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ANTIGENS OF THE ASEXUAL STAGES OF *PLASMODIUM FALCIPARUM*

G.V. BROWN+, D.J. KEMP, R.L. COPPEL, P.E. CREWTHER,
J.G. CULVENOR & R.F. ANDERS

To identify antigens of relevance to the induction of immunity to Plasmodium falciparum *malaria we have analyzed large numbers of antigen expressing clones in a λ-Amp3 library selected with serum from humans with a lifetime of malaria exposure. To review the approach, two antigens associated with the membrane of infected cells are described: MESA (a variable antigen associated with the surface of erythrocytes infected with mature stages of* P. falciparum*) and KAHRP (the knob-associated histidine rich protein).*

An approach to the seroepidemiology of malaria using cloned antigens as substrates is described using the ring-infected erythrocyte surface antigen (RESA) as an example.

The antigens of the asexual stages of *Plasmodium falciparum* are numerous and the targets of protective immunity are not known. One approach to the isolation of potentially important antigens is to produce monoclonal antibodies that can be used for affinity purification of the relevant molecules. We have used a different strategy that involved analysis of large numbers of antigens in an expression library selected with serum from humans with a lifetime of malaria exposure.

To review some of the features of our general approach to this problem, two antigens associated with the membrane of infected cells are described: MESA (a variable antigen associated with the surface of erythrocytes infected with mature stages of *P. falciparum*) and KAHRP (the knob-associated histidine rich protein).

A valuable tool for studying the seroepidemiology of malaria is the use of cloned antigens as substrates to measure specific antibody responses. This approach is illustrated using the ring-infected erythrocyte surface antigen (RESA) as an example.

MATERIAL AND METHODS

A λgt11-Amp3 (λ-Amp3) bacteriophage expression library was used to express proteins of *P. falciparum* in *Escherichia coli* as polypeptides fused to the C-terminus of β-galactosidase. Expressed antigens were detected by colony immunoassays using a pool of human immune serum from individuals living in an endemic area of Papua New Guinea (reviewed in Kemp et al., 1986). To characterize parasite proteins corresponding to cloned fragments, monospecific antibodies were prepared in three ways: (i) immunizing mice with bacterial lysates; (ii) immunizing rabbits with purified fused polypeptides, or (iii) preparing polyclonal human antibodies from pooled plasma using the bacterial clone coupled to Sepharose as an affinity-adsorbent (Crewther et al., 1986). Polyclonal but monospecific antibodies prepared in this way are highly specific for the antigen of interest and have been used for characterization of the corresponding parasite protein by immunofluorescence, immunoelectronmicroscopy and immunoblot analysis of stage-specific antigens. The antibody preparations have also been tested for inhibition of parasite growth *in vitro.*

Antibody to portions of individual malaria antigens – *E. coli* expressing fused polypeptides were harvested, treated with lysozyme, Triton X-100 and DNAase then the insoluble fused polypeptides were solubilized with 2% SDS, 2mM dithiothreitol, 0.1M phosphate buffer, pH 6.8. Soluble proteins were fractionated by gel filtration and fractions containing the fused polypeptide were depleted of non-protein material on a column of hydroxylapatite. The fused polypeptides eluted from the hydroxylapatite with 0.5M phosphate buffer, pH 6.8 were 50-90% pure depending on the abundance of the fused polypeptide in the original clone. Flexible polyvinylchloride microtitre plates (Dynatech Laboratories, Alexandria, Virginia, USA) were coated overnight with fused polypeptides at 2μg/ml. Sera were depleted of antibody to *E. coli* by overnight absorption with a preparation of bacteria containing bacteriophage but no inserted malaria genes, then incubated for 2h at a standard dilution (usually 1:1000) in the wells of plates coated with parasite antigen or

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The Walter and Eliza Hall Institute of Medical Research – Victoria 3050, Australia. +Corresponding author.

β-galactosidase alone. The plates were washed before addition of sheep antihuman IgG (Fab'2) fragments of affinity-purified (anti-γ). After 2h incubation, unbound antibody was washed off before iodinated protein A was added. After 2h, plates were washed to remove unbound protein A then cut up using a hot wire for determination of bound radioactivity using an autogamma spectrometer.

RESULTS

The Knob-Associated Histidine-Rich Protein (KAHRP) – Clones D10 and E12 of Papua New Guinean *P. falciparum* isolate FC27 were obtained by limiting dilution *in vitro* and maintained in long-term culture in candle-jars using essentially the method of Trager & Jensen (Trager & Jensen, 1976). By electronmicroscopic examination, knobs were identified on the surface of erythrocytes infected with trophozoites and schizonts of D10 but not on the surface of erythrocytes infected with mature forms of E12.

It was already known from DNA hybridization studies that cDNA sequence SD17, derived from another *P. falciparum* isolate NF7, hybridized with genomic DNA of the knobby clone D10, but not with the knobless clone E12 (Corcoran et al., 1986). Sonicated SD17 cDNA was inserted into λ-Amp3 and a clone hybridizing with SD17 and producing a fused polypeptide was selected for further study. This clone, designated Ag617, was then used as an affinity adsorbent for preparation of monospecific human antibodies from a pool of immune serum.

In immunofluorescence assays with acetone-methanol fixed parasitized cells the antisera produced a speckled pattern of fluorescence over mature parasites. Counterstaining with the nuclear stain propidium iodide confirmed that the fluorescence was not localized to merozoites.

Immunoelectronmicroscopy confirmed and extended these observations (Culvenor et al., 1986). Sections of parasitized infected cells incubated with human antibodies to Ag617 and then with protein A-colloidal gold produced specific labelling of large protruding knobs, particularly on the cytoplasmic side (Fig. 1). Attempts to label intact infected cells have so far been unsuccessful.

Stage-specific immunoblotting with human antibodies to Ag617 showed that the corresponding parasite antigen was dominant in mature stages of the parasite and varied in molecular weight among isolates from Mr 85,000 to Mr 105,000.

All the features strongly suggest that Ag617 is the knob associated histidine-rich protein described by Kilejian (Kilejian, 1979) and studied later by others (Hadley et al., 1983; Leech et al., 1984) and preliminary nucleotide sequencing demonstrates histidine-rich regions in Ag617.

Size polymorphisms have been noted in other *P. falciparum* proteins and shown to reflect different numbers of tandemly repeated peptides in alleles of the genes of different isolates (Kemp et al., 1986). The phenomenon has been noted in a family of histidine-rich proteins that includes a small polymorphic histidine and alanine-rich protein (SHARP) (Stahl et al., 1985) and preliminary investigations suggest that the same mechanism occurs in KAHRP. Apart from size polymorphisms seen with KAHRP and SHARP, complete deletions of the genes for these proteins and a third histidine-rich protein have been detected in various isolates. The availability of these clones enables the mechanism of gene deletion to be studied and may provide a means for investigating the relationship between the knob proteins and molecules involved in cytoadherence.

The Mature Parasite-Infected Erythrocyte Surface Antigen (MESA) – MESA is a high molecular weight polypeptide (Mr $\cong$ 250,000) associated with the membrane of erythrocytes containing trophozoites and schizonts (Coppel et al., 1986). Antibodies from human serum affinity purified on the MESA cDNA clone also reacted with two smaller proteins (Mr $\cong$ 82,000 and 86,000) that could be fragments of the larger protein or cross-reactive products. In merozoites, prominent bands were detected at Mr $\cong$ 125,000 and 115,000 but these did not persist to the ring stage. The high molecular weight antigen was only partially soluble in Triton X-100 suggesting that at least part of the protein is attached to the erythrocyte cytoskeleton.

In immunoblots, the antibodies reacted with equal intensity to molecules in FC27, a Papua New Guinea isolate, and NF7, a West African isolate but reactivity with isolates V1 and K1 from Vietnam and Thailand respectively was less intense. The dominant specificity varied considerably in apparent molecular weight among isolates (differences in Mr of $\cong$ 30,000 dalton). Analysis of subclones proved that the original clone contained at least two distinct antigenic determinants with the strain-specific component for FC27 contained in a hexapeptide sequence repeated many times.

Immunoelectronmicroscopy with protein A-gold localized the antigen in trophozoites and schizonts, particularly at the limiting membrane of the parasite. Gold particles were prominent

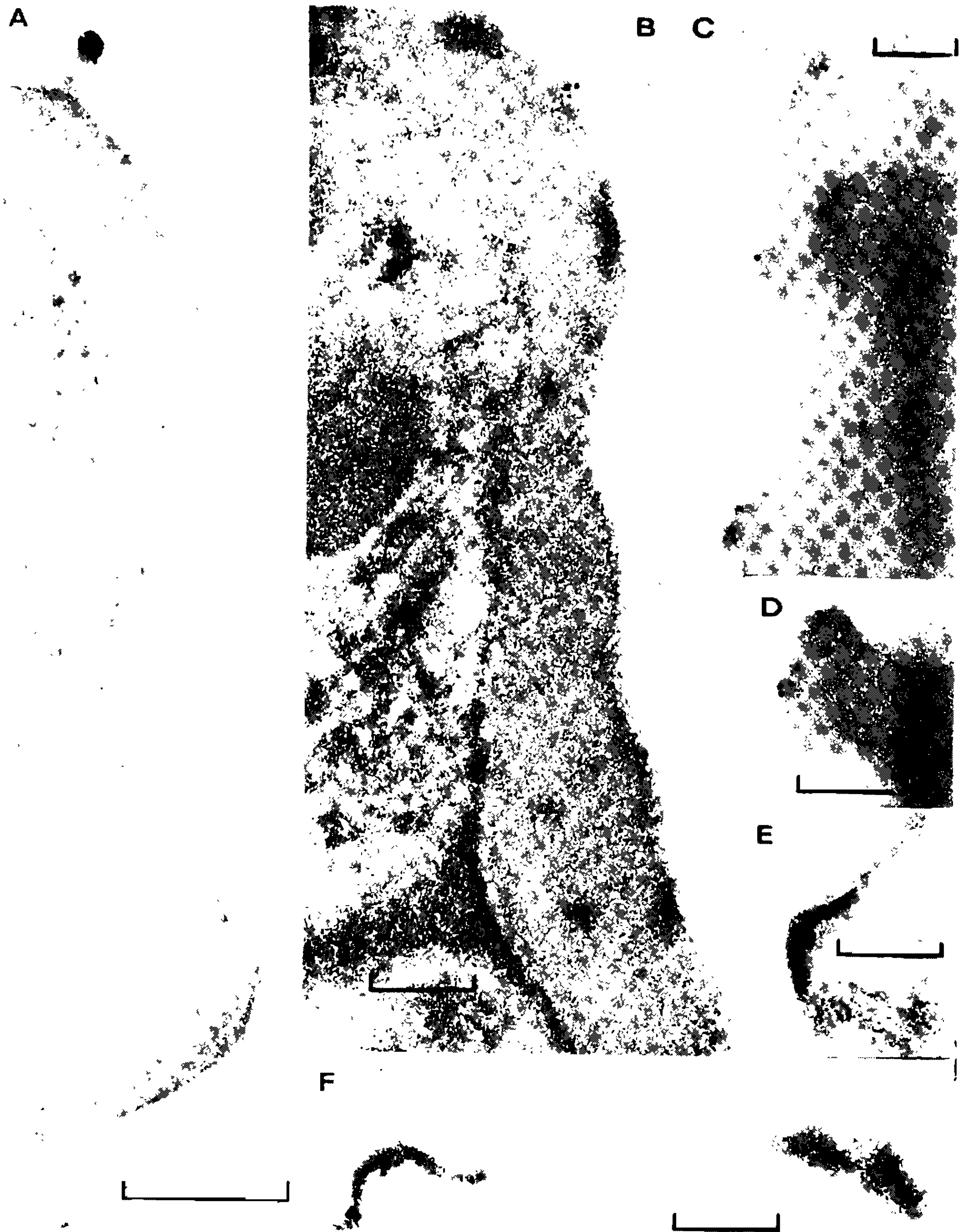

Fig. 1 – **Localization of the knob protein. Immunolabelling of isolate ItG2 with anti-Ag617 and protein** A-gold. A: Trophozoite-infected erythrocyte labelled with 10nm gold showing label only over two protruding knobs. Bar equals 0.5μm. B: Schizont-infected erythrocyte with 4nm gold over erythrocyte plasma membrane knobs and parasitophorous vacuole. Bar equals 0.1 μm. C and D: Knob detail of a trophozoite-infected erythrocyte labelled with 4nm gold. Bar equals 0.1 μm. E and F: Knob detail of erythrocyte plasma membrane of a lysed schizont-infected cell showing 4nm gold particles associated with knob material on the cytoplasmic side. Bar equals 0.1 μm.

in membrane lined vesicles in the erythrocyte cytoplasm and were also seen in caveolae at the erythrocyte surface.

A number of high molecular weight variant molecules associated with mature parasites have been described previously including the S-antigens, and the precursor to the major merozoite surface antigens. MESA is similar in a number of characteristics to the strain-specific antigens presumed to be involved in cytoadherence (Leech et al., 1984). The antigenic diversity, molecular weight, and solubility properties are similar but studies on cytoadherent lines will enable definitive experiments to confirm the relationship, or to establish whether MESA belongs to yet another group of variant antigens.

Seroepidemiology with defined antigens – Malaria antigens expressed as fused polypeptides in *E. coli* have been used as substrates in radioimmunoassays (RIA) or enzyme-linked immunosorbent assays (ELISA) to determine antibody responses to single antigens in individuals experiencing episodes of parasitaemia. Total ELISA values (using sonicated infected cells as substrate) did not change after patent parasitaemia, whereas in many cases antibody to individual molecules was boosted then fell over the following six months (Brown et al., 1986). It is well known that the total anti-malarial antibody level is an index of *exposure* to malaria and provides no indication of individual protection against this disease. Candidate vaccine antigens can now be tested in a specific way to determine whether there is an inverse correlation between antibody levels and disease susceptibility.

Seroepidemiology using different antigenic determinants of RESA – The ring-infected erythrocyte surface antigen (RESA) (Coppel et al., 1984) is transferred to the erythrocyte around the time of merozoite invasion (Brown et al., 1985). This molecule corresponds to the dominant antigen reacting with anti-malarial antibodies eluted from the surface of air-dried, lightly glutaraldehyde fixed parasitized cells (Wåhlin et al., 1984). Antibodies from some immune human sera, affinity-purified on the cloned antigen show a variable but small degree of inhibition of parasite growth *in vitro* (Brown G.V., unpublished data) and antibodies affinity-purified from the cell surface produce marked inhibition (Wåhlin et al., 1984).

RESA contains two separate regions of repetitive sequences (Cowman et al., 1985) and fused polypeptides corresponding to each region have been obtained for immunization studies and for use as substrates in immunoassays of the immune response to different parts of the molecule.

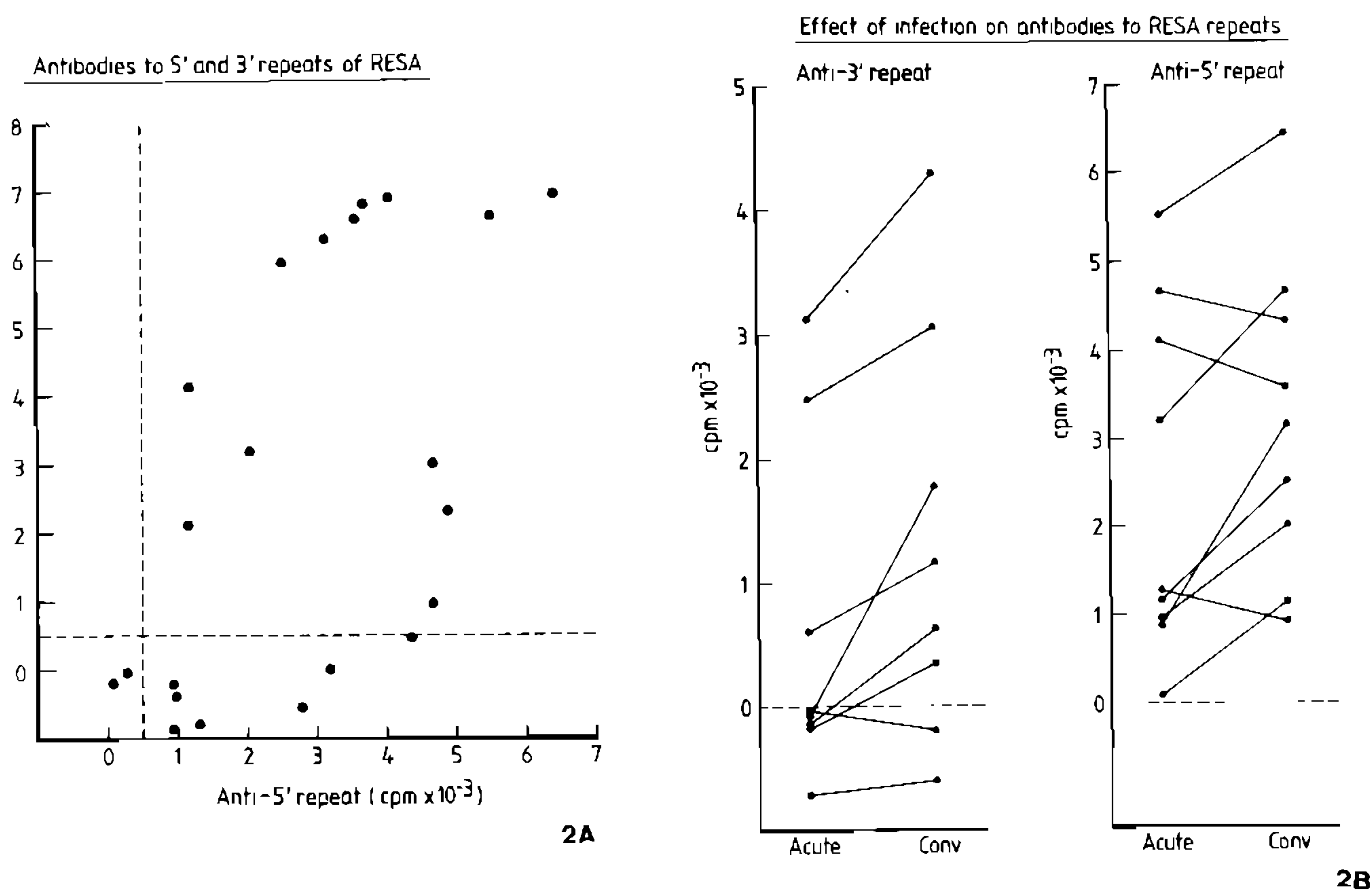

Fig. 2 – Antibody response to different repeat regions of RESA. A: Correlation between 5' repeat and 3' repeat in a group of individuals. B: Antibody response to acute infection.

It is clear that in most cases there is a correlation between the antibody response to the 5' repeat and the 3' repeat region but in some individuals the antibody response to one repeat region is dominant (Fig. 2A). Similarly we can measure the antibody response to acute infection in a number of individuals. Once again, different individuals respond differently; usually, infection causes a boost in antibody to both determinants, but in other cases, there is no measurable increase to either repeat region (Fig. 2B). If protection is afforded by antibody response to only one of these regions, it is apparent that measurement of reactivity to the whole protein is not a precise measure of protection. It is likely that seroepidemiology with individual antigenic determinants will, in the future be the best measure of protective immunity.

CONCLUDING REMARKS

With the goal of identifying candidate vaccine molecules from the very large number of *P. falciparum* proteins cloned in *E. coli* we selected antigen-expressing clones from a λ-Amp3 library using a pool of serum from humans living in an endemic area. Human antibodies affinity-purified on antigen expressing clones have been used for localization of many of these antigens by immunoelectronmicroscopy, immunofluorescence and immunoblotting – including a knob-associated protein (KAHRP) and an antigen of mature parasites (MESA) described here.

The availability of large amounts of individual cloned antigens enables seroepidemiology to be performed with increased precision by analysis at the level of individual antigenic determinants.

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