10% polyacrylamide gel on which total cell-free products were analysed.
A = cell-free system + no RNA
B = cell-free system directed by *Trypanosoma brucei* RNA
C = cell-free system directed by *P. knowlesi* RNA.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (*12*).

Specific P. knowlesi *antisera*

Schizont-infected cells isolated according to Brown et al. (*6*) were freeze-thawed 3 times as a source of antigen. A 3-kg rhesus monkey was inoculated intramuscularly with 2.0 ml of this concentrated cell material incorporated in an equal volume of Freund's complete adjuvant, followed 14 days later by a similar inoculum in incomplete adjuvant. The immunity of the monkey was subsequently boosted on two separate occasions by injections of alum-precipitated antigen. Sera from 2 monkeys were used, one immunized with antigen from parasites of the same variant type (NK1) as used for a source of RNA, the other with antigen from a different variant (NK4), isolated by the technique described earlier (*6*).

RESULTS

RNA was extracted from *P. knowlesi* as described in the "Methods" section, and was translated in a reticulocyte cell-free system. The total products of this system were analysed by SDS-polyacrylamide gel electrophoresis and the results are shown in Fig. 1, together with those for controls in which no RNA or *Trypanosoma brucei* RNA were added to the cell-free system.

Cell-free assays were set up as above using *P. knowlesi* RNA, and specific malaria products were precipitated with anti-*P. knowlesi* serum. The products were analysed by gel electrophoresis and subsequent autoradiography (Fig. 2). Controls were included as described in the legend to Fig. 2.

Total counts in material precipitated from 100 μl of cell-free assay systems by means of trichloracetic acid or antisera are shown in Table 1.

Table 1. Radioactivity in material precipitated from the cell-free system by means of trichloracetic acid (TCA) or antisera

RNA	cpm[a] in TCA precipitates (A)	cpm[a] in *P. knowlesi* antiserum precipitates (B)	cpm[a] in normal monkey serum precipitates (B)	B/A x 100 (%)
P. knowlesi	397 140	51 030	—	12 8
P. knowlesi	308 590	—	15 580	5 0
No RNA	76 900	4 220	—	5 5
No RNA	79 340	—	4 660	5 9

[a] Counts per minute.

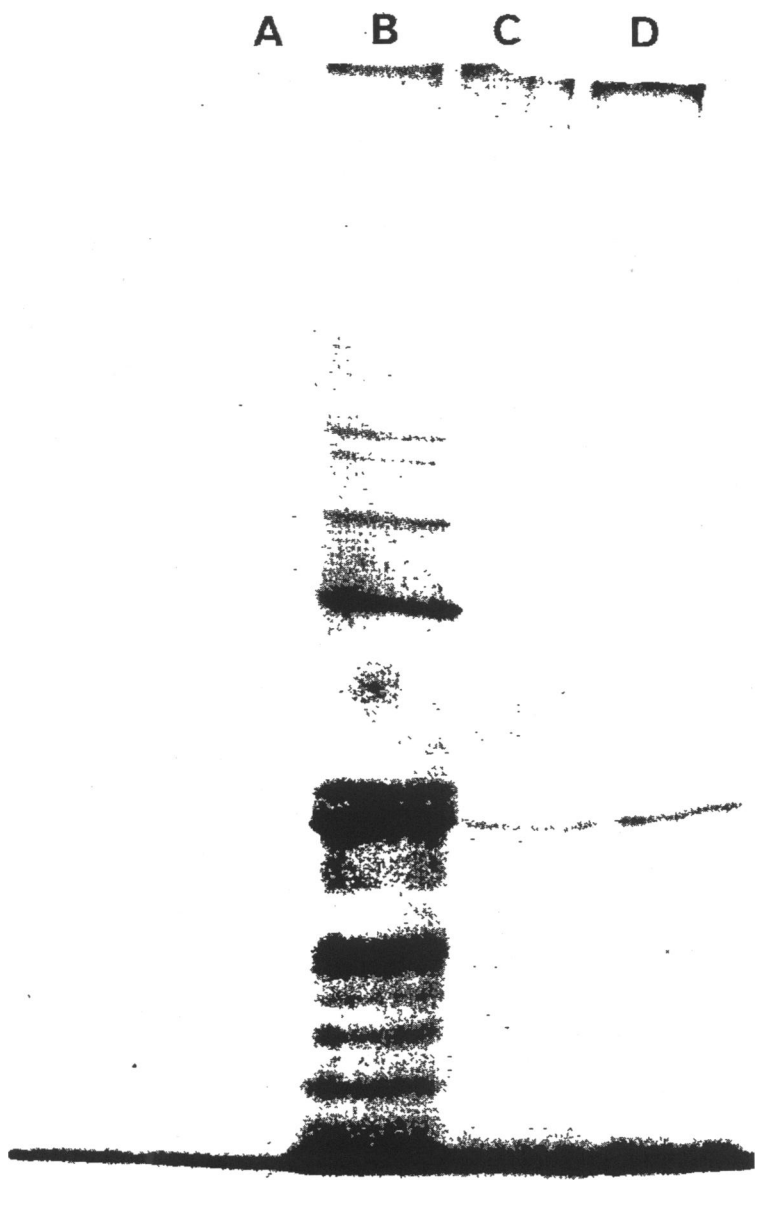

Fig. 2. Autoradiograph of an SDS–7 5% polyacrylamide gel on which various serum-precipitated products of the cell-free system were analysed.

A = cell-free system + no RNA/*P. knowlesi* antiserum
B = cell-free system + *P. knowlesi* RNA/*P. knowlesi* antiserum
C = cell-free system + *P. knowlesi* RNA/normal monkey serum
D = cell-free system + *P. knowlesi* RNA/trypanosome antiserum.

A comparison has been made between the results obtained in the cell-free system using *P. knowlesi* RNA and the results McColm obtained by *in vitro* labelling (with (^{3}H)-isoleucine) of *P. knowlesi* parasites (*13*). Specific products of the cell-free system precipitated by malaria antiserum and total products from the *in vitro* labelling with (^{3}H)-isoleucine were analysed by gel electrophoresis (Fig. 3).

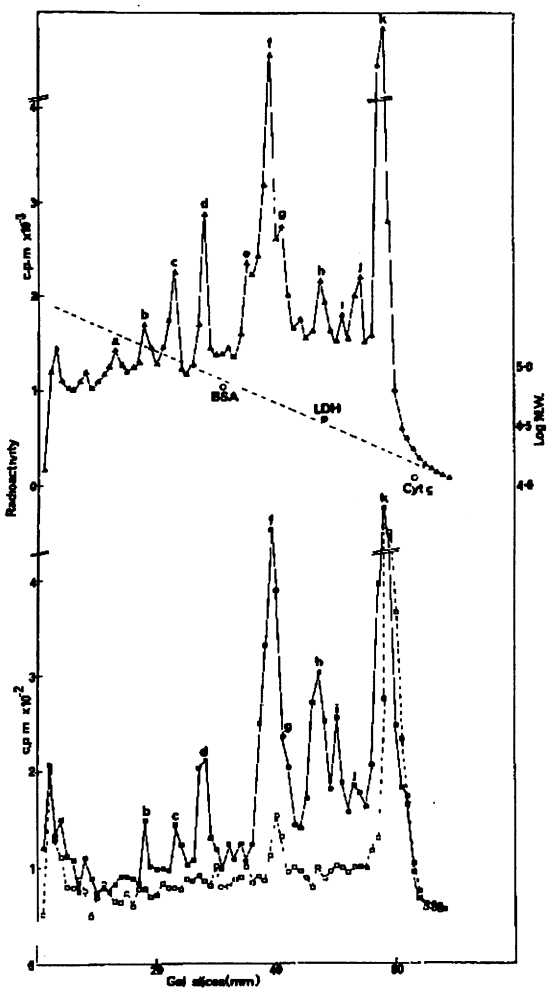

Fig. 3. A comparison of *in vitro* labelled protein from parasitized red cells (*13*) and antisera precipitated products from the cell-free system.

Triangles: total protein from parasitized cells labelled *in vitro* with (^{3}H)-isoleucine; black squares; cell-free system + *P. knowlesi* RNA/*P. knowlesi* antiserum; open squares; cell-free system + *P. knowlesi* RNA/trypanosome antiserum.

Molecular weight markers: BSA — bovine serum albumin — MW 68 000; LDH — lactate dehydrogenase — MW 35 000; Cyt *c* — cytochrome *c* — MW 12 400.

Cell-free assays were set up using *P. knowlesi* RNA from one variant and homologous and heterologous antisera were used to precipitate malarial products. The results did not reveal any significant differences between the precipitated products.

DISCUSSION

The rabbit reticulocyte cell-free system is a very efficient protein synthesizing system, which is capable of processing a whole range of mRNAs. Pretreatment of the reticulocyte lysate with a Ca^{++}-dependent nuclease (*10*) before the translation begins greatly reduces endogenous background activity. As can be seen in Fig. 1, total RNA from *P. knowlesi*-parasitized cells stimulates the incorporation of (^{35}S)-methionine into a range of polypeptides. These polypeptides are compared with those obtained when RNA from *T. brucei* was added to the cell-free system. The patterns are distinct for the particular RNA used, although some common bands can be seen.

Many of the polypeptides synthesized in the cell-free system are antigenic, as shown in Table 1 and Fig. 2, where products were precipitated with trichloracetic acid, specific *P. knowlesi* antiserum, or normal monkey serum. These antiserum-precipitated products also compare well with the results obtained by McColm (*13*) when parasitized red cells were labelled *in vitro* with (^{3}H)-isoleucine. A range of polypeptides with molecular weights ranging from over 100 000 to around 20 000 were found in both the cell-free assays and after *in vitro* labelling of parasitized cells. The major product had a molecular weight of about 50 000–55 000 (peak f in Fig. 3). This correlation is important as it is essential that the cell-free system should provide a reasonably accurate view of the protein synthesis that occurs within parasitized cells.

Having shown that the mRNA assaying system was satisfactory we tried to detect the synthesis of variable antigen by using RNA from one variant and precipitating products using homologous or heterologous antiserum. This did not, however, yield any conclusive results. No easily detectable differences were observed between the two polypeptide patterns in SDS-polyacrylamide gels.

It has been assumed throughout that the variable antigen is a polypeptide portion from either a glycoprotein or a protein. It could, however, be another component, such as a carbohydrate, but this seems unlikely as carbohydrates lack the adaptability of polypeptides. Even one different amino acid in a polypeptide could cause a change in conformation of a protein, and so lead to a change in antigenicity. It is for this reason that the variable antigen is believed to

be a polypeptide, and therefore the cell-free protein-synthesizing system is an ideal method of assaying for it.

Another problem that we have come across is the likelihood that the antibody (or antibodies) to the variable antigen(s) is a nonprecipitating antibody, or is directed at a configuration formed by combined parasite antigen and erythrocyte membrane components, in which case it would be impossible to visualize the product on an SDS-gel. The solution to this problem is to compare a gel on which total cell-free products have been analysed with a gel on which antiserum-precipitated products have been analysed, and look for a missing band. In Fig. 3 certain bands appear to be absent from the antisera-precipitated cell-free products, i.e., bands "a" and "e", but this apparent difference is due, in part anyway, to the gel-slicing technique. Autoradiographic comparisons provide a more accurate method for comparing results. Apart from the possibility that the variable antigen is not precipitable by antibody, there is also the possibility that only a small quantity of antigen is produced and that it is masked by other proteins present. It is hoped that by combining improved RNA extraction techniques with an increase in the quantity of material analysed on SDS-gels (i.e., by overloading the gel for most proteins), minor bands may become visible.

Despite the difficulties in detecting variable antigens in the cell-free system, these results indicate the parasite origins of many antigens from infected erythrocytes. Further work is still going on using this indirect method of investigating malarial antigens, and work is also in progress using the more direct approach of extraction, fractionation, and analysis of parasite material.

RÉSUMÉ

SYNTHÈSE DE POLYPEPTIDES DE *PLASMODIUM KNOWLESI* DANS UN SYSTÈME ACELLULAIRE

Les résultats préliminaires d'épreuves visant a caractériser les antigènes de *Plasmodium knowlesi*—dont certains peuvent varier selon le stade du parasite—sont exposés. De l'ARN messager a été extrait de *P. knowlesi* et transféré dans un système à base de réticulocytes de lapin—système particulièrement efficace pour la synthèse des protéines—après lyse des cellules et avec adjonction de (^{35}S) méthionine. L'analyse des produits du système par électrophorèse en gel de dodécyl-sulfate de sodium-polyacrylamide avant et après une épreuve de précipitation avec un antisérum spécifique de *P. knowlesi* a permis de déceler la présence d'un grand nombre de polypeptides d'un poids moléculaire variant entre plus de 100 000 et environ 20 000. On a constaté une bonne corrélation entre ces résultats et ceux obtenus par McColm après marquage *in vitro* de *P. knowlesi* par la (^{3}H) isoleucine. Dans les deux systèmes, le polypeptide principal a un poids moléculaire se situant vers les 50 000–55 000. Bien que les essais n'aient pas encore permis d'observer la synthèse d'un antigène variable, il y a tout lieu de penser que celui-ci est un polypeptide en raison de l'adaptabilité de ce composé. Quoi qu'il en soit, l'origine parasitaire de nombreux antigènes de l'érythrocyte infecté est dès à présent établie par ces expériences.

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