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ATTEMPTS TO DEFINE *PLASMODIUM FALCIPARUM* ANTIGENS WITH HUMAN MONOCLONAL ANTIBODIES

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Twenty-four lymphoblastoid cell lines were established from peripheral blood lymphocytes transformed by EB virus, of healthy Africans, living in an hyperendemic malaria area and hyperimmune to P. falciparum. *Fifteen cell lines secreted antibodies against antigens of the asexual blood stages. Six IgG and nine IgM clones were selected for analyzing their specificity by immunoprecipitation and immunoblots. Antibodies of two clones recognized a 230-240 kDa parasite protein and those of two other a 115 kDa* P. falciparum *polypeptide. Products of oligoclonal cell lines identified an additional 66 kDa antigen. The other clones reacted only in immunofluorescent assays by binding to antigens of the trophozoite stage.*

Human lymphoblastoid cell lines (LCLs) producing monoclonal antibodies of defined specificities have been obtained in different antigenic systems by immortalization of human B lymphocytes with Epstein-Barr virus (EBV) (Steinitz et al., 1977). These antibodies recognize haptens (Steinitz et al., 1979), bacterial (Steinitz et al., 1981) viral (Crawford et al., 1983; Seigneurin et al., 1983; Stricker et al., 1985), and parasite antigens (Monjour et al., 1983; Lundgren et al., 1983; Monjour et al., 1984).

As malaria is a major health problem in tropical areas, human monoclonal antibodies produced by LCLs' from hyperimmune subjects might represent important tools for the characterization and the isolation of parasite antigens recognized by the immune system of man. Furthermore, some of these antibodies might express inhibitory properties *in vivo*, and be clinically useful for passive serotherapy in chemo-resistant infections.

So far, the majority of monoclonal antibodies used for characterization of *Plasmodium falciparum* antigens have been raised in mice (Perrin et al., 1980; Schofield et al., 1982; Hall et al., 1983).

Murine hybridoma, however, may define *P. falciparum* epitopes differing from those involved in the acquisition of natural immunity in man. Stable human cell lines producing monoclonal antibodies specific for the erythrocytic stages of *P. falciparum* have been described (Monjour et al., 1983; Lundgren et al., 1983). These antibodies, synthetized by lymphocytes obtained from hospitalized malaria patients, were not characterized by immuno-precipitation and immunoblot assays.

In this report, we describe five different human LCLs immortalized by EBV, established from lymphocytes of hyperimmune African donors, living in a holoendemic area for *P. falciparum* infection, without clinical symptoms of malaria. These lines secrete monoclonal antibodies which are directed against different antigenic determinants of red blood cell (RBC) stages of *P. falciparum.*

MATERIALS AND METHODS

EBV infection of B lymphocytes from hyperimmune subjects – Peripheral blood lymphocytes of young adults living in a malaria endemic rural area near Bobo-Dioulasso (Republic of Burkina Faso) were isolated from heparinized blood just after the rainy season. All subjects had elevated serum antibody titers against *P. falciparum*, as measured by an immunofluorescent antibody (IFA) test. The mononuclear cells were isolated from the buffy coat by centrifugation on a "Ficoll Hypaque(R)" gradient and resuspended at 5×10^6 cells in 1ml of EBV deriving from the

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supernatant culture medium of B 95.8 cell line ($10^3 TD_{50}$/ml) (Miller & Lipman, 1983). After 1 to 2hrs., the cells were resuspended at 10^6 cells/ml in fresh RPMI 1640 medium containing 20% foetal calf serum, penicillin 100U/ml, streptomycin 10μg/ml, glutamine 2mM, with or without 0.2μg/ml of cyclosporin A (Sandoz Ltd., Basel, Switzerland). These procedures were carried out at the laboratories of the O.R.S.T.O.M. in Bobo-Dioulasso.

Cloning of EB virus transformed cell lines – Cells from culture bottles producing anti-*P. falciparum* antibodies after four weeks in the initial flask were cloned by limiting dilution with 1, 10 or 100 cells in 96 well tissue culture plates, which contained a feeder layer of 10^5 irradiated lymphocytes (2,000 rad) from cord blood or EBV seronegative donors. The cultures were left without any medium change for three weeks and then tested by IFA for the presence of anti-*P. falciparum* antibodies. Positive cultures were propagated first in 24 well tissue culture plates and thereafter in 50ml tissue culture flasks.

Indirect immunofluorescence antibody assay (IFA) – The specificities of the monoclonal antibodies, and of the different sera of African subjects corresponding LCLs, were determined by IFA.

Acetone-fixed parasitized RBC, from an European patient infected during an African safari and from *in vitro* cultures (UPA strain), presenting all erythrocytic stages, were used as antigenic preparations and normal RBC as control. For IFA, each slide was treated with 50μl of cell supernatants for 30 min at 37°C thereafter, with fluorescein isothiocyanate conjugated goat anti-human immunoglobulins (Igs) (Biomerieux, Lyon, France) and stained with Evans' blue before examination under fluorescence microscopy.

Immunoglobulin analysis – The IgG, IgM and IgA content of cell culture supernatants was determined with a sandwich-enzyme-linked immunosorbent assay (ELISA) (Renversez et al., 1983), using monospecific antisera (Dakopatts, Glostrup, Denmark). Surface Igs on the LCLs were detected by direct immunofluorescence on fresh cellular preparations using fluorescein-labelled F $(ab')_2$ of monospecific antibodies to human μ, γ, α, K and λ chains (Biosys, Compiègne, France; Institut Pasteur Production, Paris, France). Intracytoplasmic Igs were detected with the same antibodies on cells fixed with methanol/acetone (v/v) at –20°C for 1 min. The monospecificity of these reagents had been confirmed with well-characterized myeloma, Waldenstrom's macroglobulinaemia or chronic lymphocytic leukaemia cells.

Epstein-Barr nuclear antigen (EBNA) was detected by anti-complement immunofluorescence (Reedman & Klein, 1973), early antigen (EA) and viral capsid antigen (VCA) by indirect immunofluorescence (Henle & Henle, 1966).

Preparation of ^{35}S-methionine-labelled *P. falciparum* extracts – Five ml of a 5% human RBC suspension with 10% parasitaemia (UPA strain) were incubated at 37°C in RPMI, free of methionine, supplemented with glutamine, bicarbonate and 10% normal A (+) human serum. The parasites were labelled for 6hrs. with ^{35}S-methionine (20μCi/ml, sp. act = 800Ci/mM). The infected cells were then pelleted, washed four times with cold phosphate buffered saline (PBS) and disrupted with 1ml of 0.5% Nonidet P40 (NP 40) (W/v) in TNE (0.01M Tris, pH 7.4, 0.1M NaCl 0.005M EDTA) with 0.5mM phenyl-methylsulfonyl fluoride.

SDS polyacrylamide gel and immunoprecipitation analyses – Fifty μl of ^{35}S-methionine *P. falciparum* lysate containing 4×10^5 cpm were incubated for 1hr. at 0°C with 5μl of IFA-positive sera or with 10 to 50μl of tenfold concentrated supernatants of positive LCLs'. The mixtures were subsequently treated with *Staphyloccus aureus* protein A for 1hr. The precipitates were washed twice with TNE supplemented with 1% NP40, twice with TNE supplemented with 0.5% Np 40, once in a buffer containing 0.5M NaCl and 0.01M sodium phosphate pH 8.0 and once in distilled water. The precipitates were then dissolved in sample buffer (10% glycerol (v/v), 2.5% SDS (w/v) 5% β-mercaptoethanol (v/v), 0.002% bromophenol blue (w/v) and 0.04M Tris-HCl, pH 6.3), and heated at 100°C for 3 min prior to electrophoresis on a 7% SDS polyacrylamide slab gel. The gels were treated for fluorography, dried and exposed for autoradiography using Kodak X-Ray films (Eastman Kodak Co).

Immunoblotting – Red blood cells from *P. falciparum* cultures (24% parasitaemia), containing all the erythrocytic stages of the parasite, were washed with PBS and lyzed with six volumes of 0.05% NP40 in TNE containing 0.5mM PMSF. The lysates were cleared by centrifugation for 30 min at 2°C. The antigens were added to the sample buffer (2x) and heated at 100°C for 3 min, and then run on a 7% polyacrylamide slab gel. The separated proteins were transferred electrophoretically to a nitrocellulose sheet.

The blots were treated at 20°C with monoclonal culture supernatants for 1hr. After four vigourous washings in 10% newborn calf serum (NBCS)/0.2% Triton X100 (w/v), the blots were

incubated for 40 min with secondary antibodies, peroxidase-conjugated F (ab')$_2$ fragments prepared from sheep for the anti-human IgG (H & L chains) reagent or from goat for anti-human IgM (Biosys, Compiègne). Following five extensive washings with NBCS/PBS/Triton X100, the blots were incubated with 3.3'-diaminobenzidine-HCl/Tris-HCl buffer, pH 7.4.

RESULTS

Establishment of lymphoblastoid cell lines – LCLs were established in one to two weeks after EBV infection. From the 40 different donors, 24 lines were immortalized into LCLs. Failure to establish a greater number of line was probably due to the absence of a CO_2 incubator in the first steps of cultures which were done in Africa. Among these 24 LCLs, supernatants of 15 gave positive polyclonal IFA reactivities after one month of culture. The effect of cyclosporin A was tested on LCLs derived from same donors. In 11 subjects the antibody titer was higher in the LCL established in presence of cyclosporin A.

Five different LCLs were cloned by limiting dilution. Secreting clones derived from one cell were obtained for three lines: B11 (six clones), B38 (five clones) and B39 (four clones). Secreting clones were not elicited with one cell per well for the B4 and B18 lines. With 10 cells per well, these sublines, however, regularly produced antibodies to *P. falciparum*.

Characterization of the LCLs and of the antibody they produce – The six clones derived from the B11 line synthetized approximately 15μg IgG/ml/10^6 cells/24hrs., consisting of λ chains for four clones and K chains for two. The nine clones obtained from the B38 and B39 lines produced only IgM, either K or λ, at a rate of 5-20μg IgM/ml/10^6 cells/24hrs. The B4 and B18 viable sublines obtained from cloning with 10 cells secreted oligoclonal K and λ IgG. Their initial synthetic rate of 1-4μg IgG/ml/10^6 cells/24h decreased after six months to 0.5-1.0μg. All cells from the different LCLs contained EBNA, 2% of the cells of each line were positive for EA, and none contained VCA.

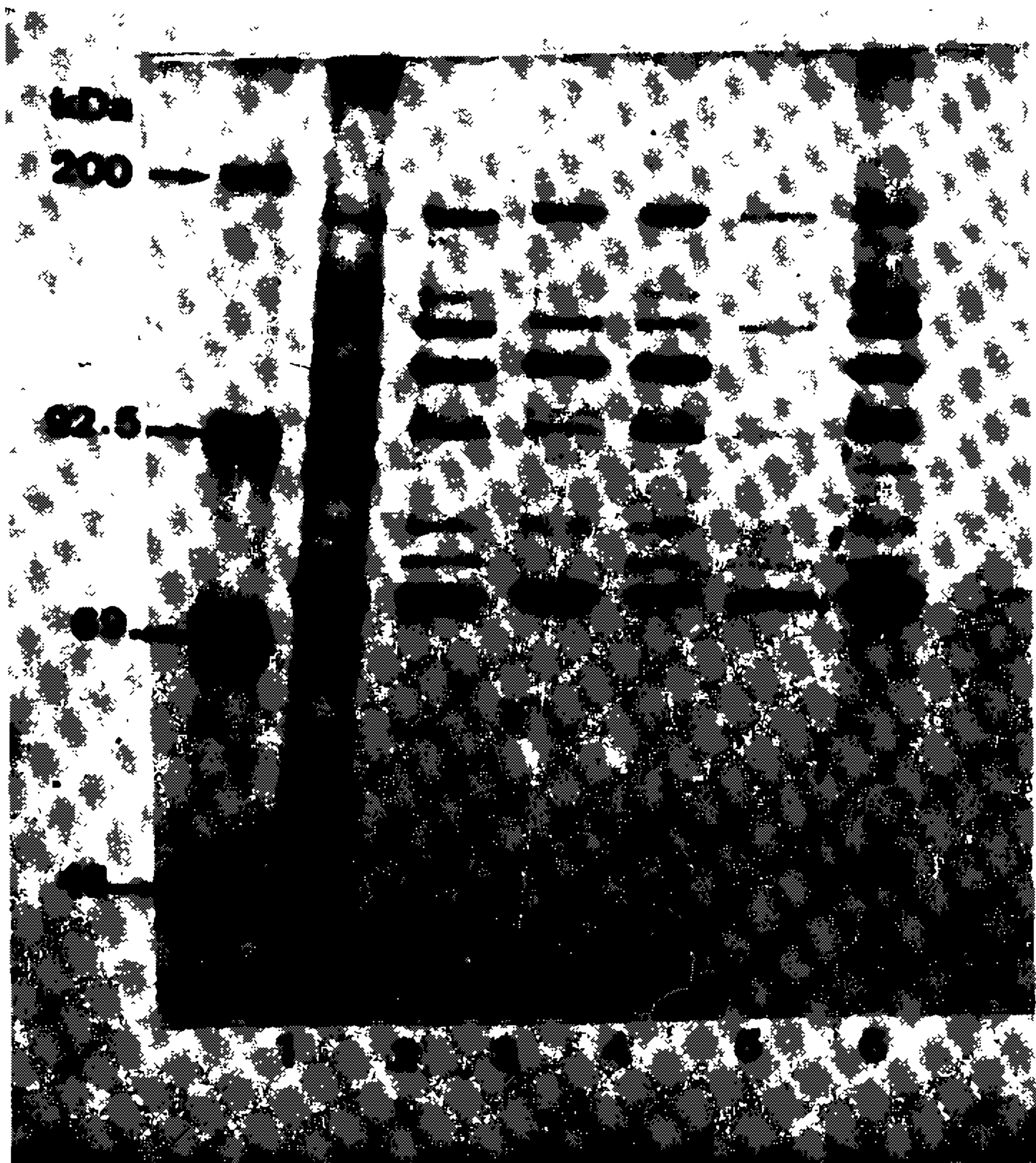

Fig. 1: Autoradiogram of 7% SDS-PAGE analyses of ^{35}S-methionine labelled *P. falciparum* antigens immunoprecipitated with the five different sera from the subjects were the LCLs were derived; total proteins (lane 1), B4 (lane 2), B11 (lane 3), B38 (lane 4), B39 (lane 5), B18 (lane 6).

Distinctive staining patterns in IFA differentiated these mono- or oligoclonal antibodies. All B38 and B39 clones gave a distinctive trophozoite pattern, B11 and B4 antibodies reacted strongly with the ring form of the parasite, whereas those of B18 produced a patchy staining pattern of parasitized RBC. On control, non parasitized, RBC preparations no reactivity was visible with the 15 monoclonal reagents, consistent with a parasite-specific antibody.

Molecular specificities of the anti-*P. falciparum* antibodies – Analyses by immunoprecipitation of biosynthetically-labelled *P. falciparum* lysates and by immunoblotting permitted to follow the effect of the cloning procedures on the specificities recognized by the anti-*P. falciparum* antibodies.

The immunoprecipitation patterns obtained with native sera were typical of those of immune subjects living in malaria holoendemic areas. At least eight common antigens of the *P. falciparum* UPA strain were identified by all sera. The intensity of the precipitating reactions and the number of additional bands varied among sera (Fig. 1). The initial polyclonal IFA-positive IgG from supernatants of B4, B11 and B18 LCLs revealed *P. falciparum* antigenic components of 66, 230-240 and 115kDa, which were barely visible or absent with supernates of B38, B39 and of an IFA-negative subline of B11 (Fig. 2). No antibody selection was yet detectable. Monoclonal IgG antibodies secreted by two clones derived from the B11 LCL interacted with a 230-240kDa protein, that was also detected, provided a slight overload, by the serum antibodies of donor B11 (Fig. 3). Among the B39 monoclonal IgM, featuring identical IFA patterns, two clones products recognized in immunoblots a 115kDa polypeptide (Fig. 4); some minor precipitating bands of M_r above and below 115kDa were also present, indicating possible proteolytic degradation. Neither the B38 oligoclonal supernates nor the five B38 monoclonal IgM reacted with parasite extracts in immunoblotting assays, developped with anti-IgG and anti-IgM reagents.

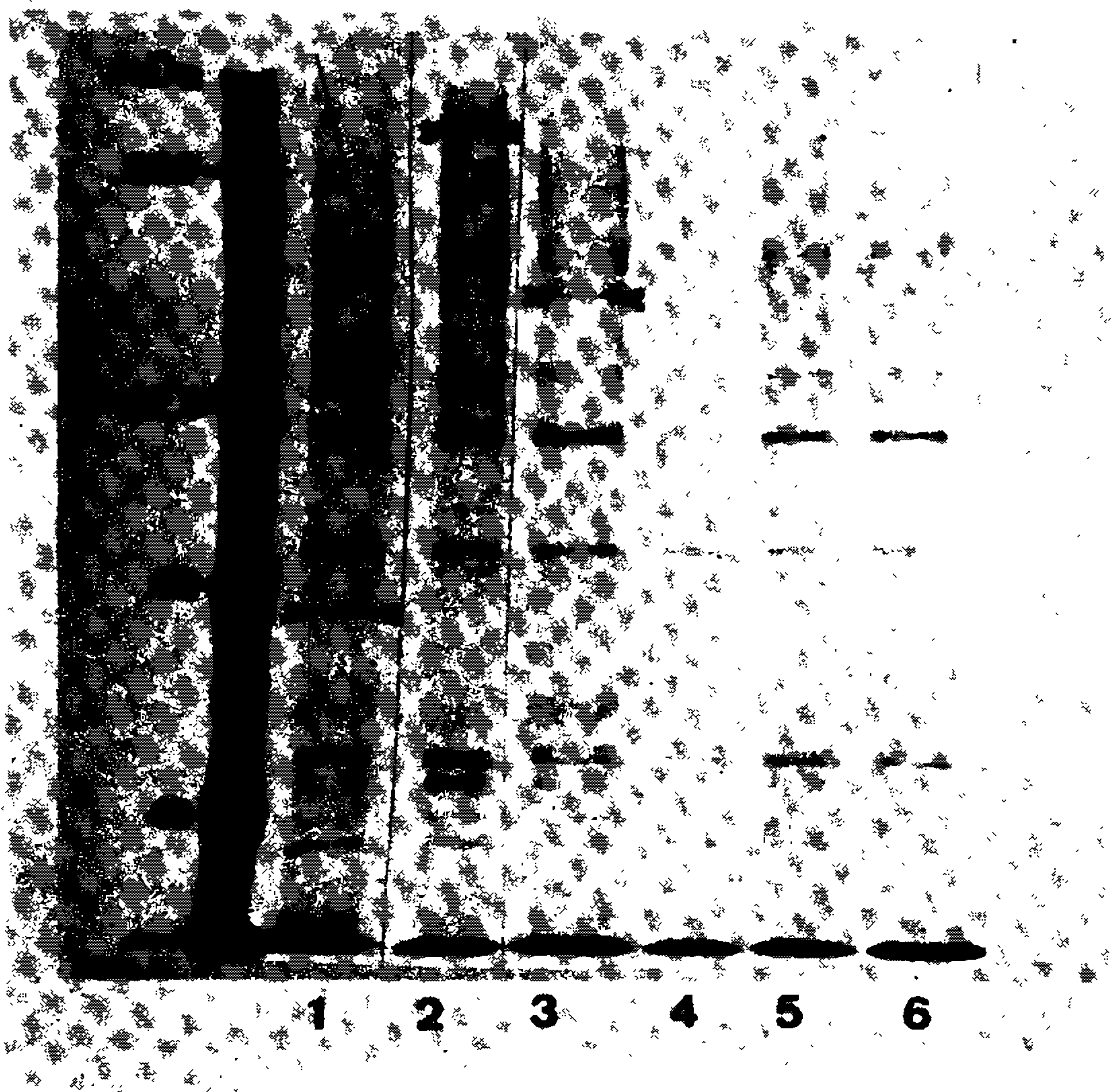

Fig. 2: Autoradiogram of 7% SDS-PAGE analyses of ^{35}S-methionine labelled *P. falciparum* antigens immunoprecipitated with the five different initial polyclonal cell lines supernatant IFA positive: B4 (lane 1), B11 (lane 2), B18 (lane 4), B39 (lane 5) and an IFA negative control supernatant (lane 6).

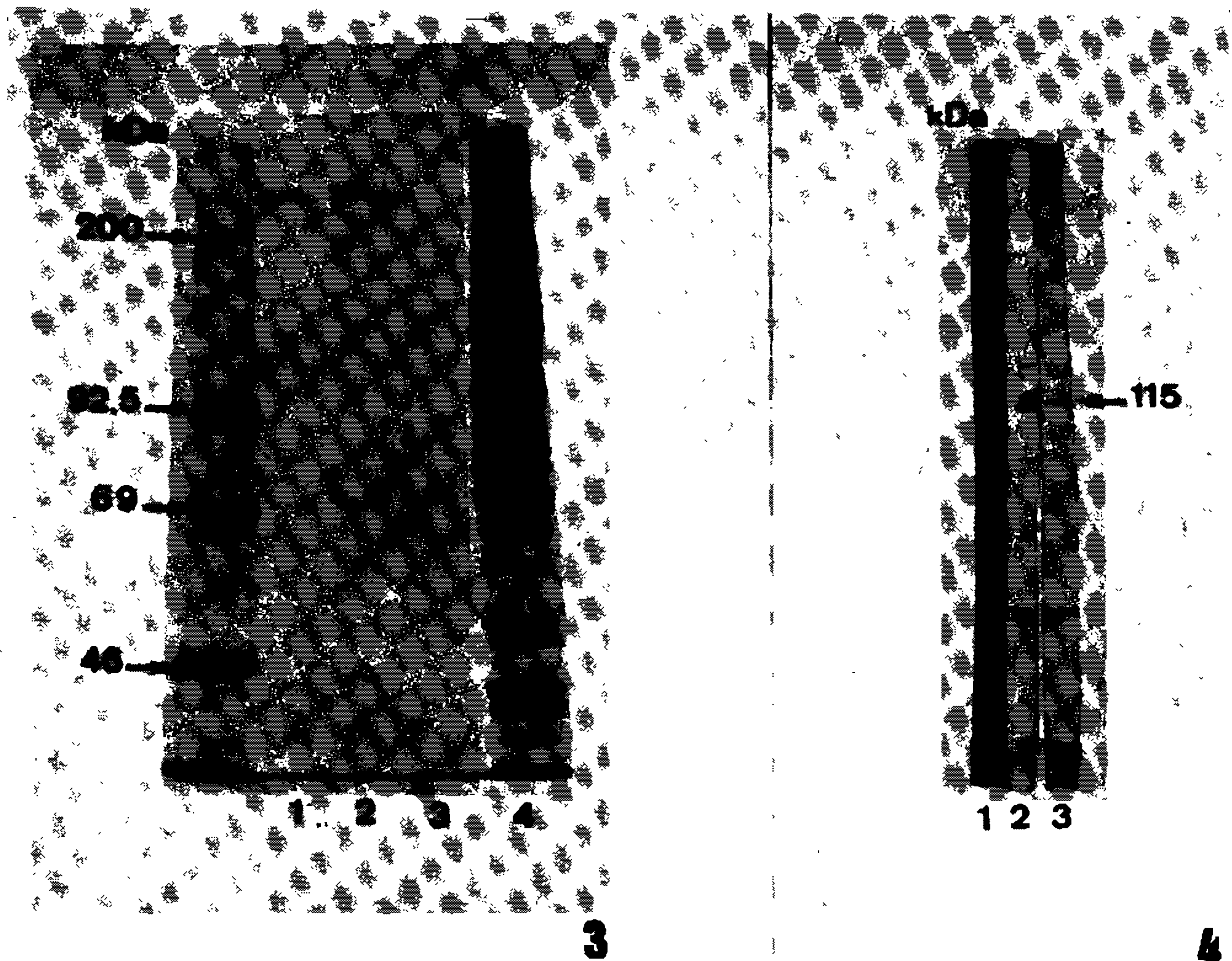

Fig. 3: Autoradiogram of 7% SDS-PAGE analyses of ^{35}S-methionine labelled *P. falciparum* antigens immunoprecipitated with two different IFA-positive clones of B11: B11$_5$ (lane 1), B11$_9$ (lane 2), negative serum (lane 3) positive serum of B11 subject (lane 4). Fig. 4: Immunoblot of 7% SDS-PAGE of *P. falciparum* antigens with positive human serum (lane 1), B39 (53) IFA-positive supernatant (lane 3), revealed by anti-human IgM secondary antibody.

DISCUSSION

Human LCLs producing anti-*P. falciparum* antibodies were obtained by EBV immortalization of B lymphocytes of African subjects living in a hyperendemic area, studied at time of low and chronic parasitaemia. Lundgren et al., 1983, ourselves (Monjour et al., 1983-1984) and Schmidt-Ullrich et al. (1986), have previously described some LCLs secreting anti-*P. falciparum* antibodies, established from B lymphocytes of malaria patents. The properties of these monoclonal antibodies out of four were, however, not studied in details. In this report, mono- or oligoclonal antibodies are shown to react with different antigens of the RBC asexual stages of *P. falciparum*.

An unexpected finding was the relatively short period of time (one to two weeks) required for establishing EBV LCLs derived from lymphocytes of malaria hyperimmune subjects when compared with European donors (four to five weeks), and using the same transforming dose of EBV (Seigneurin et al., 1983). The mitogenic factors liberated by *P. falciparum* infected erythrocytes and active on lymphocytes (Greenwood, Oduloju & Platt-Mills, 1979; Kataaha, Facer & Holborow, 1984), probably enhance the EBV immortalizing properties. Of interest was also the observation that of the 24 established LCLs, 15 secreted, without any prior selection or specific stimulation, parasite-specific Igs. This ratio was high relative to previous experience: out of 40 LCLs from herpes simplex virus (HSV), hyperimmune donors, only four secreted HSV monoclonal antibodies (Desgranges et al., in preparation). It is likely, that after the rainy season, a large number of B lymphocytes are engaged in the synthesis of antibodies against *P. falciparum*. Besides, Weidanz (1982) noticed that infection of man and of experimental animals with plasmodium often induced a profound decrease of cell-mediated immunity, accompanied by a polyclonal B lymphocyte activation and auto-immune phenomena. The fact we could establish LCLs, with and without cyclosporin A, for nearly each African donor, all highly EBV seropositive, indicates that the T lymphocytes of these subjects had lost normal control over transformed B-lymphocytes (Rickinson et al., 1981; Moss et al., 1983; Whittle et al., 1984). The higher specific Igs synthesis rate which occurred in the lines to which cyclosporin was added, is due to the capacity of this drug to increase non specific polyclonal Igs synthesis (Delespesse et al., 1983).

The monoclonal antibodies described herein, initially selected from cell line supernates reacting with acetone-fixed parasitized RBC, detected only trophozoites and erythrocytic schizonts and did not recognize sporozoites or liver schizonte (data not shown). In immunoprecipitation assays, the Igs secreted by two clones identified a 230-240kDa antigens and those synthetized by two other clones a 115kDa antigen; oligoclonal IgGs' from B4 sublines gave a faint reaction with a 66kDa polypeptide. The products of the B38 clones, which failed to precipitate ^{35}S-labelled extracts of the UPA strain or to interact in immunoblot, may contain an antibody directed against either an antigen absent in this plasmodial laboratory strain, or *P. falciparum* components present at a low level in extracts of non-synchronized cultures. The findings that among the 35-40 polypeptides recognized by the donors' sera, no more than two, possible three, antigens were identified by 15 monoclonal reagents, derived from five different donors, deserves further study; it may correspond to a bias in the initial selection procedures, or may have occurred as a random phenomenon.

The availability of stable human B cell lines, producing *P. falciparum* specific antibodies, should be helpful in correlating *in vitro* effects with antibody functions expressed *in vivo*, such as immunoprophylaxis in susceptible non-human primates. In addition, it should be of interest to investigate whether LCLs may be obtained from lymphocytes of patients with other parasitic diseases, also associated with B cell hyperreactivity, with the same propensity as that observed for subjects hyperimmune to *P. falciparum*.

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