frequency in almost all populations studied. PLGN*4 [7] however, has only been seen in Asian populations [5, 7, 8] but was originally identified under the names $PLG^2$ [8] and $PLG^2$ [5]. Although PLGN*4 was not seen in our Indian, Eskimo and Mexican-American populations their gene frequencies for PLG*1 are consistent with Mongoloid ancestry. The variant PLG*D was observed at polymorphic levels in the Black populations of our study and the very low frequency seen in our local White population probably represent Black admixture. As the techniques reported in the literature become more comparable and a uniform nomenclature is established, the PLG marker system will become a valuable tool for genetic analysis and population studies.

Received June 6, 1983

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# High resolution comparison of *Plasmodium knowlesi* clones of different variant antigen phenotypes by two-dimensional gel electrophoresis and computer analysis

Antigenic variation of the schizont-infected cell agglutination-antigen on erythrocytes infected by *Plasmodium knowlesi* is due to the expression of different malarial proteins. Here we examined whether antigenic variation is accompanied by multiple phenotypic changes in the proteins synthesized by *P. knowlesi*. The malarial proteins of two clones, one of which was produced by antigenic variation from the other clone *in vivo*, were labeled by [$^3$H]isoleucine uptake during *in vitro* parasite growth. The proteins were separated by two-dimensional gel electrophoresis, detected by fluorography and compared using the GELLAB computer system. In the molecular weight range 35 000 to 230 000 and pH range 4.55 to 6.10, we found only four qualitative differences and two robust quantitative differences among approximately 500 proteins characterizing the protein phenotype of these clones. One clone exhibited four minor protein spots absent from the other. We conclude that antigenic variation in *P. knowlesi* represents the differential expression of a very small number of malarial genes.

## 1 Introduction

Chronic infection of rhesus monkeys with asexual blood stages of the malaria parasite *Plasmodium knowlesi* is characterized by successive populations of parasites which express different forms of a variant antigen on the surface of infected erythrocytes [1]. The variant antigen is identified by specific antibody-mediated agglutionation of erythrocytes infected by mature forms (schizonts) of the intracellular parasite, hence the acronym SICA (schizont-infected cell agglutination) to describe this antigen [1, 2]. The capacity of *P. knowlesi* to express different SICA phenotypes on infected cells is thought to enable this parasite to evade variant-specific parasiticidal immune respones [3, 4]. Using cloned *P. knowlesi* parasites of different SICA phenotypes and variant-specific agglutinationg sera, we identified the SICA antigens as proteins synthesized by the malaria parasite of apparent molecular weight (Mr) 190 000–225 000, depending on the particular clone [5].

One of the *P. knowlesi* clones (Pk1(B+)1+) was cloned from a population of parasites (Pk1(B+)) derived by antigenic variation *in vivo* of another clone (Pk1(A+)) [6]. The SICA antigen phenotypes of Pk1(A+) and Pk1(B+)1+ can be distinguished by two assays for variant-specific antibody bound to the surface of infected cells (agglutination and indirect immunofluorescence using fluorescein-conjugated anti-rhesus monkey immunoglobulin) [6] as well as by biochemical identification of different variant antigens on immunoprecipitation analyses

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**Abbreviations:** **SICA:** Schizont-infected cell agglutination; $M_r$: Molecular weight; **2-D:** Two-dimensional; **SDS:** Sodium dodecyl sulfate; **PBS:** Phosphate buffered saline; **DTT:** Dithiothreitol; p*I*: Isoelectric point



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[5]. However, we do not know whether expression of different variant antigens constitutes the only difference in phenotype of these related clones. Evidence is accumulating for considerable phenotypic lability of malaria parasites both *in vivo* [6] and when cultured *in vitro* [7], raising the possibility that the obligatory step of expanding cloned parasites *in vivo* prior to experimentation introduces multiple phenotypic alterations. In this report we compare the phenotypes of Pk1(A+) and Pk1(B+)1+ by comparing the proteins that they synthesize during *in vitro* maturation from immature (ring-stage) to mature (schizont-stage) parasites.

A detailed comparison of the proteins synthesized by two different cells is currently best achieved by two-dimensional (2-D) gel electrophoresis [8], whereby several hundred individual protein spots can be resolved on a single gel. Although a qualitative comparison can be performed visually, computer technologies have enormously enhanced the qualitative and quantitative analysis of every spot on different gels, especially when multiple gels are run for each sample to provide statistical veracity. Accordingly, we have used the GELLAB computer analysis system [9–17] to analyze Pk1(A+) and Pk1(B+)1+ proteins labeled by [$^3$H]isoleucine uptake and separated on multiple 2-D gels. We report that these clones of different SICA phenotype have very few other differences in protein phenotype (4 qualitative differences of a spot missing from one clone were detected as well as 2 robust quantitative differences). A modification to part of the GELLAB system is also presented which allows a more efficient computer search for missing spots in pairwise comparisons of different spots.

## 2 Materials and methods

### 2.1 Cell culture and biosynthetic radiolabeling

Rhesus monkey erythrocytes infected by ring-stage parasites of *P. knowlesi* clones Pk1(A+) and Pk1(B+)1+ were obtained by thawing cryopreserved stocks [18]. The cells were resuspended in RPMI 1640 containing 10 % of the normal level of isoleucine (made using the Select-Amine Kit, Grand Island Biological Co., Grand Island, NY 14072) and the following additional constituents: 2 g/l D(–) glucose, 20 mM 4-(2-hydroxyethyl)-1-piperazine-N′-2-ethanesulfonate (HEPES), 0.25 % w/v sodium bicarbonate, 15 % v/v normal rhesus monkey serum, 25 μg/ml gentamycin, 10 μg/ml hypoxanthine, L-[4,5-$^3$H]isoleucine (200 μCi/ml, 106 Ci/mM, Amersham Corp., Arlington Heights, IL). The concentration of parasitized cells was $2 \times 10^7$/ml and final hematocrit 2–5 %. The erythrocyte suspensions (5 ml) were incubated 20–22 h at 37 °C in sealed 25 cm$^2$-tissue culture flasks (Corning Glass, Corning, NY 14830) which were initially gassed with 3 % $O_2/N_2$, 6 % $CO_2$ and 91 % $N_2$. Radiolabeled schizont-infected erythrocytes were harvested by centrifugation of the cultures (550 *g* × 10 min), resuspension in phosphate-buffered saline (PBS: 150 mM NaCl, 20 mM sodium phosphate, pH 7.2) to 20 % hematocrit and loading onto step gradients of 56 % and 40 % Percoll/PBS (Pharmacia, Uppsala, Sweden). Isotonic Percoll stocks of 56 % and 40 % Percoll were made by adding 0.1 vol. of 10× concentrated PBS to Percoll (*i. e.*, 90 % Percoll) and then diluting this solution appropriately with PBS. The step gradients consisted of 5 ml of 56 % Percoll under 3 ml of 40 % Percoll in 15 ml centrifuge tubes. Two milliliter volumes of erythrocyte suspension were loaded per tube. After centrifugation (800 *g* × 20 min) at 23 °C, a dark brown band consisting of >95 % intact schizont-infected erythrocytes was collected from the 40–50 % Percoll interface. The cells were washed three times in PBS and resuspended to $1 \times 10^8$/ml. Two aliquots of suspension (10 μl) were taken for analysis of trichloracetic acid insoluble radioactivity. Another aliquot was taken for analysis by one-dimensional sodium dodecyl sulfate (SDS-polyacrylamide gel electrophoresis (<20 μl) and the remainder pelleted. The cell pellet was resuspended with vortexing in 1 % w/v SDS, 100 mM dithiothreitol (DTT) containing 2 mM phenylmethylsulfonyl fluoride (1 volume) at approximately $1 \times 10^9$cell/ml. Solid urea was added to 9 M final concentration followed by 1 volume of 9 M urea, 100 mM DTT, 4 % w/v Triton X-100 and 4 % Ampholines (pH 3.5–10, LKB Produkter, Sweden). The samples were vortexed intermittently during 10 min incubation on ice and solubilized material recovered by centrifugation (Beckman Airfuge, Fullerton, CA 10 000 *g* × 15 min). The cell extract was stored for up to 3 days at minus 70 °C before isoelectric focusing.

### 2.2 2-D electrophoresis

10–20 μl samples of solubilized schizont-infected erythrocytes containing $1–4 \times 10^6$ trichloracetic acid precipitable counts/min were analyzed by 2-D electrophoresis according to the method of O'Farrell [8]. Replicate analyses were performed for each sample. The Ampholines were from LKB Produkter, Sweden, in a volume ratio of pH ranges 5–8 and 3.5–10 of 2.0/0.5. The pH gradient on isoelectric focusing was linear from pH 4.5 to 6.8. The second-dimension SDS-polyacrylamide gel electrophoresis was performed on 7.5 % acrylamide gels using the discontinuous buffer system of Laemmli and Favre [19]. The gels were fixed and stained with Coomassie Brilliant Blue R-250 [20] to identify the molecular weight standards and then processed using En$^3$Hance (New England Nuclear, Boston, MA 02118) and dried for fluorography [21].

### 2.3 The GELLAB system for semiautomatic 2-D gel analysis

GELLAB is a 2-D polyacrylamide gel electrophoresis image analysis system implemented as a set of programs running on DEC system-10 or -20 computers. The 2-D gels, visualized as autoradiographs, are assigned sequential accession numbers in the system and then converted to digital images by a TV camera connected to the computer. These gel images are then analyzed by the spot extraction program called SG2DRV [11, 15]. The spots extracted from each gel are given arbitrary sequential numbers and are characterized by a set of features: *x* (isoelectric point) and *y* (molecular weight) location, area, integrated density, *etc.* Between 12 and 15 landmark spots, being well-defined and morphologically distinctive spots on all gels, were identified on each gel using interactive graphics computer techniques similar to the FLICKER program [17]. Using the gel-segmented spot lists and landmarks, all possible spots are paired using the CMPGEL programm [12] comparing each gel and a representative gel or Rgel [13–14]. For the results reported here we examined four gels, two for each *P. knowlesi* clone. The composite spots of such paired spot sets (each set being the corresponding spot across all gels in the

data base) are called representative spot or Rspot sets. A composite gel or CGL data base was then constructed using the CGELP program [14]. As each Rspot set was formed it was assigned a new arbitrary sequential numeric name. The CGELP interactive program then permitted partitioning the data base by spot features or gel classes. CGELP also provides extensive statistical, numerical and display tools for data base analysis. For purposes of analysis, Rspot sets were required to have a mean area of at least 25 pixels, and member spots were required to be consistenly present or absent in all gels of a given class. Only spots with pairing labels of 'sure pair', 'possible pair', or 'unresolved spot', including the Rspot data base, were considered.

#### 2.3.1 Quantitative spot search in GELLAB

There are various qualitative statistical tests both parametric (t-Test and f-test) and nonparametric (Rank sum tests) that can be used in GELLAB. For each particular problem they can be applied at the desired level of statistical confidence. In this study we used the t-Test to determine whether the mean densities of spots were significantly different between the two parasite phenotypes. For each spot in each gel class the mean and standard deviation of normalized spot density was computed. These values were then used with the t-Test table to determine whether differences in mean density were statistically significant at 99 % or 95 % confidence level.

#### 2.3.2 Missing spot search in GELLAB

In addition to the standard qualitative spot difference seaches found in the CGELP, a new missing spot search was developed to facilitate the work described here. A spot was said to be missing if it was present in one class of gel and totally absent from another. This constraint was made even more robust by requiring that, in the class of gels in which the spot appears, it meet particular Rspot set feature criteria in order to even be considered (for example, the subset of Rspot gels in the same class in which the spot had to be present could be at 80 % of the possible number of gels for that class; the CV of spot area should be less than some small value; *etc.* [14–16]).

Care must always be taken in verifying missing spots found with such a search because what is termed a qualitative change (*i. e.*, missing spot) could in reality be a quantitative change which is below the detection level of the combined autoradiograph-film scanner-spot segmenter process.

#### 2.3.3 Rmap and mosaic-derived images

Having found a set of potentially interesting spots using GELLAB, it is useful to visualize them both globally (*i. e.*, in a total "picture" within a single gel by labeling the interesting spots) and locally (*i. e.*, in a display of the same subregion surrounding one particular interesting spot from multiple gels, the subregions being placed in adjacent panels). Derived images which implement these concepts are called Rmaps and mosaics, respectively, and are illustrated in Fig. 2 and 3, respectively.

The Rmap shows the spots of interest in context of the entire isoelectric point(p*I*)-$M_r$ space. The mosaic facilitates manual verification of true or false positive events which were detected by a GELLAB search. By comparing 2 Rmaps of spots in a search for differences representative of the two classes being compared, false positive differences can be quickly eliminated manually, thus allowing analysis of mosaic images of only those spots which appear to be real changes in the 2 Rmaps.

## 3 Results

### 3.1 Optimization

After 20–24 h in culture, approximately 60 % of the parasites had matured from ring-stages to multinuclear schizont-infected cells containing 2–8 nascent parasites. By density gradient centrifugation we recovered only multinucleated-infected erythrocytes which progressively decrease in density as the parasite multiplies. This step removed from the analysis any viable less mature parasites and nonviable or unhealthy parasites that failed to recover from cryopreservation and thawing and which might synthesize abnormal proteins. The purified schizont-infected cells of Pk1(A+) and Pk1(B+)1+ were indistinguishable in parasite morphology or maturity at the time of solubilization for isoelectric focusing. These clones incorporated approximately equal trichloracetic acid insoluble radioactivity from [$^3$H] isoleucine when normalized for equal numbers of schizont-infected erythrocytes. After cryopreservation, thawing and *in vitro* culture, both clones still expressed the same SICA phenotype when tested with variant-specific agglutinating sera [6]. We elected to solubilize these cells directly in 1 % SDS, 200 mM DTT, rather than attempt to fractionate them into membrane and cytoplasmic components with the attendant risks of proteolysis and loss of material. By addition of solid urea and excess Triton X-100 we could provide an 8-fold ratio by weight of Triton X-100 to SDS as recommended for preventing any effects of SDS during isoelectric focusing [22]. Since the predominant protein in extracts of parasitized erythrocytes prepared in this manner is globin, the Coomassie Blue staining patterns of these 2-D gels visualized only a small proportion of the proteins actually present. It was evident from these staining patterns that none of the proteins present in these samples other than globin were in excessive amount so as to cause spot distortion [8]. Fortunately, globin migrates to a region at the bottom of the gel away from the majority of other proteins so that even localized distortion near globin was tolerable.

### 3.2 Visual analysis of 2-D gel patterns of labeled proteins

The [$^3$H]isoleucine-labeled proteins of schizont-infected erythrocytes from Pk1(A+) and Pk1(B+)1+ were compared in 3 biosynthetic radiolabeling experiments, each involving multiple gel runs of each sample and a range of flurographic exposure times. No differences in the positions of major proteins or in their composition could be detected by visual comparisons. It was clear that the majority of malarial proteins synthesized by these clones were identical on 2-D electrophoresis. Examples of the gel patterns for these clones are shown in Fig. 1.

Visual assessment of the 2-D gel patterns indicated that te reproducibility of multiple gel runs in parallel of a particular

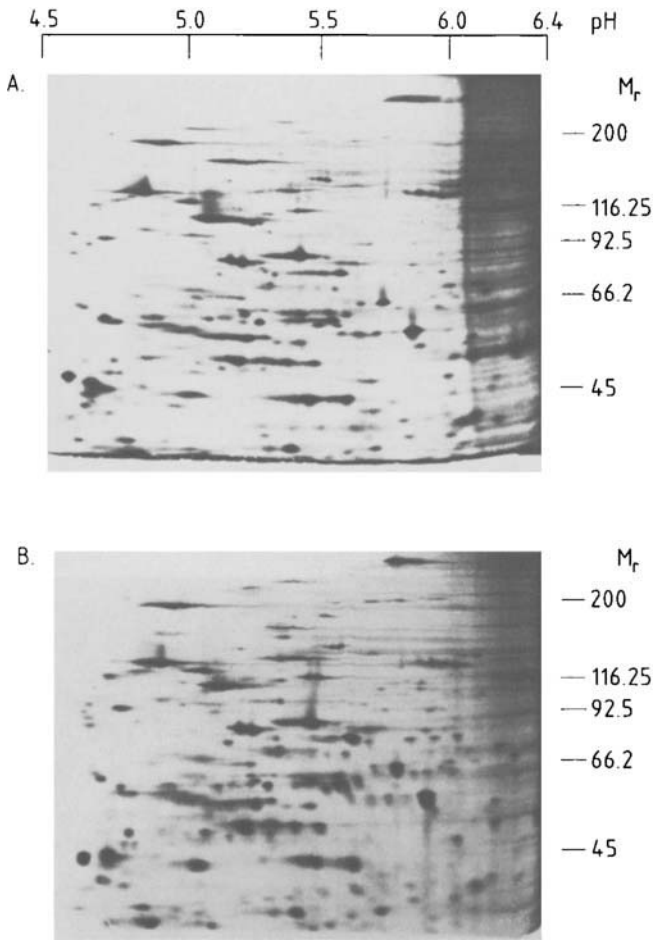


*Figure 1.* Fluorographs of malarial proteins labeled by [$^3$H]isoleucine uptake during parasite maturation from ring to schizont stage. Comparison of clone Pk1(A+) (Top panel: A) and clone Pk1(B+)1+ (Bottom panel: B) which was derived from Pk1(A+) by antigenic variation *in vivo*. Relative molecular masses ($M_r$) were determined by co-electrophoreses of a mixture of proteins of known $M_r$ (Biorad Lab., Richmond, CA) and are indicated on the right of both panels in kilodalton. A replicate isoelectric focusing gel was divided into 2 mm sections, each of which was eluted with 1 ml of degassed distilled water for determination of the pH scale shown across the top panel.

sample was excellent. Gel runs of samples focused on isoelectric focusing gels run at different times were more difficult to compare visually, there being localized expansion and/or contraction of different zones of the 2-D gels. Visual assessment also indicated deficiencies in the 2-D gel system. There was excessive streaking, high background radioactivity, and poor spot resolution at the extreme cathodal side of the gel (Fig. 1). A reliable computer analysis of this region is precluded since the computer program segments streak into multiple spots and thereby generate false complexity [16]. Secondly, proteins of $M_r > 150\,000$ tended to run as streaks rather than as discrete spots. These were not excluded from analysis since their morphology and occurrence were highly reproducible characteristics (Fig. 1). Thirdly, at the anodal edge of the 2-D gel and bottom edge adjacent to the bromophenol blue dye-marker, considerable variation was seen even with duplicate runs of the same sample. This resulted from differences in pH gradient at the anodal extremity, or differences in size-fractionation close to the dye marker. The majority of spots in these edge zones were found to be different in computer-comparison of replicate gels of Pk1(A+) and Pk1(B+)1+ and could be rejected visually as false positives. The effective $M_r$ range for comparison of proteins of the two clones was approximately 230 000 to 35 000 and effective p*I* range 4.55 to 6.10.

### 3.3 Computer analysis of missing spots

The total numer of spots from the segmented gel image was 600 to 800, depending on the gel and fluorographic exposure time. The total number of spots detected visually was approximately 300. After excluding the cathodal extremity of the gel where streaking was most marked, the number of segmented spots was approximately 400–500. With the missing spot search designed to identify spots present in all gels of one clone and missing from all gels of the other clone, only four spots were identified that exhibited a marked difference in radioisotope incorporation between Pk1(A+) and Pk1(B+)1+ (Fig. 2a–b). The Rspot numbers of these spots were 138, 157, 317 and 700. Each spot was present in Pk1(A+) but absent from Pk1(B+)1+. There were no spots present in Pk1(B+)1+ that were not also present in Pk1(A+). The apparent Mr's and approximate p*I* of these spots were as follows [Rspt[≠] ($M_r$, p*I* in parenthesis): Rspot[138] (51 000,6.05); Rspot[157] (41 000,4.95); Rspot[317] (45 000,4.50) and Rspot[700] (66 500,5.35). Two of these spots (317 and 138) focused at the extremes of the pH gradient in regions where variability in the pH gradient between individual gels is most apparent. They have been included in this analysis since they were seen in all Pk1(A+) gels but no Pk1(B+)1+ gels, and the entire set of gels appeared otherwise identical in these particular regions judging from the spacing of adjacent spots that did not vary (Fig. 2a–b).

Two additional spots represented robust quantitative changes found using a t-Test at .99 (Rspt[686]: $M_r$ 95 000, p*I* 5.3) and .95 (Rspot[186]: $M_r$ 65 000, p*I* 5.32) significance and are shown in Fig. 2d. Fig. 2c shows the set of spots used to normalize the measured density of each gel (the sum of measured density of these spots for each gel is used to divide any given spot measurement for that gel).

The authenticity of these spots as real differences in the 2-D gel pattern was confirmed by visual examination of mosaic images of just the regions around each spot. In this way all gels of each class (Pk1(A+) versus Pk1(B+)1+) could be compared in the same image. Fig. 3 illustrates four such mosaic images for the four missing spots discussed above. Fig. 4 illustrates mosaic images of the two robust t-Test changes. Mosaic analysis enabled us to reject several other spots projected to be different on the basis of the computer search, since these differences obviously resulted from computer mismatching of smeared or streaked spots. All other spots shown in Figs. 1 and 2 fell into one of three classes: they were indistinguishable in p*I*/$M_r$ with each gel of the two clones; they exhibited an apparent alteration in p*I* and/or $M_r$ on different gels of one clone, but one of these p*I*/$M_r$ values shared coordinates with all gels of the other clone; they exhibited alterations in p*I*/$M_r$ on gels from both clones. According to the stringent criteria we established for defining different proteins of Pk1(A+) and Pk1(B+)1+ (see section 2), all these other spots could be listed as shared spots, or as spots which display heterogeneity independent of the parasite phenotye, *i. e.* within a particular sample. We do not have an explanation for such heterogeneity aside from suggesting that minute

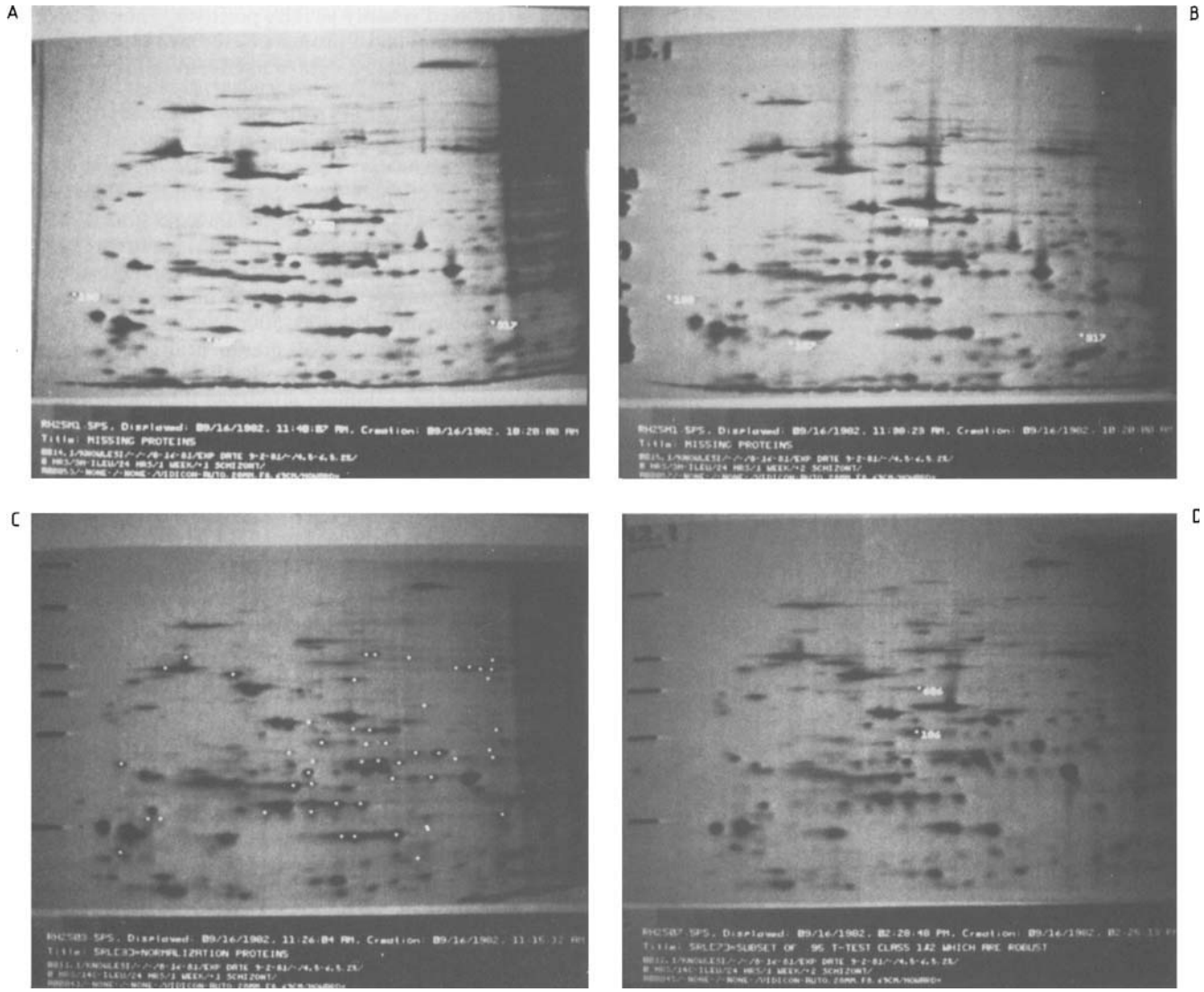


*Figure 2.* R maps of missing spots (qualitative changes) and quantitative changes found by GELLAB analysis of clones Pk1(A+) and Pk1(B+)1+. The missing spots are shown on a gel of Pk1(A+) proteins (a) and on a gel of Pk1(B+)1+ proteins (b). The t-test was used to find quantitative changes with a data base normalized by the set of spots shown on a Pk1(A+) gel in (c). These spots were present in all gels, being non-saturating and stable. R spots found using the t-test are shown on a Pk1(B+)1+ gel in (d). These four gels constituted the data base for GELLAB analysis.

differences and imperfections in the geometry of the isoelectric focusing gel and relationship of this gel to the second-dimension gel might be responsible.

It should be noted however, that none of the quantitatively more important proteins easily identified by visual inspection of the gel in the region $M_r$ 230 000–35 000 and p*I* 4.55–6.10 exhibited significant differences between replicate gels of either clone, or between gels of different clones. This result was also obtained in two experiments comparing [$^{35}$S]methionine-labeled proteins of these clones by 2-D electrophoresis and visual analysis.

## 4 Discussion

### 4.1 Comparison of parasite phenotype using 2-D gels

The two clones of *P. knowlesi* examined are phenotypically quite distinct on the basis of variant-specific antibody-mediated agglutination of schizont-infected erythrocytes [5, 6], on indirect immunofluorescence assay for variant antigen expression on the surface of infected cells [6], and also on biochemical analysis of variant-antigen phenotype [5]. However, we have shown that the protein phenotypes of these clones, as defined by 2-D gel analysis of malarial proteins labeled by [$^3$H]isoleucine incorporation, are very similar. Of the approximately 300 individual proteins of these parasites which have $M_r$ in the range 35 000–230 000 and p*I* in the range 4.55–6.10, only four were determined to be qualitatively different and two quantitatively different proteins was synthesized by Pk1(A+) but absent from 2-D gels of Pk1(B+)1+. These proteins exhibiting qualitative changes had $M_r$ and p*I* values as follows: 51 000, 6.05; 41 000, 4.95; 45 000, 4.50 and 66 500, 5.35. The two proteins exhibiting quantitative changes had $M_r$ and p*I* values of: 95 000, 5.3 and 65 000, 5.32. We have shown that the SICA-variant antigens of these clones have $M_r$ in the range 185 000–230 000 [5]. Our previous analyses of SICA antigens on one-dimensional SDS-polyacrylamide gels included analysis of proteins in the $M_r$ range of the four spots

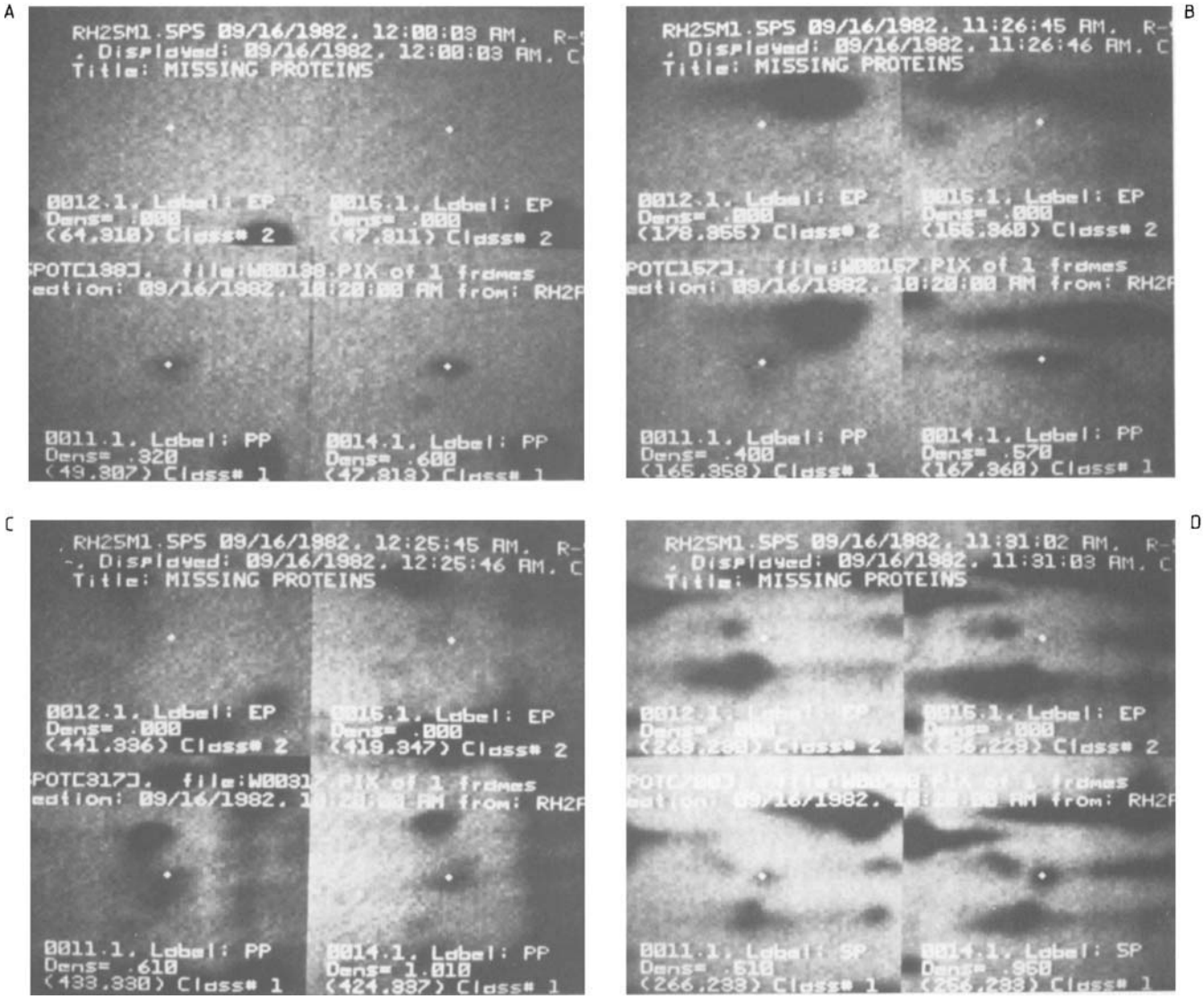


*Figure 3.* Mosaic images of the four missing spots (qualitative changes) found using GELLAB. Each mosaic shows the same gel region for two Pk1(B+)1+ gels (class 2) in the upper panels and two Pk1(A+) gels (class 1) in the lower panels. The center of the following spots has been labeled with a white dot by the computer: Block a, R138; block b, R157; block c, R317, block d, R700.

identified here and failed to identify molecules of this size which immunoprecipitate in a variant-specific manner. However, it is quite possible that these spots represent either proteolytic degradation products of the Pk1(A+) variant antigens ($M_r$ 210 000 and 190 000) or proteins in volved in the expression and/or processing of this variant antigen. The absence of comparable spots that are unique to Pk1(B+)1+ is perplexing, as we would intuitively expect two cloned variants of *P. knowlesi* (one being derived by antigenic variation from the other) to have analogous complements of proteins. Proteolysis of some high molecular weight protein(s) in Pk1(A+), but less so or not at all with Pk1(B+)1+, would account for this observation.

Regardless of the nature of these six proteins found to be differently synthesized by Pk1(A+) versus Pk1(B+)1+, it is clear that quantitatively these proteins are all extremely minor components of the total malarial proteins. Furthermore, since only six proteins were different of the approximately 500 we have detected by analysis of malarial proteins on acid-range gels as well as with pH 4.6–6.5 gels, the qualitative differences in protein phenotype represent about 1 % using one-dimensional gels and [$^3$H]proline, [$^3$H]histidine, [$^3$H]arginine, [$^3$H]isoleucine or [$^{35}$S]methionine as precursors of malarial proteins failed to find any differences, qualitative or quantitative, in proteins synthesized by Pk1(A+) and Pk1(B+)1+ (Howard and Barnwell, unpublished data). It is therefore apparent that conversion of the SICA-antigen phenotype from expression of one specificity (Pk1(A+) with variant antigens of $M_r$ 190 000 and 210 000) to another specificity (Pk 1(B+)1+ with variant antigens of $M_r$ 200 000 and 205 000) [5] does not involve a large change in the nature of malarial proteins other than the variant antigens. Indeed the variant antigens of each clone which do change could not be detected by one-dimensional gel analysis of total biosynthetically radiolabeled proteins from these clones, indicating that the variant antigens themselves are quantitatively minor products of proteins synthesis (Howard and Barnwell, unpublished data). The variant antigens were not detected on these 2-D gels. We do not yet know whether this is due to their focusing beyond the anodal or cathodal extremes of the isoelectric focusing gel or whether they represent such a small proportion of the radioactivity that

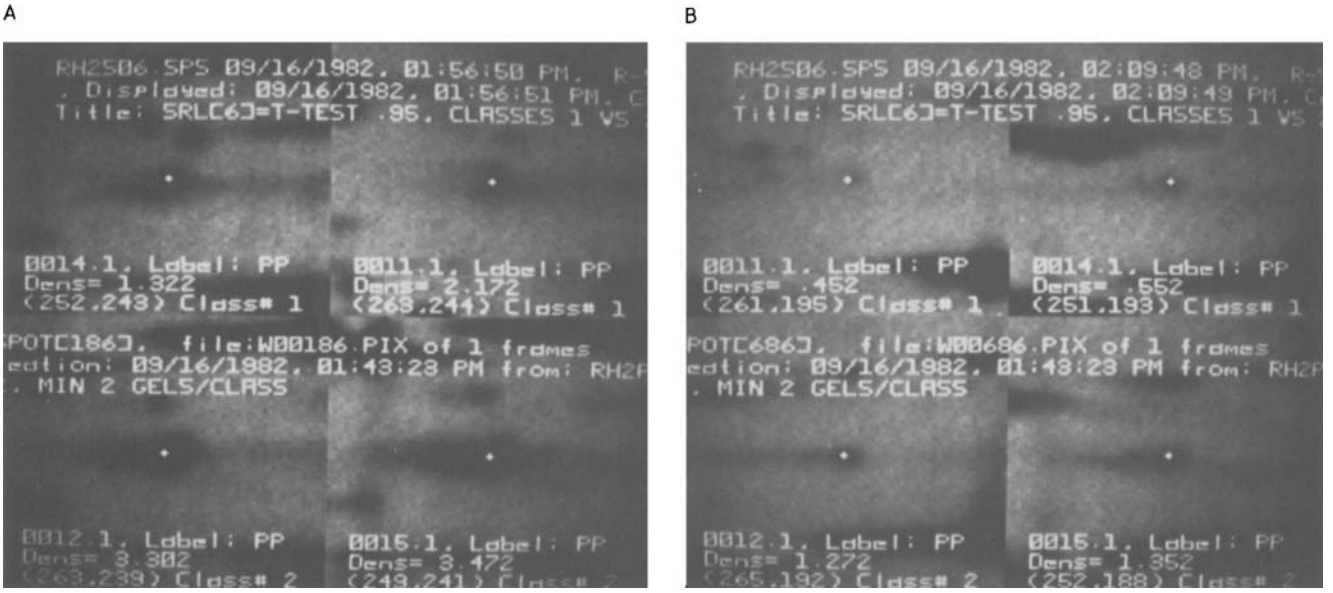


*Figure 4.* Mosaic images of the two spots exhibiting quantitative changes found using GELLAB. Each mosaic shows the same gel region for two Pk1(A+) gels (class 1) in the upper panels and two Pk1(B+)1+ gels (class 2) in the lower panels. The center of the following spots has been labeled with a white dot by the computer: block a, R186, 0.95 t-test significance; block b, R686, 0.99 t-test significance. The normalized density (Dens) of each spot is given in the computer labeling of each segment of these mosaics.

without immunoprecipitation these are masked by more predominant proteins.

### 4.2 P. knowlesi *vs* P. falciparum

These results with *P. knowlesi* clones are markedly different to those obtained by 2-D gel analysis of noncloned isolates of *P. falciparum* derived from different geographic locations [23]. Fourteen proteins varied with these *P. falciparum* isolates. The variations were sufficiently large in p*I* and/or $M_r$ and the proteins which varied represented such a high proportion of the total protein radioactivity that they were readily identified by visual gel comparisons [23]. It was possible to identify proteins specific for West African versus South East Asian isolates, and others which varied in isolates collected within a particular area. These *P. falciparum* isolates are almost certainly different genotypically as well as phenotypically. In contrast, the genetic relationship between the *P. knowlesi* clones examined here is very close, one cloned being derived by antigenic variation from the other. It is not yet known whether antigenic variation by these clone parasites results from differential gene expression of a repertoire of SICA antigen genes or by somatic mutation of a more limited number of variant antigen genes. Our results show that whatever the genetic mechanism, it does not involve a gross change in the nature of proteins synthesized. The *P. knowlesi* clones Pk1(A+) and Pk1(B+)1+ are of the Malaysian H-strain, which on the basis of immunological data [24] will share strain-specific antigens that are different from antigens which characterize other *P. knowlesi* strains, such as the Philippine or Hackery strains [1, 3, 24, 25]. We predict on the basis of the 2-D analysis of *P. falciparum* isolates, that different *strains* of *P. knowlesi* will exhibit differences in protein phenotype on the order of those seen with *P. falciparum*. Antigenic variants within a *P. knowlesi* strain clearly do not exhibit such differences.

In conclusion, the 2-D protein phenotypes of Pk1(A+) and Pk1(B+)1+ are almost identical, indicating that the expression of different malarial variant antigens on the surfac of infected cells is not linked to major alterations in protein synthesis, and most probably reflects a relatively small and discrete genetic rearrangement.

*We thank J. Barnwell for assistance in preparation of the cloned parasites and V. Kao for technical assistance.*

Received April 15, 1983

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# Detection of α-amylase isoenzymes by means of two-dimensional electrophoresis followed by blue starch staining

α-Amylase isoenzymes present in human body fluids were separated by two-dimensional electrophoresis in the absence of denaturing agents and detected by blue starch staining. Human body fluid samples (5 sera, 2 salivas, 1 pancreatic juice and 1 urine) were subjected to the analysis. Two to six activity spots of α-amylase were detected for each sample. The spots were classified into four main species by their isoelectric points (p*I*) 6.9, 6.5, 5.8 and 5.3. All the α-amylase isoenzymes showed apparent molecular weights above 140 000.

## 1 Introduction

The charge heterogeneity of α-amylase isoenzymes in human body fluids has been studied by means of one-dimensional electrophoretic techniques such as cellulose acetate membrane electrophoresis [1], polyacrylamide gel electrophoresis [2, 3] and isoelectric focusing [4]. On the other hand, the presence of high-molecular weight species of α-amylase in human serum was reported by Wilding *et al.* [5] and the binding of α-amylase with immumoglobulin molecules was suggested, employing ultracentrifugation. However, the simultaneous analysis of p*I* and molecular weight of the α-amylase isoenzymes has not been performed previously. Recently, Manabe *et al.* [6] described a technique of two-dimensional electrophoresis in which no denaturing agent was used. We applied this technique for the analysis of human body fluid α-amylase isoenzymes. After electrophoresis, the gels were stained for α-amylase activity using blue starch as a substrate. The pIs and apparent molecular weights of the α-amylase isoenzymes were estimated simultaneously.

## 2 Materials and methods

### 2.1 Materials

Blue starch tablets were purchased from Pharmacia Diagnostics (Uppsala, Sweden). Ampholine (pH 3.5-10) was obtained from LKB Produkter AB (Bromma, Sweden). Acrylamide, N,N′-methylenebisacrylamide (Bis) (both special grade for electrophoresis), glycine, Tris base and ammonium persulfate were from Wako Pure Chemical Industries (Tokyo, Japan). N,N,N′,N′-tetramethylethylenediamine and Coomassie Brilliant Blue R-250 (both special grade for electrophoresis) were from Nakarai Chemicals (Kyoto, Japan). Agar was from Nissui Seiyaku (Tokyo, Japan). Sepharose 4B was from Pharmacia Fine Chemicals (Uppsala, Sweden). Filter papers were from Toyo Roshi (Tokyo, Japan).

### 2.2 Samples

Human sera were obtained conventionally. Human macroamylasemia serum was kindly provided by Dr. T. Shinoda of Tokyo Metropolitan University. Human pooled serum was purchased from Nippon Pharmacy (Tokyo, Japan). Human saliva was collected without preliminary stimulation. Human pancreatic juice was obtained by cannulation of the pancreatic duct and diluted 100-fold with distilled water. Human urine, concentrated about 3000-fold, was kindly provided by Dr. S. Jitzukawa of Kumpukai Yamada Hospital. Sucrose was added to the sample solutions to give a concentration of 40 % w/v and the mixtures were stored at -20 °C. Taka-amylase was prepared from Taka-diastase as described by Yamakawa and Okuyama [7].

### 2.3 Two-dimensional electrophoresis

Two-dimensional electrophoresis in the absence of denaturing agents was performed as described previously. First-dimension isoelectric focusing was performed on gel columns 14 cm × 0.5 cm I. D. A 4 % acrylamide (0.2 % Bis) solution containing 2 % Ampholine, pH 3.5-10, and 0.05 % ammonium persulfate was used to prepare the focusing gels. The electrode solutions were 0.04 M NaOH (cathode) and 0.01 M

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Abbreviations: Bis: N,N′-Methylenebisacrylamide; p*I*: Isoelectric point; SDS: Sodium dodecyl sulfate



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