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An asparagine-rich protein from blood stages of *Plasmodium falciparum* shares determinants with sporozoites

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ABSTRACT

We describe a cDNA clone derived from mRNA of asexual blood-stages of the malaria parasite Plasmodium falciparum. This clone, designated Ag319, expresses a P.falciparum antigen fused to β-galactosidase in Escherichia coli. Human antibodies from Papua New Guinea were affinity-purified by adsorption to extracts of Ag319 immobilized on CNBr-Sepharose. The antibodies reacted predominantly with P.falciparum polypeptides of Mr 220,000 and 160,000, and a number of ill-defined lower molecular weight species. Antibodies reacted in indirect immunofluorescence with all asexual blood-stages although the antigen appeared to be most abundance in the schizont. Surprizingly the antibodies also reacted with sporozoites. The amino acid sequence predicted from the complete nucleotide sequence of this clone is remarkable because 40% of the residues are Asn, and so the antigen has been termed the Asparagine-Rich Protein (ARP). Like other P.falciparum antigens, ARP contains tandemly repetitive sequences, based on the tetrapeptide Asn-Asn-Asn-Met and we have confirmed that these represent natural epitopes by reaction of the corresponding synthetic peptides with human antibodies. Surprisingly, ARP is also rich in Asn outside the tandem repeats.

INTRODUCTION

A considerable number of polypeptides from the malaria parasite Plasmodium falciparum are natural immunogens in man. The construction of libraries expressing P.falciparum antigens in Escherichia coli has enabled the identification of cDNA clones expressing many of these antigens and hence facilitated their detailed examination at the molecular level (1-5). Interestingly, most of the antigens studied to date contain tandemly-repeating oligopeptides, often in extensive stretches (6-10).

In some of these repetitive antigens, the sequence repeats contain very limited subsets of amino acid residues and consequently skew the overall amino acid compositions. For example the Histidine Rich Protein (HRP) of P.lophurae contains 73% His (11) because it consists largely of repeats, most commonly decapeptides containing 8 His residues (12,13). A small histidine- and alanine-rich protein (SHARP) from P.falciparum contains

hexapeptide and pentapeptide repeats (14) and has an overall predicted composition of 30% His and 29% Ala. The composition of constituent repeats explains the high level of Asn (31%) in the circumsporozoite protein of P.falciparum (3) and high levels of Ala and Gly (15) in the corresponding protein of P.cynomolgi.

We describe here the isolation and some properties of a cDNA clone encoding a portion of a P.falciparum antigen which is present in all stages of the asexual life cycle in the blood. This antigen or a cross-reacting molecule is also present in sporozoites. From the cDNA sequence, we deduce that this protein contains a limited number of tetra- and octapeptide repeats but also has a very unusual composition outside the tandem repeats. As Asn constitutes 40% of the total amino acid residues, we have termed it the Asparagine-Rich Protein (ARP).

MATERIALS AND METHODS

Parasites and cloned DNA molecules

P.falciparum isolates FCQ27/PNG (FC27), IMR143, IMR144 and MAD71 were obtained through collaboration with the Papua New Guinea (PNG) Institute of Medical Research. NF7, originating from Ghana, and K1 originating from Thailand were obtained from D. Walliker, Edinburgh University. All isolates were maintained in long-term asynchronous culture as described by Trager and Jensen (16). Construction of P.falciparum cDNA clones from NF7 mRNA (17), cloned in λgt10 was as described (1) except that cDNA molecules greater than 2 kb long were selected. The cDNA inserts were isolated from the λgt10 library and recloned in λgt11-Amp3 (λAmp3) as described (1).

Sera

Sera were obtained with informed consent from individuals from Madang, Papua New Guinea through Dr. M.P. Alpers and associates, Papua New Guinea Institute of Medical Research.

Affinity purification of human antibodies on fixed and air-dried merozoites

Merozoites were harvested over a 2 hr period of culture from medium containing FC27 parasites synchronised twice with sorbitol treatments (18). Erythrocytes were removed by centrifugation and sieving (19) and the merozoites were washed in 2 changes of triethanolamine buffer (20). Glutaraldehyde was added to a final concentration of 1% (v/v) and the parasites were incubated at 0°C for 5 minutes. They were immobilised by suction on to nitrocellulose filters and incubated with human antibodies isolated from

a pool of Papua New Guinea sera. Unbound material was removed by washing and specifically bound antibodies were eluted with 0.2M glycine-HCl, pH 2.8.

Colony immunoassays

Replicas of antigen-positive clones were grown overnight at 30°C, induced at 38°C, and lysed (21). Sera were absorbed to remove anti-E.coli reactivity, diluted 1:500 in 3% bovine serum albumin/ Tris saline at pH 9.6 and applied to filters that were incubated with ^{125}I protein A from Staphylococcus aureus and autoradiographed overnight.

Affinity purification of human antibodies on lysates of E.coli clones that express ARP.

Induced 50 ml cultures of antigen-positive clones were prepared as described previously (22). The pelleted bacteria were sonicated and soluble bacterial proteins were conjugated to CNBr-activated Sepharose (Pharmacia, Sweden). Antibodies from a pool of human sera collected from individuals living in Papua New Guinea were affinity purified on the immobilized antigen as described (23).

Immunoblots

Protein extracts of cultures of P.falciparum were prepared and fractionated on 7.5% polyacrylamide/NaDodSO$_4$ gels. Proteins from the gels were transferred electrophoretically to nitrocellulose, incubated in 5% non-fat milk powder in PBS and reacted with affinity purified human antibodies (23). The filters were incubated with ^{125}I-labeled protein A and autoradiographed.

Immunofluorescence

Thin smears of parasitised erythrocytes from asynchronous cultures of P.falciparum (isolate FC27) were fixed with 90% acetone/10% methanol and reacted with antibodies against ARP. Human antibodies affinity-purified on Ag319-Sepharose or serum from a rabbit immunized with the purified fused polypeptide of Ag319 were employed. Fluorescein-conjugated sheep anti-human Ig or goat anti-rabbit Ig antisera were used as the second reagent. Parasite nuclei were counterstained with propidium iodide and the slides were mounted in 90% glycerol/10% PBS containing p-phenylenediamine for viewing under U.V. illumination.

Immunofluorescence with glutaraldehyde-fixed and air-dried parasite preparations were performed according to the method of Perlmann et al. (24). Sporozoites from mosquito salivary glands were air-dried on slides fixed with 90% acetone/10% methanol and treated with affinity-purified human anti-ARP antibodies as described previously (25) Intact sporozoites were treated with azide, then assays performed as described previously (26).

Hybridization experiments

Phage DNA was digested with EcoRI and size-fractionated on a 1% low-melting agarose-gel. The insert was recovered by phenol extraction, sub-cloned in pUC-9, purified and then nick-translated. Hybridizations were in 0.75 M NaCl/0.75 M Na citrate/50% formamide/50 μg ml^{-1} salmon sperm DNA/ 10 μg ml^{-1} poly (C)/0.02% Ficoll/0.2% polyvinyl-pyrollidone/0.02% BSA at 42°.

Nucleotide sequence determination

The dideoxy chain termination method (27) was employed for sequence determinations. The inserts of Ag319 and fragments generated from the inserts by digestion with restriction endonucleases, were cloned into M13mp8 and mp9 (28).

RESULTS

An expression library constructed from mRNA of the Ghanaian isolate NF7

We have described previously the isolation of a number of cDNA clones expressing P.falciparum antigens by serological screening of an expression library constructed in λgt11-Amp3 (λAmp3) (1). The library contained cDNA sequences 0.6-2.0 kb in length, derived from the Papua New Guinea isolate of P.falciparum FCQ27/PNG (FC27). In an attempt to identify further antigens a new cDNA library was constructed from the Ghanaian isolate NF7. Because many antigens of P.falciparum are relatively large polypeptides, cDNA molecules ≥2.0 kb in length were selected for cloning in λAmp3. In order to maximize the chance of detecting weakly-positive clones, randomly selected clones were picked in triplicate into geometrical arrays before immunological screening. Instead of using affinity-purified immune human antibodies or a single immune human serum as previously employed (1,21,22) we screened with a pool of 10 immune human sera from PNG adults. From a total of ~3500 recombinants that were picked into the primary unselected arrays, 103 gave positive reactions in colony immunoassays with this serum pool. These 103 colonies were repicked in triplicate into a single array on one nitrocellulose filter and designated the NF7 array.

Identification of ARP by reaction with antibodies purified on fixed merozoites

Antibodies purified from a pool of PNG sera by binding to merozoites that had been fixed with glutaraldehyde and immobilized (Materials and Methods), were eluted and reacted with the array of antigen-expressing colonies described above. One colony, designated Ag319 (Fig. 1A) was selected

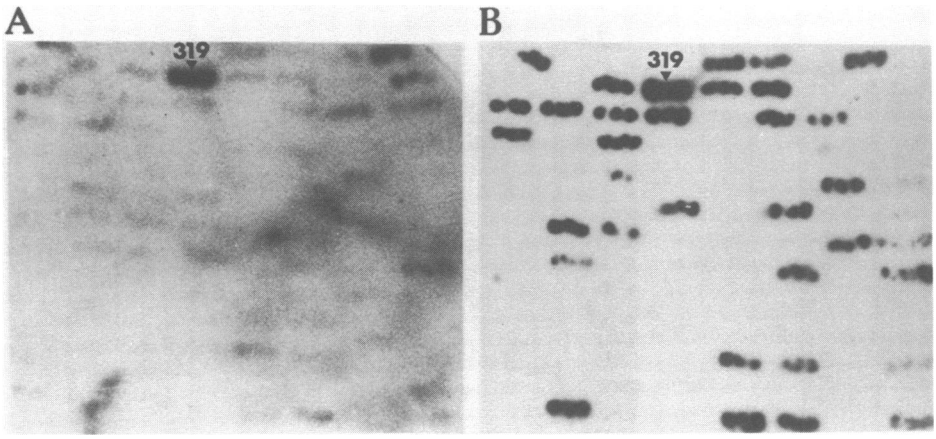

Figure 1 Detection of Ag319 by colony immunoassay. Human antibodies were reacted with the NF7 array (see Materials and Methods). A: Human antibodies eluted from lightly glutaraldehyde-fixed and air-dried monolayers of merozoite preparations. B: Human antibodies affinity-purified on bacterial lysates of clone Ag319. 32 further clones in this array expressing Ag319 sequences are detected.

for further study because it reacted much more strongly than any others in the NF7 array. A protein extract from Ag319 was immobilized on CNBr-activated Sepharose and used as an adsorbent for affinity-purification of human antibodies from PNG serum as described elsewhere (23). These purified antibodies were then reacted with the NF7 array. The antibodies purified on Ag319 reacted with a total of 32 clones from this array (Fig. 1B). These antibodies did not react with any clones from the two arrays that we have described previously, containing 78 (22) and 133 (21) clones respectively (data not shown). Hence we conclude that Ag319 represents an antigenic specificity that was not isolated previously. Surprisingly, sibling analysis by hybridization with Ag319 cDNA revealed that the 32 clones of this antigenic specificity represented at least two different DNA sequence families that did not cross hybridize but nevertheless cross-react antigenically (manuscript in preparation).

Identification and localization of ARP

In order to identify the P.falciparum polypeptide represented by Ag319, the human antibodies purified on immobilized Ag319 extract were used to probe immunoblots of polypeptides extracted from P.falciparum under various conditions. In immunoblots of supernatants from saponin-lysed infected erythrocytes, complex patterns were obtained but the antibodies reacted predominantly

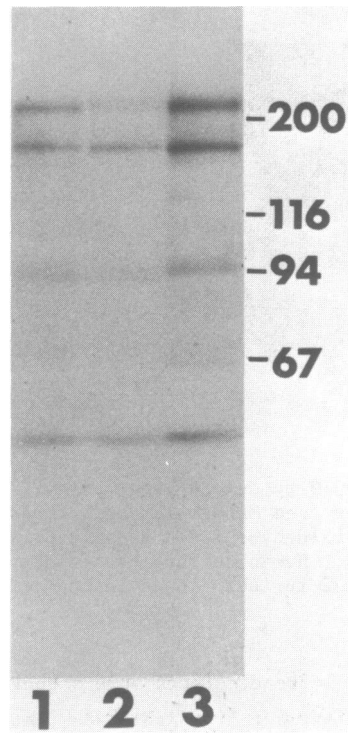

Figure 2 Immunoblots of supernatants from saponin-lysed infected erythrocytes; with human antibodies purified on Ag319. Lane 1 = NF7, lane 2 = K1, lane 3 = FC27. The apparent molecular weight of the most prominent bands were calculated to be 220 and 160 kD.

with two major polypeptides of Mr 220,000 and 160,000 (Fig. 2). Similar results were obtained with 3 different isolates of P.falciparum. Less intense bands at Mr 98,000 and 52,000 and some minor components were also present. However, solubilization of parasite antigens in Triton X-100 or in electrophoresis sample buffer containing SDS and 2ME yielded a number of ill-defined bands ranging up to around Mr 220,000 (data not shown). Antibodies to other polypeptide antigens reacted with these same parasite extracts to give sharp, single bands in parallel immunoblotting experiments (23). These results suggest that there may be a number of antigenically related polypeptides and/or that ARP is a large (Mr >200,000) polypeptide that is degraded in these extracts or processed in the parasite.

The human antibodies purified on Ag319 and a rabbit antiserum raised against the purified fused polypeptide from Ag319 were used in indirect

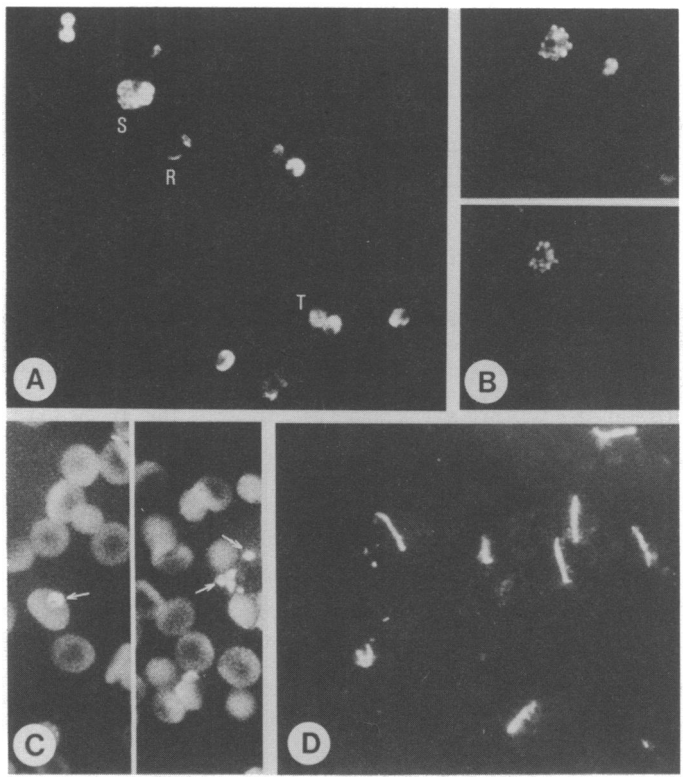

Figure 3 Indirect immunofluorescence of P.falciparum reacted with human antibodies affinity-purified against Ag319.
A Fluorescein staining of asexual blood stages in erythrocytes fixed with acetone/methanol illustrating ring (R), trophozoite (T) and schizont (S) stage parasites.
B One field of view containing a mature, segmental schizont examined for fluorescein (upper panel) and propidium (low panel) fluorescence.
C Fluorescein staining of parasites lightly fixed with glutaraldehyde illustrating a merozoite apposing an erythrocyte (left panel) and a cluster of extracellular merozoites (right panel).
D Fluorescein staining of acetone-fixed sporozoites.

immunofluorescence assays, both on acetone-fixed parasitized erythrocytes (Fig. 3A,B) and on preparations lightly fixed with glutaraldehyde and air-dried (Fig. 3C). Both antisera gave identical results and in control experiments with human antibodies purified on a λAmp3 column, no fluorescence was detected. The antibodies reacted with all stages of the asexual blood cycle, with the predominant staining in acetone-fixed smears localised to the parasites and not the host cell (Fig. 3A). The intensity of fluorescence

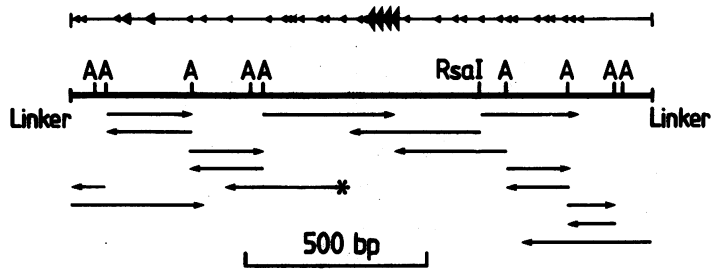

Figure 4 Structure of the cDNA clone Ag319 and the strategy for determining its sequence. The repeats are indicated on the top line by filled triangles: large and small triangles represent octamers and tetramers, respectively. Arrows below represent the extent of sequencing runs from AhaIII (abbreviated A), RsaI-sites and the linker. The arrow with an asterisk shows a run obtained with a synthetic primer corresponding to the complementary sequences from position 714-735 of Ag319.

at a given antibody dilution increased progressively between ring-stages, trophozoites and schizonts. In mature schizonts the pattern of staining was coincident with the segmentation of developing merozoites within the host cell, as determined by counterstaining of parasite nuclei with propidium iodide (Fig. 3B). ARP was not detected on parasitised cells fixed with glutaraldehyde, but the antibodies reacted with extracellular merozoites present in the same preparations (Fig. 3C).

Anti-ARP antibodies react with sporozoites

The human antibodies affinity purified on the Ag319 adsorbent were tested for reactivity against sporozoites by indirect immunofluorescence. Control antibodies purified from the same serum on Sepharose-adsorbents bearing extracts from E.coli or recombinant clones expressing other P.falciparum antigens did not react when tested on acetone-fixed sporozoites (data not shown). Antibodies to Ag319 reacted with both acetone-fixed (Fig. 3D) and non-fixed, azide-treated P.falciparum sporozoites but not with P.vivax sporozoites.

Nucleotide and amino acid sequence of ARP

The 1.6 kb cDNA insert from Ag319 and fragments generated from it by digestion with AhaIII or RsaI were subcloned in the vectors M13 mp8 and mp9 and sequenced by the dideoxy chain termination procedure (Figs. 4 & 5).

The sequence of Ag319 shown translated in Figure 5 is in phase with β-galactosidase and contains a single open reading frame that extends throughout the cDNA. Because Ag319 produces a large fused polypeptide we conclude that the frame shown is correct. The sequence exhibits a very high

Figure 5 Nucleotide and deduced amino acid sequence of Ag319. Hexa- and octapeptide repeats are underlined with thick lines, whereas the interspersed tetrapeptide repeats are underlined with thin lines.

AT-content with 50% adenine and 27% thymidine in the coding strand.

ARP contains 40% Asparagine

The insert of Ag319 codes for a relatively hydrophilic polypeptide. Surprisingly, 40% of the Ag319-polypeptide consists of asparagine and 7.6% is methionine. Commencing at position 803, there are 3 tetrapeptide (Asn-Asn-Asn-Met) and 4 octapeptide tandem repeats (Asn-Asn-Asn-Met-Asn-His-Asn-Met). A further 5 repeats of Asn-Asn-Asn-Met are interspersed along the sequence. There are also 16 tetrapeptide units composed of Asn-Asn-Asn and a variable fourth amino acid: Ile (3x), Ser (3x), Glu (3x), Thr (2x), Lys (2x), Asp (2x), Asn (2x) Tyr (2x), Gly (1x) and Phe (1x). In total 29

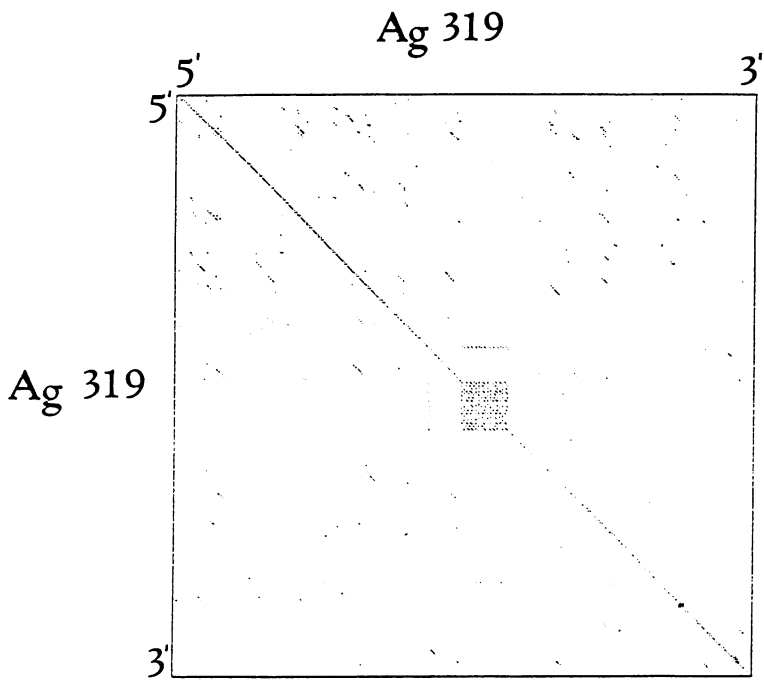

Figure 6 Protein Diagon according to the programme of Staden (29) revised by A. Kyne. The deduced protein sequence of Ag319 was compared to itself. The octapeptide repeats can be seen clearly as well as multiple internal homologies due to the interspersed Asn-rich tetrapeptides.

tetrapeptide and 5 octapeptide repeats that all have a high content of asparagine can be somewhat arbitrarily distinguished. We also noted two hexapeptide units located at positions 137-154 and 209-226 that are composed of Asn (or Asp)-Met-Asn-Asn-Ser-Asn. A computer analysis on the protein sequence of Ag319 using the Diagon program of Staden (29) clearly shows the block of repeats and the high degree of homology with the other tetrapeptide units (Fig. 6). To confirm the predicted sequence, we tested a synthetic peptide corresponding to the repeat-sequence His-Asn-Met-Asn-Asn-Asn-Met-Asn-His-Asn-Met-Asn-Asn-Asn-Met-Asn-His. Affinity-purified human antibodies reacted with this peptide in a solid-phase binding assay (data not shown).

Genomic organisation of ARP

DNA from 5 P.falciparum isolates (FC27, IMR143, IMR144 and MAD71 from Papua New Guinea and NF7 from Ghana) was cleaved with AhaIII and RsaI, size-fractionated on 1% agarose, blotted to nitrocellulose and hybridized with the Ag319 probe. As can be seen in Figure 7 ARP does not exhibit restric-

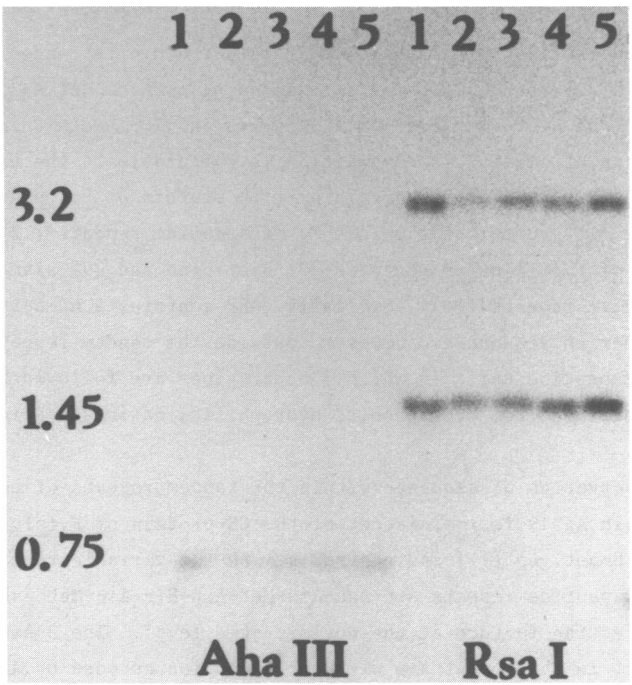

Figure 7 Hybridization of Ag319 cDNA to restriction fragments of P.falciparum DNA. DNA from the 5 isolates of P.falciparum indicated below was cleaved with Aha III or Rsa I, fractionated by electrophoresis on a 1% agarose gel, blotted to nitrocellulose, hybridized with ^{32}P Ag319 cDNA and autoradiographed. The P.falciparum isolates were: (1) FC27; (2) NF7 from Ghana; (3) IMR143; (4) IMR144 and (5) MAD71 from Papua New Guinea. Fragment sizes are indicated in kilobases.

tion fragment polymorphisms like those observed for a number of other cloned P.falciparum antigens (9,14,30). The Ag319 probe hybridized to a 0.75 kb AhaIII and to 3.2 and 1.45 kb RsaI-fragments, in accordance with the restriction map of Ag319 (Fig. 4). The small AhaIII fragments (see Fig. 4) were run off the gel and therefore cannot be seen. The block of 3 tetra- and 4 octapeptide repeats is located within a 680 bp AhaIII fragment, which is close to the size of the chromosomal fragment measured by Southern blotting. These results suggest that few if any repeats have been deleted from Ag319. This finding is important as it has been shown that approximately 100 repeats were deleted from a chromosomal clone encoding the S-antigen of P.falciparum isolate FC27 (17).

DISCUSSION

ARP is another antigen of P.falciparum that exhibits tandemly repeated units of short oligopeptides. The corresponding cDNA clone, Ag319, codes for a polypeptide that contains 40% asparagine and 7.6% methionine. This extreme skewing of amino acid composition is comparable to the histidine rich proteins of plasmodia. A histidine-rich protein of P.lophurae contains 73% histidine (11) contributed mainly by decapeptide repeats (12) and one such protein of P.falciparum contains 30% histidine and 29% alanine, again predominantly in repeats (14). Similarly, ARP contains a number of repeats of asparagine-rich sequences. However, outside the tandem repeats ARP contains 29 tetrapeptide units in which 3 asparagines are followed by a variety of residues derived from hydrophobic, hydrophilic, basic and acidic amino acids.

The conservation of sequence within the tandem repeats of tetra- and octapeptides in Ag319 is reminiscent of the CS-protein of P.falciparum (3), RESA (8) and S-antigen (17) and contrasts with the variable repeats in FIRA (9). The octapeptide repeats Asn-Asn-Asn-Met-Asn-His-Asn-Met exhibit a further interesting feature at the nucleic acid level. The 3 Asn residues flanked by Met in the repeat are with one exception encoded by AAT. However, the Asn residues preceding and following the His within the repeat are more frequently encoded by AAC. Similar silent 3rd basepair changes have been observed in other repetitive P.falciparum antigens.

Antibodies affinity-purified on immobilized lysates of Ag319 reacted with acetone fixed parasites of all stages of the asexual blood cycle. However after glutaraldehyde fixation, free merozoites but not the intracellular stages still reacted strongly with the antibodies. This is an unusual feature among the range of antibody specificities tested to date. It contrasts with antibodies against RESA (8) or Pf155 (24) that react with the surface of ring-infected erythrocytes but not with merozoites after glutaraldehyde fixation. Immunoelectronmicroscopy with anti-ARP antibodies will be necessary for more precise localization of ARP at different stages of the blood cycle.

Another interesting feature is the common reactivity to anti-ARP antibodies shown by sporozoites and blood-stage parasites. Cross-reactive antigens of these stages have not been previously cloned with the exception of the CS protein-related antigen (25,31). Because of the reactivity of anti-ARP antibodies with unfixed sporozoites, it is possible that some cross-reactivity may even exist between ARP and the CSP, particularly because the CSP also has a high Asn content (3,15). However, anti-ARP antibodies and

monoclonal antibodies against the CSP repeat did not compete with each other when binding to sporozoites. Thus anti-ARP antibodies apparently do not react with an epitope encoded by the CSP repeat (data not shown). The strong reaction of anti-ARP antibodies with P.falciparum sporozoites in immunofluorescence therefore provides a means for distinguishing P.falciparum and P.vivax in mosquitoes and may be an improvement on the anti-CSP antibody probes currently in use that rely on conservation of the tetrapeptide repeat sequence (3,15).

Recently we have found that a majority of the 32 antigen-positive clones that react strongly with anti-ARP antibodies in the NF7 array do not hybridise to Ag319 cDNA and vice versa (data not shown). Hence there must be at least two antigenically cross-reactive polypeptide sequences encoded by distinct DNA sequences. This will undoubtedly have contributed to the complexity of immunoblotting patterns and makes it inappropriate at this stage to assign more than a provisional molecular weight to ARP, i.e. to the dominant proteins recognized by anti-ARP antibody. However, these data suggest that there may be a family of asparagine-rich proteins, just as there are a number of histidine-rich proteins (32). The functions of these proteins in Plasmodia remain to be determined.

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