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Reversal of Knob Formation on *Plasmodium falciparum*–Infected Erythrocytes

Abstract. *The human malarial parasite* Plasmodium falciparum *can produce surface protrusions (knobs) on infected erythrocytes; however, long-term culturing of the parasite results in the appearance of knobless cells. In this study it was found that a knob-producing clone lost the ability to produce knobs in vitro. Furthermore, a clone not producing knobs derived from the knob-producing clone regained the capacity to produce knobby cells in vitro. Certain parasite proteins were associated with the knobby phenotype but not with the knobless type. These results indicate that the parasites change in vitro in a spontaneous and reversible manner independent of immunological selection.*

Isolates of *Plasmodium falciparum* can undergo several changes in culture (*1–5*). Since these isolates probably represent mixed populations when they are placed in culture, subsequent changes may merely reflect preferential growth of subpopulations. Indeed, antigenic differences found in separate isolates obtained from the same geographic area suggest considerable dissimilarity among strains of *P. falciparum* (*6*). Likewise, cloned strains differ from one another and the parental strain in isoenzymes and drug sensitivity (*3, 5, 7*).

Plasmodium falciparum produces knoblike protrusions on infected erythrocytes (*1, 8*) during the trophozoite and schizont stages of development. However, Langreth *et al.* (*2*) cultured knob-producing (K^+) isolates for over 2 years and obtained clones that no longer produced knobs (K^-) (*2*). It appears that the K^- strains originated from cultures that contained mixtures of K^+- and K^--infected cells (*2, 5, 7*).

To determine whether appearance of K^- variants in culture represents a change in vitro or merely the growth of a subpopulation, we cloned a K^+ strain and then screened cultures for infected cells without knobs. K^- parasites from such cells were subcloned and similarly used to select for cells with knobs. Finally, a protein was identified that is present in K^+ but not K^- parasites.

We cultured (*9*) an isolate of *P. falciparum*, strain FVO, obtained from an infected *Aotus* monkey. Before being cloned, the parasite cultures were synchronized for development (*10–12*). Then cells and parasites were grown in 25-cm^3 tissue culture flasks (hematocrit, 2.5 percent; parasitemia, 2 percent) with an appropriate gas mixture (*9*). The flasks were rocked to promote infection of erythrocytes by one parasite each. Fewer than 1 percent of the infected cells contained more than one parasite per erythrocyte, as determined by microscopic observation of Giemsa-stained thin smears. Parasites were cloned in 96-well plates by limiting dilution to an average of one parasite per 100 wells in cultures maintained at a hematocrit of 2 percent. The medium was replaced every 48 hours and fresh erythrocytes were added every 4 days (*3*). After 19 days of growth, stained thin smears were observed and 1 of 192 culture wells was found to contain infected cells. A Poisson distribution of parasites per well was assumed, so that the probability that the parasites in a positive well arose from a single infected cell is $\mu e^{-\mu}/(1 - e^{-\mu})$, where μ is the average density of parasites in the inoculum; μ was determined with the expression $\mu = \ln [1 + (\text{number of positive wells})/(\text{number of negative wells})]$. Thus the calculated probability that the K^+ clone used to select for K^- variants arose from a single parasite is 99.86 percent. Transmission electron microscopy was used to confirm that this clone was present only in knobby cells. On the basis of this observation and statistical analysis of the cloning results, we are reasonably certain that the results presented below were obtained by using a pure K^+ clone.

We used the K^+ clone (Fig. 1A) to select for spontaneously produced K^- variants by means of the following procedure. The K^+ clone was grown in culture and knobless cells were separated from knobby ones in a gelatin-containing medium (*10*) in which knobby cells settled more slowly than knobless ones. The lower phase contained uninfected cells and erythrocytes infected with ring-stage parasites or K^- tropho-

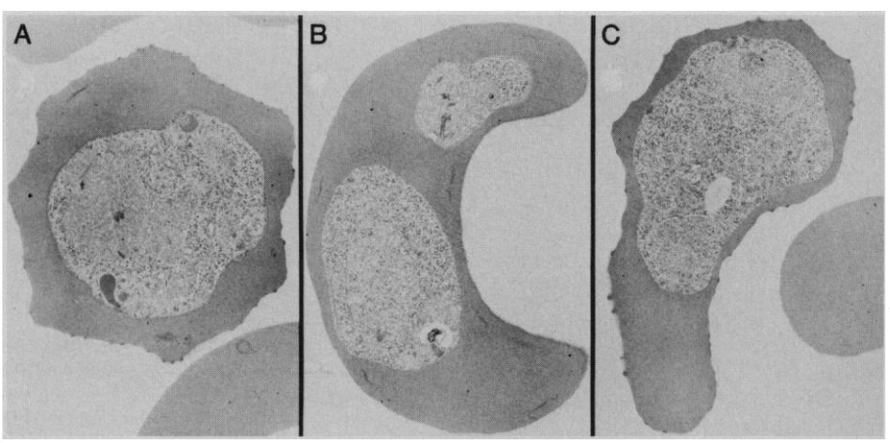

Fig. 1. Electron micrographs of erythrocytes infected with trophozoites of K^+ and K^- strains (×9000). (A) An erythrocyte infected with a K^+ clone isolated from strain FVO. (B) A cell infected with a K^- subclone derived from a mixed K^+-K^- culture after nine gelatin separations. (C) A knobby cell derived from a K^- clone after eight gelatin separations. Cells prepared for electron microscopy (*8*) from synchronized cultures were viewed with a Hitachi HU-12A electron microscope.

zoites and schizonts. This material was subcultured to enrich its population of K^- parasites. The parasites were repeatedly grown and separated in gelatin, and in each instance the lower phase was subcultured. The enrichment procedure was monitored by examining Giemsa-stained thin smears of the culture before gelatin separation and the upper and lower phases after separation. Parasitemia and developmental stages of the parasites were determined in 100 infected erythrocytes. By comparing parasitemias and developmental stages in the upper and lower phases with those in the starting culture, the respective degrees of enrichment were ascertained. A change in either or both qualities indicated a change in the culture from K^+ to K^-.

As a positive control, erythrocytes infected with a K^+ clone were gelatin-separated in parallel, but in this case the upper phase (knobby cells) was repeatedly subcultured. The culture obtained from the upper phase showed eight- to tenfold enrichment in cells infected with mature parasites at each of nine separations. Transmission electron microscopy showed that 95 percent of the infected cells had knobs after the ninth separation.

In contrast, the culture obtained by growing the lower phases after nine gelatin selections was inefficiently separated. That is, the upper phase contained many cells infected with ring-stage parasites (34 percent of all infected cells) and was enriched for parasitized cells by a factor of only 1.5 compared to the culture before separation. Transmission electron microscopy showed 41 percent of the infected cells to be knobby.

We obtained subclones of K^+ and K^- parasites by limiting dilution of the mixed K^+-K^- culture (obtained from the ninth selection for K^-) as described above, except that we diluted the culture to an average density of one parasite per ten wells. Of the 13 subclones obtained (6.8 percent of the cultures), 7 were K^+ and 6 were K^-, as determined by gelatin separation. The probability that the parasites in a positive culture arose from a single infected cell is 96.74 percent. Two clones of each type were confirmed as K^+ or K^- by transmission electron microscopy.

Since a K^- variant was obtained from a K^+ clone by growth and enrichment for knobless cells, the parasites apparently changed spontaneously during growth in vitro. To determine whether this change is reversible, we subjected a K^- subclone (Fig. 1B) to similar growth and enrichment, but this time selected for knobby cells by repeatedly subculturing the upper phase. At the seventh gelatin separation there was a 1.2-fold increase in mature parasites and a 1.5-fold enrichment in parasitized cells in the upper phase. These values increased in subsequent sedimentations, so that the culture became functionally indistinguishable from a K^+ subclone that had been grown and gelatin-separated in parallel. Transmission electron microscopy of the culture that changed from K^- to K^+ confirmed that 95 percent of the infected cells had knobs at the eighth enrichment (Fig. 1C). A sib K^- clone that was treated identically also produced knobby cells (13 percent by electron microscopic examination) at the 12th sedimentation.

Other investigators (*12*, *13*) have shown that at least one *P. falciparum* protein produced in knobby cells is undetectable in knobless ones. The original K^+ clone used in our experiments also produced at least one parasite protein that the K^- clone lacked. This protein has a molecular weight of 88K (lanes 1 and 2 in Fig. 2), similar to that previously reported (*13*). Similarly, the K^+ strain selected from cells originally infected with a K^- clone produced a protein that was absent in the K^- parental clone (lane 4 in Fig. 2). But this K^+ protein was smaller (82K; lane 3) than that of the K^+ clone (lane 1) that gave rise to the K^- parental clone. Although proteins of different molecular weights were correlated with the knobby phenotype, the proteins are probably related because (i) neither protein was present in the K^- clone; (ii) both proteins were preferentially labeled by histidine compared to methionine, as previously reported for the knob-related protein (*12*); and (iii) both proteins had a characteristic fuzzy appearance when separated by one-dimensional polyacrylamide gel electrophoresis (lanes 1 to 3 in Fig. 2).

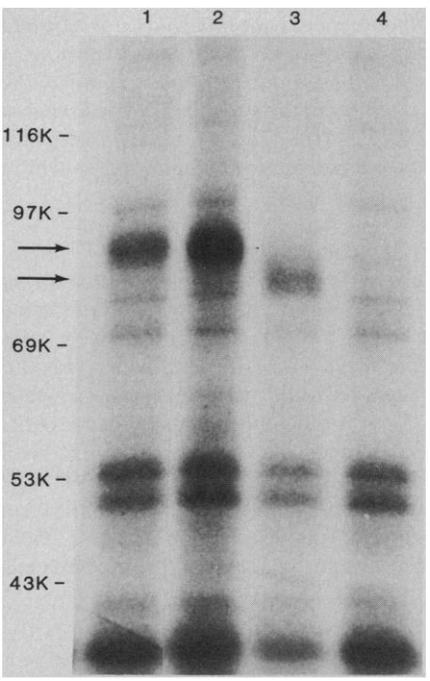

Fig. 2. Parasite proteins from K^+ and K^- strains. Parasites were labeled with [^{3}H]histidine (50 μCi/ml) (*12*), and proteins from membrane-enriched preparations (*12*, *13*) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (*19*) on 5 to 8 percent gradient gels and visualized by fluorography (*20*). A K^+-associated protein of 88K (lanes 1 and 2) is indicated by the upper arrow, while a K^+-associated protein of 82K (lane 3) is indicated by the lower arrow. Both proteins are undetectable in the K^- clone represented in lane 4. Proteins were from the following cultures: (lane 1) K^+ clone of strain FVO; (lane 2) K^+ subclone obtained from the mixed K^+-K^- culture after nine enrichments for knobless cells; (lane 3) K^+ strain obtained from a K^- clone after ten enrichments for knobby cells; (lane 4) K^- clone obtained by subcloning as for lane 2. The K^+ clone represented in lane 1 is the parent of the clones in lanes 2 and 4. The K^- clone represented in lane 4 is the parent of the strain in lane 3.

It appears that cloned strains of *P. falciparum*—at least the K^+ phenotype—can change spontaneously and reversibly in vitro. Determining rates of change from K^+ to K^- and back to K^+ is difficult because of the variables in *P. falciparum* growth and gelatin separation. If one assumes that five newly infected cells result from every parasite-infected cell at each 48-hour generation, then a single parasite would produce 1.9×10^6 parasites by the 19th day after limiting dilution when clones were first detected. If one further assumes that each gelatin selection produces a tenfold enrichment in knobby or knobless cells, depending on the type of cell chosen for subculturing, then one can estimate when a single knobby or knobless cell existed in the culture based on the percentage of such cells seen by electron microscopy. Since gelatin separation is a positive selection for knobby cells, the tenfold enrichment is a good estimate in calculating the change from knobless to knobby cells. But it may not be accurate for enrichment of knobless cells, for which gelatin separation is a negative selection. On the basis of these assumptions and data (number of generations grown, number of gelatin separations, and percentage of knobby or knobless cells determined by electron microscopy), one can calculate approximate rates of change per cell per generation. Accordingly, the estimated rate of change from K^+ to K^- was 4×10^{-6} per cell per generation. For the two clones that independently changed from K^- to K^+, the

estimated rates of change were 10^{-6} and 10^{-10} per cell per generation. The mutation rate for mammalian somatic cells is in the range 10^{-6} to 10^{-8} per cell per generation and for bacterial cells 10^{-6} to 10^{-7} per locus per replication cycle (*14*). The calculated rates of change from K^+ to K^- and from K^- to K^+ may reflect mutational events. However, because of the difficulty in calculating the rates of change for *P. falciparum* grown in vitro and the paucity of genetic information on this organism (*5*), these numbers should be interpreted with reservation.

The mechanism by which *P. falciparum* changes its K^+ phenotype is speculative. However, because the K^+ strain derived from the K^- clone produced a protein of different molecular weight than that produced by the original K^+ clone, a genetic rearrangement may have occurred in the K^+ phenotypic revertant. Indeed, genetic rearrangement has already been implicated in controlling the expression of S-antigens in *P. falciparum* (*15*) and surface proteins of *Trypanosoma brucei* (*16*). However, the observed change in molecular weight of the knob-associated protein could be explained by other genetic mechanisms, such as suppression or phenotypic reversion of a point mutation or changes in protein expression or stability.

Other investigators have suggested that the surfaces of *P. falciparum*–infected cells can change antigenically and functionally and that such changes are mediated by the spleen in infected monkeys (*17, 18*). Our results show that the K^+ phenotype can change in vitro independent of immunological selection and that this phenotypic change is reversible. These results suggest that *P. falciparum* has the potential to change considerably both in vitro and under immunological stress in vivo. By mediating surface changes in the cell that it infects, the parasite may elude the immunological response of an individual vaccinated against malaria. This potential for change should be seriously considered in developing a synthetic vaccine for malaria.

C. A. GRITZMACHER
R. T. REESE
Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037

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A Gradient of Sequence Divergence in the Human Adult α-Globin Duplication Units

Abstract. *The nucleotide sequences of the two 5′-homology blocks of human α-globin gene duplication units were determined. The sequence difference between the two blocks is essentially zero in the 5′ portions, and increases gradually toward the 3′ ends until it reaches a value of 18 percent. This gradient of sequence divergence is similar to the distribution of the frequencies of gene conversion along several loci in* Ascobolus *and yeast. Hot spots for initiation of gene correction processes appear to exist near the 5′ ends of the human α-globin duplication units. The data provide the physical evidence for polar gene correction process in a mammalian genome.*

Sequence homology among members of a eukaryotic multigene family may be maintained by two genetic recombination processes: gene conversion and homologous but unequal crossing-over (*1*). The duplicated human adult α-globin genes, α2 and α1, provide an excellent system for exploring the molecular mechanism of these processes in mammalian cells. These loci are contained within tandemly arranged, highly homologous duplication units (*2, 3*). Each unit spans approximately 4 kilobases (kb) of DNA. Despite the age of the duplication event [before the time of primate divergence (*3*)], three long blocks of sequence homology (X, Y, and Z in Fig. 1), separated by three nonhomologous sequences (I, II, and III in Fig. 1), have been preserved (*2, 4, 5*). As discussed earlier (*4, 5*), this mosaic structure suggests that gene correction between the two duplication units, by gene conversion or unequal crossing-over, or both, acts on segments of DNA. To gain further insight into this segmental correction process, we have completely sequenced DNA from the two X homology blocks which are located in the 5′ regions of the duplication units.

The map in Fig. 1A shows the general organization of the tandem duplication containing the adult α2 and α1 globin genes. The nucleotide sequences of all the α-like globin genes and their immediate flanking regions have been studied (*5–7*). Much of the intergenic DNA sequence of this gene cluster has also been determined recently (*4, 5, 8*). In the regions of the duplication units, the nucleotide sequences of the homology blocks Y and Z, the nonhomology blocks I, II, and III, and a small portion of the X blocks have been determined. From these data, Hess *et al.* (*4*) and Michelson and Orkin (*5*) have proposed the following evolutionary scenario for the generation of the present-day human α-globin gene duplication units: (i) insertion of an ancestral α-globin gene; (ii) insertion of an Alu family repeat in the 5′-flanking region of the ancestral α-globin gene; (iii) tandem duplication of a 4-kb DNA fragment containing the ancestral α-globin gene and the Alu family repeat; (iv) concerted evolution of the duplicated DNA segments by gene conversion or unequal crossing-over (or both); (v) interruption of gene correction by nonhomologous DNA insertion/deletions; and (vi) segmental sequence correction.

The major portions of the X block sequences were not determined in the above-mentioned studies. These data are of both evolutionary and clinical importance, because a complete picture of the sequence organization would provide essential information about the DNA recombination processes occurring between the two duplication units. These processes are responsible for both gene correction and the α-globin gene deletion