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The expression of *Plasmodium falciparum* bloodstage antigens in *Escherichia coli*

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A library of cDNA clones expressing proteins of the asexual blood stages of a Papua New Guinean isolate of *Plasmodium falciparum* (isolate FCQ27/PNG (FC27)) was constructed in the bacteriophage vector λgt11-Amp3. In an *in situ* colony immunoassay, human serum was used to identify colonies producing natural immunogens. Sera from donors of defined clinical status, or reactive to a defined subset of natural immunogens were used to identify clones of particular interest (for example, clones reacting with convalescent but not with acute serum or clones expressing the isolate specific S-antigen of FC27). Antisera raised by immunizing mice and rabbits with cloned antigens were used to characterize the *P. falciparum* proteins corresponding to the antigen-positive clones. Nucleotide sequence analysis of an antigen found on the surface of cells infected with ring stage parasites revealed an unusual sequence coding for eight, four and three amino acid repeats rich in acidic amino acids. The discussion centres on the use of cloned antigens as tools for the analysis of the host-protective immune response and selection of candidate vaccine molecules.

Monkeys have been immunized successfully against human malaria using parasites from *in vitro* culture (Rieckmann *et al.* 1978; Reese *et al.* 1978). However, the limitations of present culture systems that require human serum and red cells and the requirement for adjuvants make direct application of this approach unattractive for development of a vaccine for human use. Recombinant DNA technology offers a means of producing individual proteins that would be difficult to obtain from parasite preparations in the high yield and purity required for a defined antigen vaccine.

Although immunity to *Plasmodium falciparum* in man is slow to develop and is incomplete, there is evidence from passive transfer experiments (Cohen *et al.* 1961) that antibody, either alone or in combination with other effector mechanisms plays a role in immunity.

In this paper we describe how human serum has been used to identify antigens in a library of cDNA clones expressing proteins of the asexual blood stages of *P. falciparum*. Previously we have characterized sera by function *in vitro*, by the immune status of the donors and by two-dimensional electrophoretic analysis of the *P. falciparum* polypeptides with which they react (Brown *et al.* 1981, 1982, 1983). These defined sera have been screened for reactivity with *P. falciparum* clones and have enabled selection of candidate vaccine molecules. Nucleotide sequence data has revealed the unusual tandem repeat structure of two of these antigens.

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Immunological screening

Initially, a cDNA library was constructed in the plasmid pBR322 using mRNA from asynchronous cultures of the asexual blood stages of a Papua New Guinean isolate of *P. falciparum* FCQ27/PNG (FC27) established in this laboratory. Levels of antigen expression were too low to be detected by colony immunoassay of the pBR322 cDNA library but immunization of mice with lysates of *Escherichia coli* containing recombinant plasmids produced antisera with reactivity against *P. falciparum* antigens, one of which was characterized in detail (Coppel *et al.* 1984*a*). In a second and dramatically more successful approach, cDNA prepared from poly $(A)^+$ RNA of asynchronous erythrocytic stages of FC27 was amplified by cloning in bacteriophage λgt10 and then inserted into the EcoR1 site near the 3′ end of the β-galactosidase gene in bacteriophage λgt11-Amp3. Methods for construction of the cDNA expression library and isolation of clones by antibody screening have been described in detail elsewhere (Kemp *et al.* 1983; Stahl *et al.* 1984; Coppel *et al.* 1983, 1984*a*, *c*; Anders *et al.* 1984). Initially, malaria proteins fused to β-galactosidase were detected in an *in situ* colony immunoassay using affinity-purified human antimalarial antibodies. In later experiments, individual human sera were used to probe the antigen-positive colonies to allow reactivities of different antigens with defined sera to be compared (Stahl *et al.* 1984; Anders *et al.* 1984; Coppel *et al.* 1984*b*).

Seventy-eight colonies that reproducibly expressed malaria antigens were detected in an initial screen of approximately 10000 cDNA clones with human anti-malarial IgG affinity-purified on an immunosorbent prepared using a sonicate of infected red blood cells. Sibling analysis by hybridization of ^{32}P-labelled cDNA inserts from individual clones to the array of 78 colonies allowed the identification of 18 distinct sequences, each represented by between one and 15 clones, accounting for 56 of the colonies. Most of these families of sequences corresponded to distinct antigens and up to 22 further antigens may be represented by the unassigned colonies. Family assignment could be validated in some cases by showing that monospecific antibody to the protein produced by one clone reacted with all sibling clones (Anders *et al.* 1984).

The proteins expressed in antigen-positive clones were examined by one-dimensional gel electrophoresis and immunoblotting. Seventeen out of 78 of the clones produced an abundant fused polypeptide at least 5 kDa larger than β-galactosidase and readily detectable by Coomassie blue staining. At least six more cloned proteins could be detected in immunoblots probed with anti-β-galactosidase or immune human sera (figures 1 and 2). In two thirds of the clones, an abundant fused polypeptide was not detected, but immunization of mice with such clones often produced an antibody to the corresponding protein in *P. falciparum*.

Screening of a second library of cDNA clones with serum having a high titre of antimalarial antibody produced a further 133 antigen-positive colonies. Sibling analysis by hybridization identified some members of families already represented in the first array but many new antigen positive colonies were obtained.

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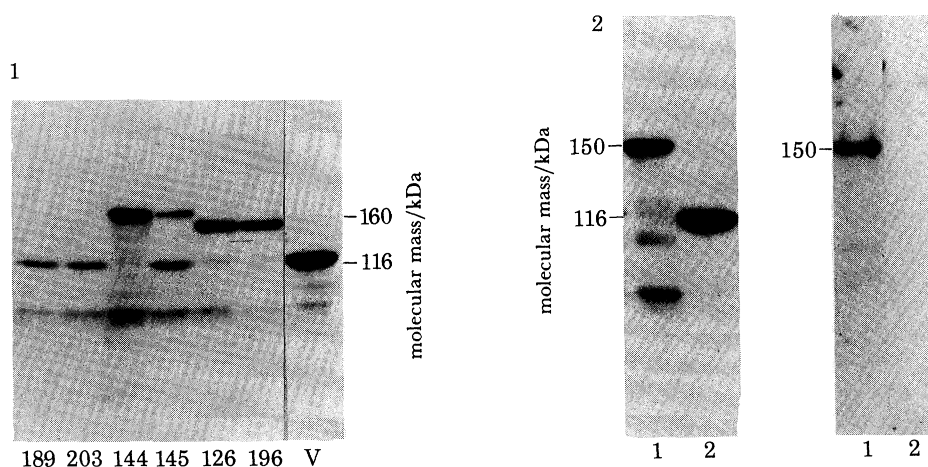

FIGURE 1. Immunoblotting analysis of proteins expressed by antigen-positive clones. Bacterial cells from thermally induced antigen-positive cultures were extracted in SDS electrophoresis sample buffer and fractionated on a 7.5% polyacrylamide gel. Proteins were transferred electrophoretically to nitrocellulose filters and probed with antibody to β-galactosidase. After reaction with ^{125}I-labelled protein A, filters were washed, dried, then exposed to X-ray film. Fused polypeptides of different lengths are visible. Native β-galactosidase (M_r 116 kDa) is present in Track V (vector alone). No fused polypeptide is visible in the first two tracks (clone Ag189 and Ag203).

FIGURE 2. Immunoblot analysis of cloned proteins. Left hand panel: rabbit antiserum with antibody to β-galactosidase has been used to probe transferred proteins from Ag13 (Track 1) or a clone expressing β-galactosidase (track 2). Right hand panel: duplicates of filters shown in the left hand panel have been probed with human serum containing antibodies to *P. falciparum*. Antimalarial antibody in human serum reacts only with the fusion protein.

Differential screening of clones with individual sera

Antibodies in the serum of individuals with acute malaria are by definition inadequate alone, either in quality or quantity, to inhibit parasite growth. Convalescent serum has antibodies boosted by acute infection and may contain 'protective' antibody. Comparison of antigens recognized by these types of sera enables identification of potentially important antigens (Brown *et al.* 1983*a*).

These clinically defined sera were used to probe identical filters containing an array of antigen-positive clones. In three out of seventeen pairs examined, seven clones showed increased reactivity with convalescent serum compared with acute serum (figure 3). Hybridization analysis showed that six of these were members of a family (Ag196) sharing the same sequence. Acute infection also stimulated a boost in antibody to members of other families.

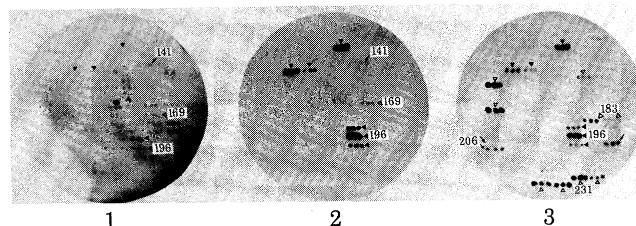

FIGURE 3. Differential screening of cloned antigens using human immune serum. 133 antigen-positive clones were picked into an array on a nitrocellulose sheet and processed for the *in situ* immunoassay. Identical replica filters were probed with serum from an individual at the time of acute infection (panel 1) and with serum from the same individual in the convalescent period (panel 2). Serum from another individual (panel 3) reacted with additional antigen positive clones. Clones designated ▾ are assigned to a single family by cDNA hybridization analysis.

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A complementary approach involves the screening of antigen-positive clones or a whole library of expressing clones with sera having known inhibitory function *in vitro*. Clones preferentially recognized by inhibitory compared with non-inhibitory sera are selected for further study as candidate host-protective antigens (for discussion see Coppel *et al.* 1984*c*).

Characterization of proteins corresponding to cloned antigens

Antisera raised against cloned malaria antigens were used to characterize the corresponding proteins of *P. falciparum* (figures 4–6 and table 1). The variety of distinct antigens (range 75–250 kDa) recognized by these antisera confirms that the clone library contains coding

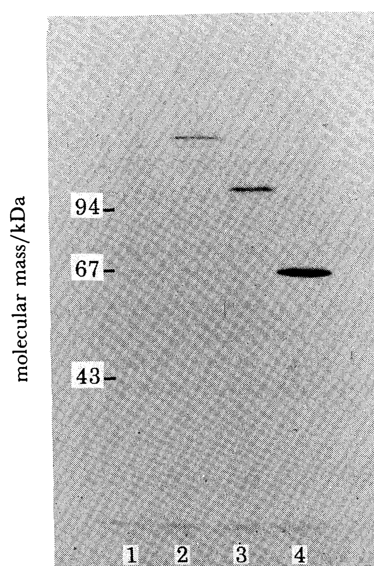

Figure 4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of immunoprecipitates of [^{35}S]methionine labelled parasites (isolate K-1) with antiserum raised by immunizing mice with clones. 1, Control; 2, Ag13; 3, Ag23; 4, Ag63.

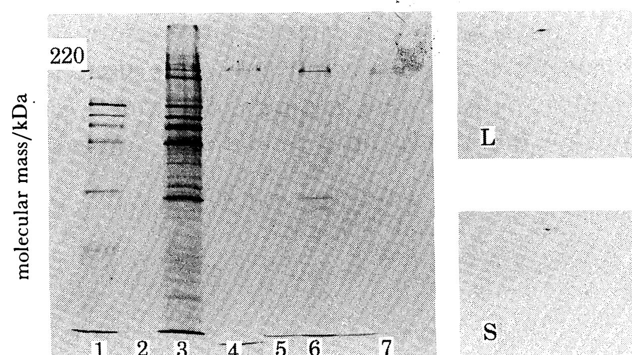

Figure 5. One- and two-dimensional gel electrophoresis of immunoprecipitates of [^{35}S]methionine biosynthetically labelled FC27 cell lysates or culture supernatants with antibodies to Ag16 raised in rabbits or mice. Track 1, supernatant, human anti-*P. falciparum*; track 2, supernatant, mouse anti-Ag16; track 3, cell lysate, human anti-*P. falciparum*; track 4, cell lysate, mouse anti-Ag16; track 5, cell lysate, pre-bleed of rabbit before immunization with purified fusion protein from clone Ag16 (FPAg16); track 6, cell lysate, rabbit anti-FPAg16; track 7, supernatant, rabbit anti-FPAg16. L, high molecular mass acidic region of two-dimensional gel corresponding to track 6. S, high molecular mass acidic region of two-dimensional gel corresponding to track 7.

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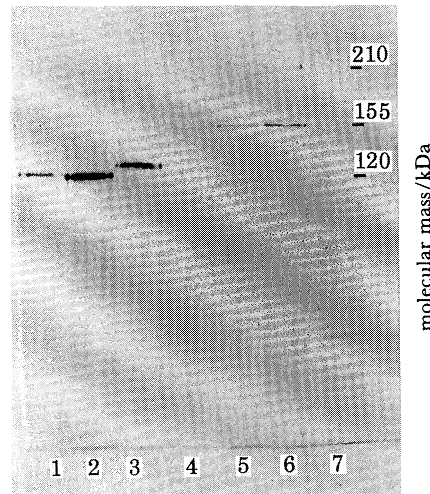

Figure 6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of immunoprecipitates of [^{35}S]methionine labelled parasites from different isolates by serum of mice immunized with Ag23 (tracks 1, 2, 3) or a member of the Ag13 family (tracks 4, 5, 6). Track 1, track 4, isolate FC27; track 2, track 5, isolate NF7; track 3, track 6, isolate K-1. Ag23 has been described in detail elsewhere (Coppel *et al.* 1984*a*) and antiserum to this clone binds to proteins of different molecular mass in different isolates.

Table 1. Characteristics of parasite antigens corresponding to some families of antigen-positive clones

clone family	M_r of parasite antigen	immunofluorescence† ring	troph.	schizont	isolate specificity
Ag: 7	> 250	−	−	+	n.s.
13	210, 155	+	−	+	n.s.
16	200	−	−	+	s.
23	120	−	+	+	n.s.
44	85	−	+	+	.
57	165	.	.	.	.
61	80	.	.	.	.
63	75	+	+	+	n.s.
120	110	.	.	.	.
169	40	.	.	.	
196	110	−	−	+	

s., isolate specific.
n.s., not isolate specific.
† Immunofluorescence assay performed using fixed infected cells.

sequences for many different antigens, some of which have already been described (Coppel *et al.* 1983; Coppel *et al.* 1984*b*, *c*; Anders *et al.* 1984; Stahl *et al.* 1984). In some cases, antisera precipitated more than one polypeptide. For example, different antisera to clones of the Ag13 family (that is, sharing the Ag13 sequence, as defined by hybridization) all precipitated a polypeptide of 155 kDa, and some but not all reacted with an additional polypeptide of 210 kDa. Repeated immunization of mice apparently increased the antibody response to the protein of high molecular mass. This result could be explained by sequence homology or precursor–product relation between the two proteins.

Antiserum to a clone designated Ag16, which encodes part of the S-antigen (Coppel *et al.*

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1983), reacted with two polypeptides of high molecular mass in both the culture supernatant and a detergent extract of infected cells (figure 5). Different proportions of the two molecules were present in the two fractions, with larger amounts of the higher molecular mass protein in the parasitized cells than the supernatant. This observation suggests that a higher molecular mass S-antigen precursor may be processed during or after release.

Indirect immunofluorescence studies (i.f.a.) showed that most of the parasite antigens corresponding to proteins expressed in *E. coli* were expressed predominantly in mature parasites. Antisera to members of the Ag13 family bound to mature parasites but also reacted strongly with the surface of cells containing immature (ring-stage) parasites. The stage-dependence of antigen detection was confirmed for two of the antigens (Ag13 and Ag16) by immunoblotting analyses using antigen preparations from synchronized cultures.

In general, each antiserum precipitated a polypeptide from the three *P. falciparum* isolates examined (FC27, the isolate from which the cDNA was derived, K-1 from Thailand, and NF7 from Ghana). The target antigen corresponding to the clone designated Ag23 varied by 10 kDa among the isolates examined (figure 6). A target antigen corresponding to Ag16 (the clone encoding a portion of the S-antigen of FC27) was not detected in the two heterologous isolates. Such clones will provide valuable markers for typing isolates.

Antigens containing tandem repeats

Antisera to clone Ag16 recognized an isolate-specific antigen (S-antigen) of 200 kDa. These characteristics suggested that Ag16 may correspond to the S-antigen of isolate FC27 (Anders *et al.* 1983). This was confirmed by the reactivity of antisera to clone Ag16 with heat-stable antigens in the culture supernatants of FC27 and E12, a cloned line derived from FC27 having an S antigen of a larger size (Coppel *et al.* 1983). Sequencing of the cDNA insert of Ag16 revealed a most unusual structure. This sequence (which could encode about 15% of the S-antigen polypeptide chain) was composed entirely of 23 tandem repeats of an 11 amino acid sequence (Coppel *et al.* 1983).

The single cDNA insert of clone Ag13 (a ring-stage infected erythrocyte surface antigen (r.e.s.a.)) has also been sequenced (Coppel *et al.* 1984*b*). An initial sequence of 69 nucleotides from the start of the cloned segment is followed by five exact repeats of a 24 base pair (eight codons) sequence, then many repeats of a subset of four of the eight codons, occasionally interspersed by a subset of three of the four codons. As is also seen with the S-antigen, variations occur in the third nucleotide of some codons, but these have no effect on the predicted amino acid sequence.

Tandemly repeated subunits that we have described in the S-antigen and r.e.s.a., together with the observation that the circumsporozoite protein of *P. knowlesi* has repeating units (Godson *et al.* 1983) suggest that these rather unusual proteins are a characteristic of plasmodia. The function of these molecules remains unknown, but the strict conservation of amino acid sequence suggests a selective advantage in retaining that structure.

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Discussion

From these studies we conclude that large numbers of parasite antigens can be expressed in *E. coli* using the bacteriophage vector λgt11-Amp3. Antigen-positive clones with single inserts can be assigned to families by hybridization sibling analysis and antisera to individual members grouped in this way react with the same biosynthetically labelled parasite protein. Failure of detection of a fused polypeptide by immunoblotting of clones does not necessarily imply that antiserum to the antigen cannot be raised in a mouse. In some cases, antisera recognize more than one polypeptide and for Ag13 this is commonly seen after repeated boosting with the cloned antigen.

Polyspecific antisera to monoclonal antigens provide a ready means of identifying the corresponding antigen of the parasite (immunofluorescence, immunoelectronmicroscopy and immunoprecipitation) and also provide reagents for *in vitro* assessment of candidate vaccine molecules. For functional tests *in vitro*, antisera raised by immunizing rabbits with purified fused polypeptides provide the most suitable reagents. Where antibodies to *E. coli* do not present a problem (for example, for i.f.a.), antisera are raised with convenience by immunizing mice with crude lysates of bacteria that express the fused protein.

Immune human sera that have been defined, by function *in vitro* or by clinical status of the donors, provide powerful reagents for direct screening of cDNA clone libraries. By using this approach we have screened large numbers of clones to find those expressing a portion of the S-antigen and an antigen that is dramatically more reactive with convalescent sera than with sera taken at the time of acute infection (Stahl *et al.* 1984).

Selection of *P. falciparum* antigens for immunization experiments

Cloned antigens produced by recombinant DNA (some of which are shown in table 1) may be used in various ways for the assessment of the human immune response to malaria and the selection of candidate vaccine molecules.

(i) Each fused polypeptide may be used as the substrate in an immunoassay to search for antibody specificities correlating with protection against clinical disease: for example, antibody specificities detected in sera from individuals studied longitudinally as they develop immunity as a result of repeated infection.

(ii) Each fused polypeptide may be used for isolation from human immune serum of polyclonal but monospecific antibodies to individual antigens. 'Natural' antibodies to the cloned antigens could be assessed *in vitro* or by passive transfer experiments.

(iii) Each cloned antigen may be tested for its ability to provoke a response in lymphocytes from donors of different immune status. Antigens that stimulate only the lymphocytes of immune individuals are candidates for the induction of a cell-mediated protective immune response.

(iv) Polyspecific antisera to cloned polypeptides can be used to localize antigens. Surface antigens of the merozoite or the infected red cell are most likely to be important in host protection.

(v) Antisera may be used to screen for variation among isolates as a non-variant molecule or the constant portion of a variant molecule is to be given highest priority.

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(vi) Correlation of immune response to a cloned antigen with anti-parasite function *in vitro* may aid selection.

(vii) Homology of a cloned antigen with a known 'host-protective' molecule in model systems would favour its inclusion.

Immunodominant variant antigens may be important for homologous protection. Indeed, the selective pressure of the host immune response is the most likely reason for the wide variation of an antigen. We have previously shown that unlysed mature parasites express a high molecular mass basic antigen (Brown *et al.* 1983*c*) and that this antigen (Pf220) varies among isolates of *P. falciparum* (Brown *et al.* 1983*b*). If this family of molecules has an invariant region accessible to the immune system, a clone corresponding to that region would encode a candidate vaccine molecule.

Immunization experiments with S-antigens will be necessary to determine their capacity to induce isolate-specific protective immune responses. However, because of the remarkable diversity of this antigen system (presumably reflecting different repeating units of amino acids) these proteins are unlikely to provide heterologous protection and would therefore be of little use as vaccine molecules unless an invariant immunogenic portion of the molecule can be identified.

Ag13 (r.e.s.a.), the other antigen in which we have identified extensive repeat sequences, may be a more promising candidate for inclusion in a vaccine. It is common to the three isolates of *P. falciparum* so far examined (NF7 from West Africa, FC27 from Papua New Guinea and K1 from Thailand), and because of its location on the surface of the infected cell is accessible to immune attack. The function of the molecule on the surface of the red cell is not known but it is apparently transferred to the red cell surface at the time of merozoite invasion. Antibodies to r.e.s.a. are commonly found in the serum of individuals living in endemic areas and it is possible that the removal of erythrocytes infected with ring-stage parasites (for example, by opsonization or antibody-dependent cytotoxicity) may be a previously unsuspected component of the protective response to malaria infection.

The availability of these cDNA clones should facilitate the construction of vaccinia recombinants that express the native polypeptide and allow vaccine testing that does not require Freund's adjuvant. Availability of large amounts of individual antigens is a major step forward for the study at a molecular level of the mechanisms such as immunosuppression, polyclonal B cell activation and massive lymphokine release that are known to occur during malaria infection.

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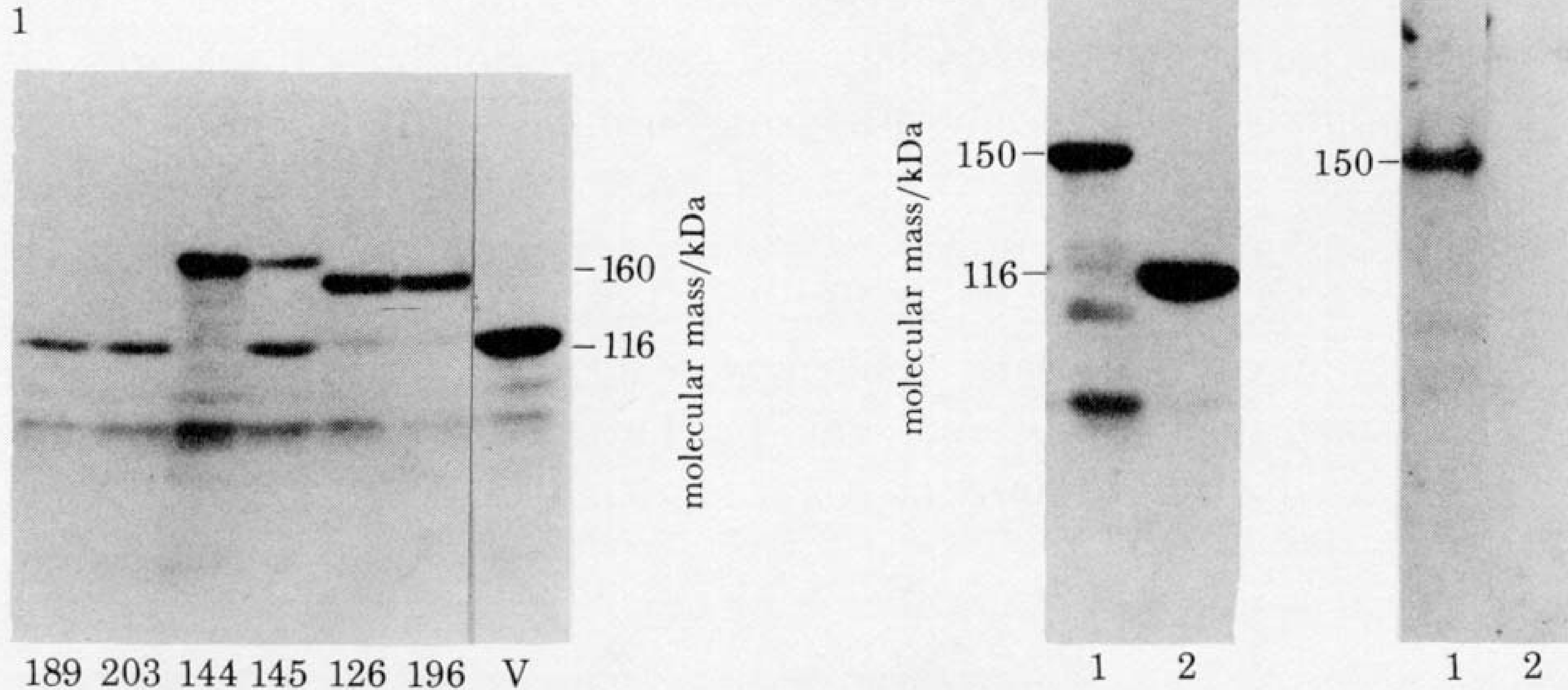

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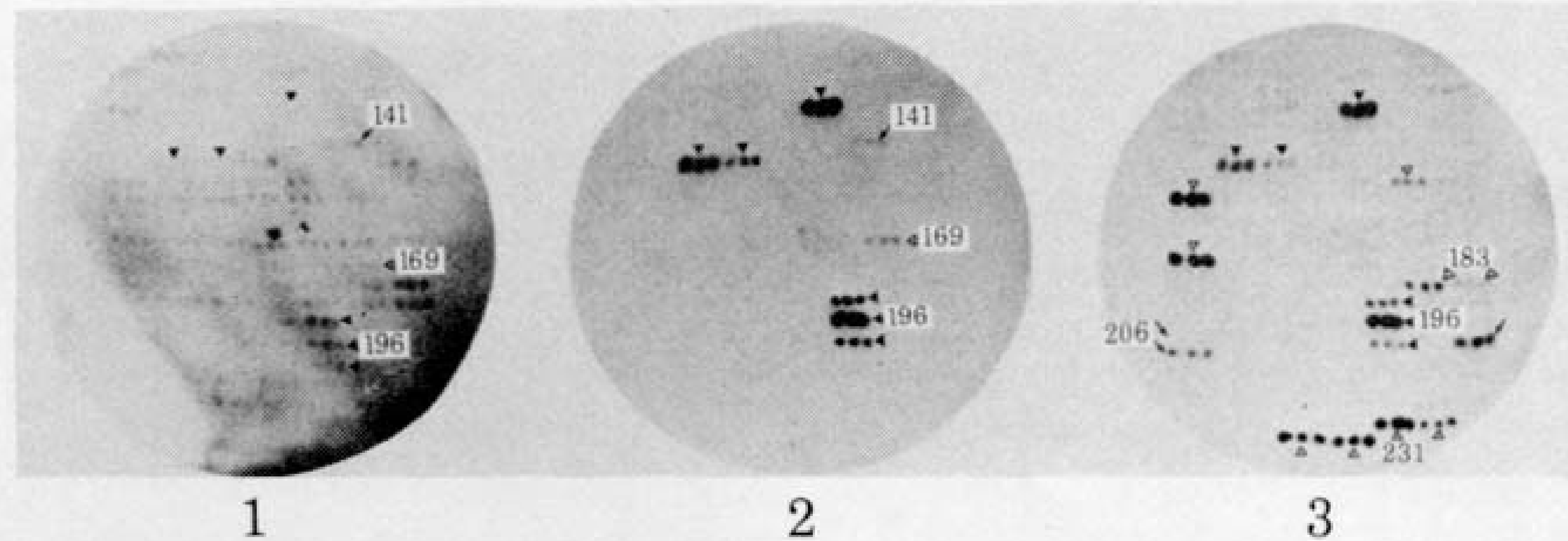

GURE 3. Differential screening of cloned antigens using human immune serum. 133 antigen-positive clones were picked into an array on a nitrocellulose sheet and processed for the *in situ* immunoassay. Identical replica filters were probed with serum from an individual at the time of acute infection (panel 1) and with serum from the same individual in the convalescent period (panel 2). Serum from another individual (panel 3) reacted with additional antigen positive clones. Clones designated ▾ are assigned to a single family by cDNA hybridization analysis.

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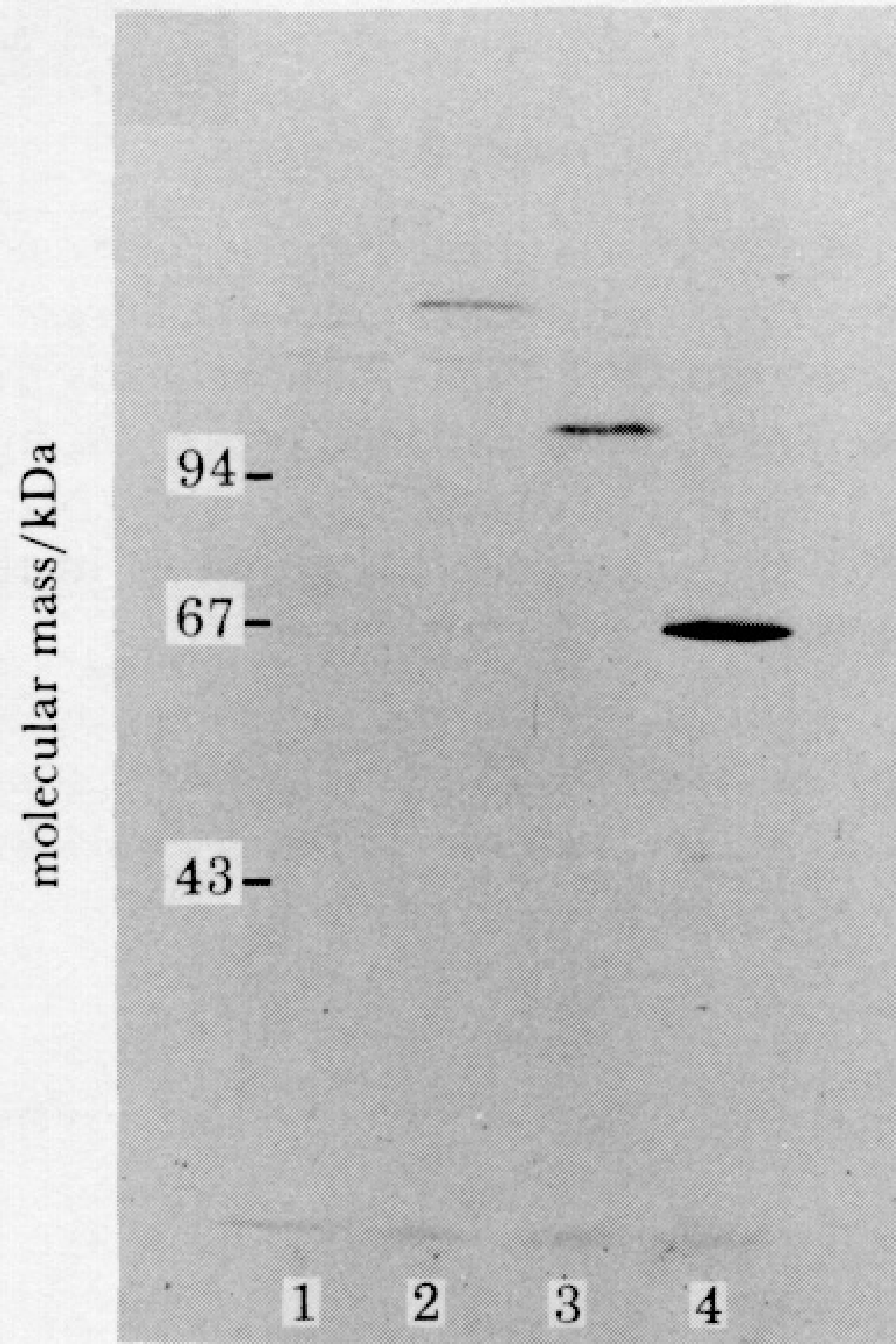

GURE 4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of immunoprecipitates of [^{35}S]methionine labelled parasites (isolate K-1) with antiserum raised by immunizing mice with clones. 1, Control; 2, Ag13; 3, Ag23; 4, Ag63.

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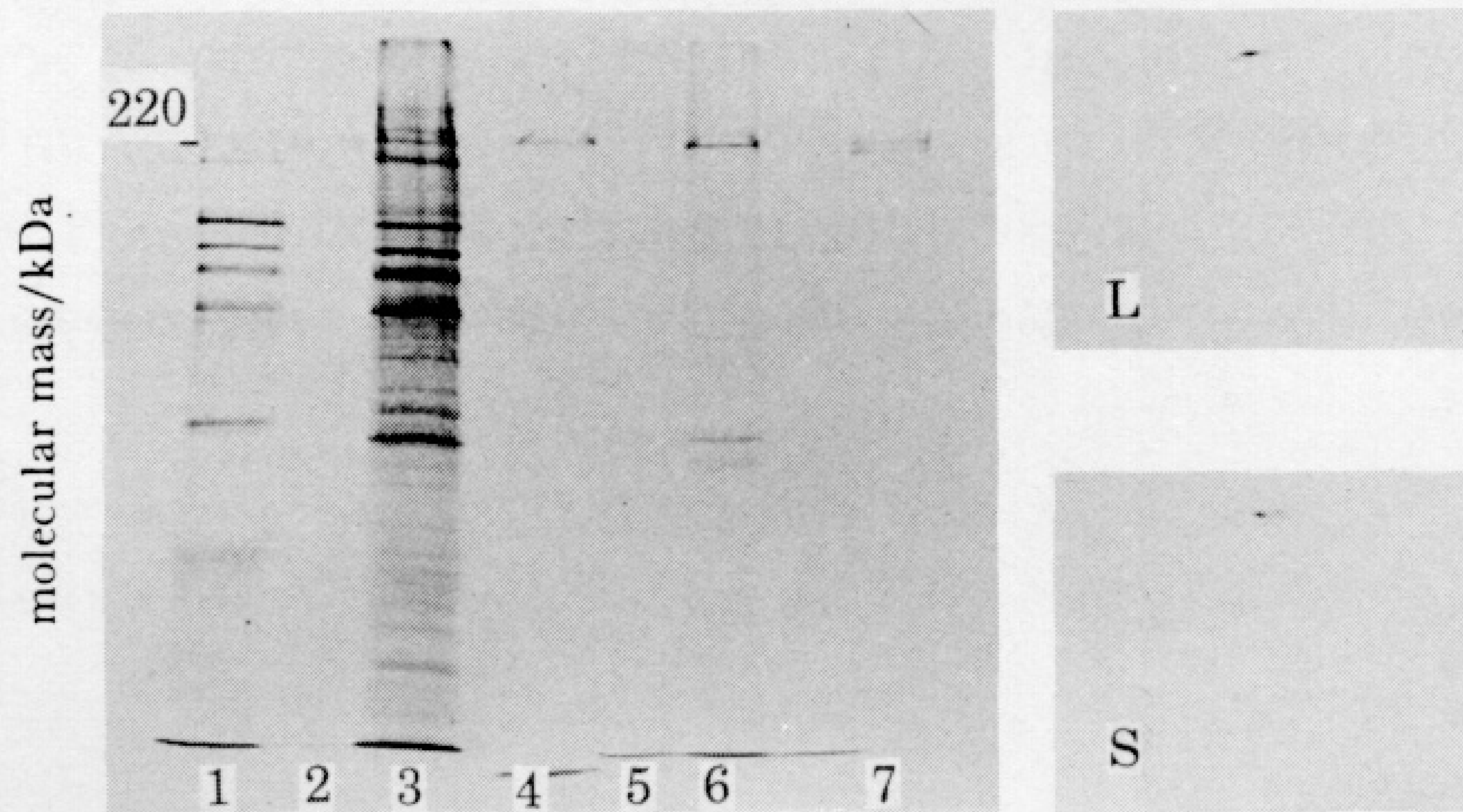

GURE 5. One- and two-dimensional gel electrophoresis of immunoprecipitates of [^{35}S]methionine biosynthetically labelled FC27 cell lysates or culture supernatants with antibodies to Ag16 raised in rabbits or mice. Track 1, supernatant, human anti-*P. falciparum*; track 2, supernatant, mouse anti-Ag16; track 3, cell lysate, human anti-*P. falciparum*; track 4, cell lysate, mouse anti-Ag16; track 5, cell lysate, pre-bleed of rabbit before immunization with purified fusion protein from clone Ag16 (FPAg16); track 6, cell lysate, rabbit anti-FPAg16; track 7, supernatant, rabbit anti-FPAg16. L, high molecular mass acidic region of two-dimensional gel corresponding to track 6. S, high molecular mass acidic region of two-dimensional gel corresponding to track 7.

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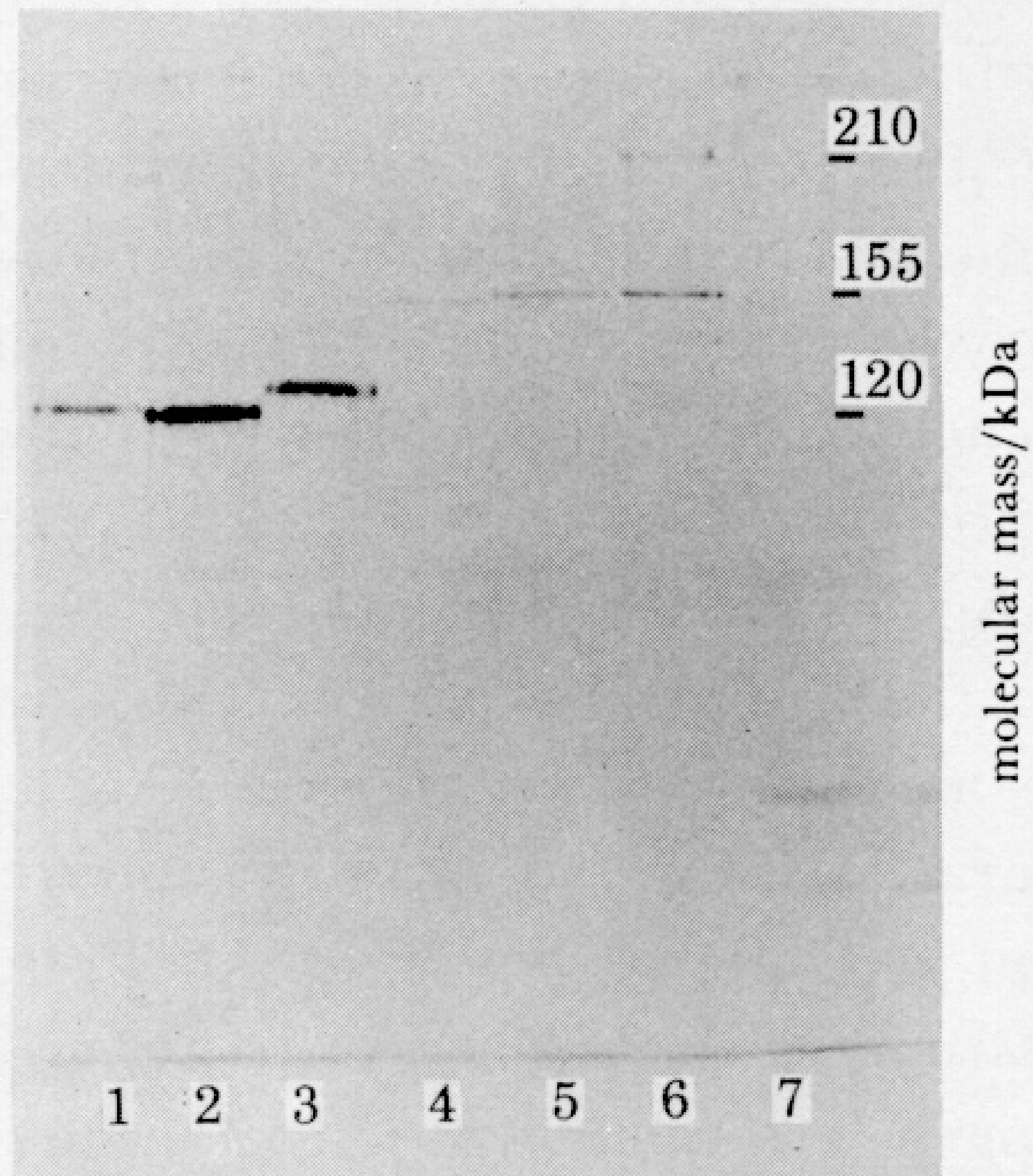

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FIGURE 6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of immunoprecipitates of [^{35}S]methionine labelled parasites from different isolates by serum of mice immunized with Ag23 (tracks 1, 2, 3) or a member of the Ag13 family (tracks 4, 5, 6). Track 1, track 4, isolate FC27; track 2, track 5, isolate NF7; track 3, track 6, isolate K-1. Ag23 has been described in detail elsewhere (Coppel *et al.* 1984*a*) and antiserum to this clone binds to proteins of different molecular mass in different isolates.