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Concentration and separation of erythrocytes infected with *Plasmodium falciparum* by gradient centrifugation*

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Concentration of infected erythrocytes was achieved in cell suspensions derived from long-term culture of Plasmodium falciparum *growing asynchronously in human erythrocytes. This new procedure involves the slow centrifugation (at 33* g*) of erythrocyte suspensions through 5% Ficoll solutions. Mature asexual erythrocytic forms are preferentially retained in the gradient solution (top fraction). After further gradient centrifugation of these parasitized cells, the concentration of mature forms is increased 15- to 31-fold and a mature form parasitaemia of 71–81% is obtained in the final erythrocyte suspension. Furthermore, at least 75% of the total number of the mature forms can be retrieved by this method. Parasitized cells that are not retained in the gradient are sedimented to the bottom of the tube (bottom fraction) and consist predominantly of ring forms. Parasites from both the top and bottom fractions are viable and have been used to initiate short-term synchronous cultures. By providing purified parasite preparations, this simple procedure will facilitate immunological, chemotherapeutic, and biochemical studies with* P. falciparum.

Recent advances in the long-term propagation of *Plasmodium falciparum* in human erythrocytes (*1*, *2*) will facilitate biological studies with human malaria parasites and increase the likelihood of developing a malaria vaccine. Before some of these investigations can be started, however, more concentrated preparations of cultured parasites are needed. In some primate plasmodia, including *P. vivax*, separation of parasitized erythrocytes from infected blood has been achieved by gradient centrifugation (*3–5*). Although substantial enrichment of gametocytes of *P. falciparum* is possible (*6*, *7*), concentration of asexual erythrocytic forms of this species has been much less satisfactory (*5*, *7*).

Concentration of parasitized erythrocytes is easier with the more mature asexual forms of various plasmodial species because they are less dense than newly-infected or noninfected erythrocytes. For example, the characteristic "brown layer" of pigment-containing schizonts can be readily harvested from the blood of rhesus monkeys with synchronous infections of *P. knowlesi* (*8*). Similar concentration of mature forms of *P. falciparum* derived from *in vivo* infections has not been obtained (*7*, *9*), presumably because the difference in density between normal and parasitized cells is smaller. Since parasites lose their synchronous development when they are maintained in continuous *in vitro* culture (*1*), concentration of the relatively low proportion of mature forms found in culture might conceivably be even more difficult to achieve. If, on the other hand, substantial concentration of mature forms were possible, immunological and biochemical studies could be undertaken to determine which components are unique to a certain developmental stage of the parasite. They would also provide mature parasites from which schizont and merozoite antigens of *P. falciparum* could be obtained for immunization studies.

Investigations were therefore initiated in our laboratory to determine whether a difference could be detected in the buoyant density of infected and noninfected erythrocytes obtained from malaria cultures (*10*). This led to studies directed towards the isolation of mature forms of *P. falciparum* by

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centrifugation through a Ficoll gradient. The results of these investigations and the viability of parasites after gradient centrifugation are described in this paper.

MATERIALS AND METHODS

Parasites

The Oak Knoll (FVO) strain of *P. falciparum* (*11*) was used in these studies. Parasites were originally obtained from D. Davidson [a] and continuous culture of these parasites was established at our laboratory by using the candle-jar method (*1*). When the parasitaemia in the culture dishes reached 2–7%, 0.2–5.0 ml of erythrocytes were collected and placed in 15-ml conical centrifuge tubes containing phosphate buffered saline (PBS), pH 7.2. The sedimented cells were washed three times and resuspended in a small amount of PBS, thin blood smears were prepared from the suspension, and the concentration of erythrocytes was adjusted to a packed cell volume (PCV) of 18–22%. Erythrocyte suspensions were then processed in Ficoll gradients according to the procedure described below.

Preparation of Ficoll solutions

Stock solutions of 50% (w/w) Ficoll were prepared by dissolving Ficoll powder [b] in PBS, pH 7.2. After storage overnight at 4°C, various dilutions were prepared for gradient centrifugation studies. The specific gravity of the stock and experimental solutions was determined pycnometrically (*12*) and compared to a standard curve in order to confirm the correct density of gradient solutions at room temperature.

Concentration procedure

Nine millilitres of Ficoll solution were pipetted into a 15-ml conical centrifuge tube. One millilitre of the erythrocyte suspension containing parasitized cells (PCV 18–22%) was then layered on the gradient and centrifuged at 40 or 33 *g* for 15 min. After centrifugation, erythrocytes were collected separately from the entire gradient column and from the bottom of the tube. The two erythrocyte fractions were washed three times in PBS, the sediment was resuspended in a roughly equivalent volume of PBS, erythrocyte counts were made, and thin smears prepared.

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[b] Sigma Chemical Co., St Louis, MO, USA.

Quantification and expression of results

Erythrocytes were counted by means of a Coulter type B particle counter. Thin blood smears were dried in air, fixed with methanol, and stained with 10% Giemsa solution in phosphate buffered water (pH 7.2) for 10 min. The percentage of parasitized cells was determined by counting 500 erythrocytes and the stage of asexual development was assessed by examining at least 100 parasitized erythrocytes.

In this study, uninucleate parasites containing pigment (trophozoites) and multinucleate parasites (schizonts) were both designated as mature forms (MFs) and considered separately from earlier developmental stages. The yield of MFs, expressed as a percentage, was the number recovered from the gradient after centrifugation divided by the number in the parasitized blood layered onto the gradient before centrifugation.

Viability of parasites

The viability of parasites after gradient centrifugation was determined by *in vitro* cultivation. Freshly-collected normal human erythrocytes were added to parasitized erythrocytes from the top and bottom fractions to reduce the proportion of infected cells to about 1%. Sufficient erythrocytes were added to RPMI 1640 medium containing 10% human serum to give a 2.5% erythrocyte suspension. Fifty microlitres of this suspension was placed in each well of a microtitration plate (*13*). Cultures were grown by the candle-jar method (*1*) for 4 days and the medium was changed at least once each day. Thin blood smears from replicate wells were prepared frequently to monitor intracellular maturation of parasites and cyclical reinvasion of erythrocytes.

RESULTS

Centrifugation of erythrocyte suspensions in a 2–10% continuous Ficoll gradient resulted in the formation of two distinct cell fractions. About 10% of the cells were suspended in the gradient column and the remainder were in the soft pellet below the column. Erythrocytes containing MFs were concentrated in the top fraction of cells and were suspended throughout the gradient column. No appreciable difference in the concentration of MFs was observed in the upper, middle, and lower thirds of the column. The sedimented cells at the bottom of the tube contained most of the ring forms and usually had only a few MFs.

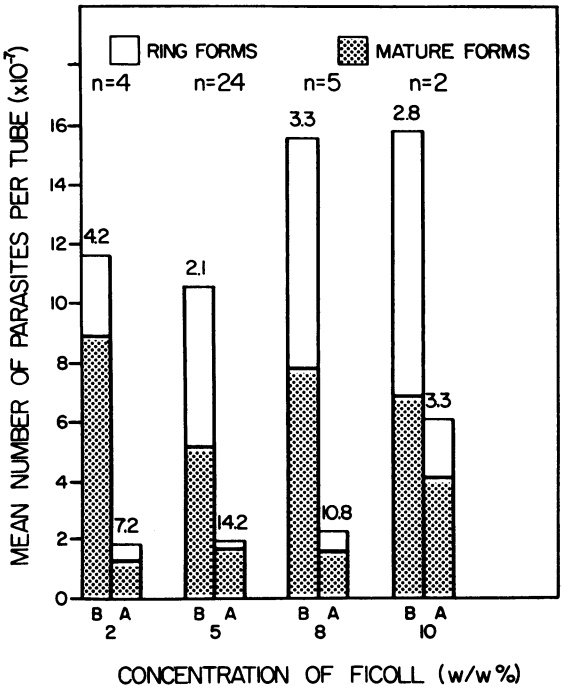

Fig. 1. The yield and concentration of mature forms of *P. falciparum* in the gradient column after centrifugation (at 40 *g*) of erythrocyte suspensions through different Ficoll concentrations. The bars represent the mean number of ring and mature forms per tube before (B) and after (A) centrifugation. Numerals above bars represent the percentages of erythrocytes that contain mature forms. n = number of samples.

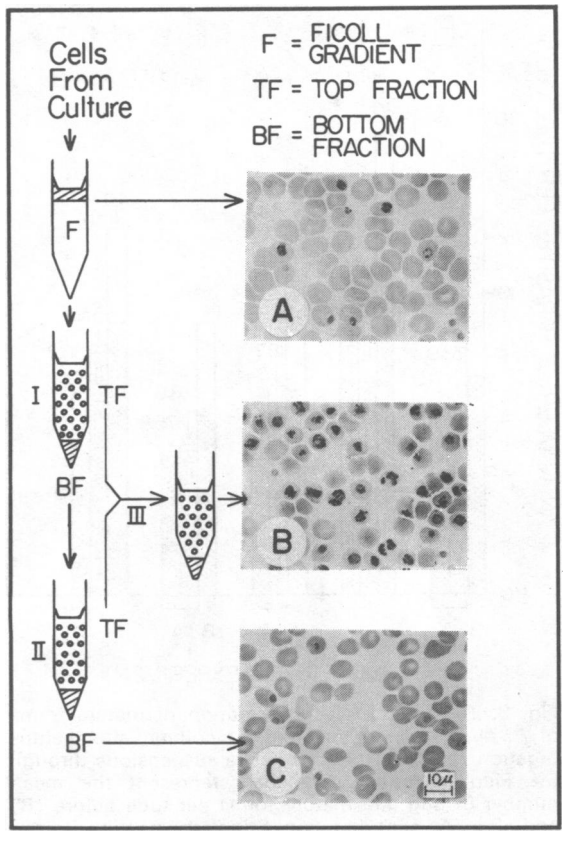

Fig. 2. Schematic representation of the gradient centrifugation procedure for concentrating asexual erythrocytic forms of *P. falciparum* from continuous culture. The three microphotographs show representative thin smears of: A, parasites from culture specimen; B, concentrated mature forms from final top fraction (centrifugation III); C, ring forms from bottom fraction (centrifugation II).

Fig. 1 shows the mean concentrations and yields of mature and ring forms in the top fraction after erythrocyte suspensions had been centrifuged at 40 *g* through different concentrations of Ficoll gradient. At a Ficoll concentration of 2%, the yield of MFs was low and less than a 2-fold increase in the concentration of MFs was obtained. The yield of MFs was greater at a concentration of 5% and, at this higher specific gravity of Ficoll, a 7-fold increase in the concentration of MFs was observed. At concentrations of Ficoll of 8% and 10%, enrichment of parasites became progressively more difficult to achieve.

In view of the encouraging results obtained with 5% Ficoll, further studies were carried out to determine whether more MFs could be retrieved by additional gradient centrifugation. The procedure, outlined in Fig. 2, consisted of further centrifugation of cells from the bottom fraction of the first gradient, pooling of the top fractions from the first and second gradients, and further concentration of MFs by centrifugation of the pooled fractions. Samples were centrifuged at 33 *g* instead of 40 *g* because in other experiments (results not shown) the lower force enhanced the yield and concentration of MFs.

Fig. 3 shows the yield and concentration in five separate experiments with centrifugation at 33 *g*. In each experiment, erythrocytes from different culture plates were pooled and, depending on the quantity of material available, cell suspensions were layered onto gradients in 4–12 centrifuge tubes. Initial parasitaemia in the different experiments

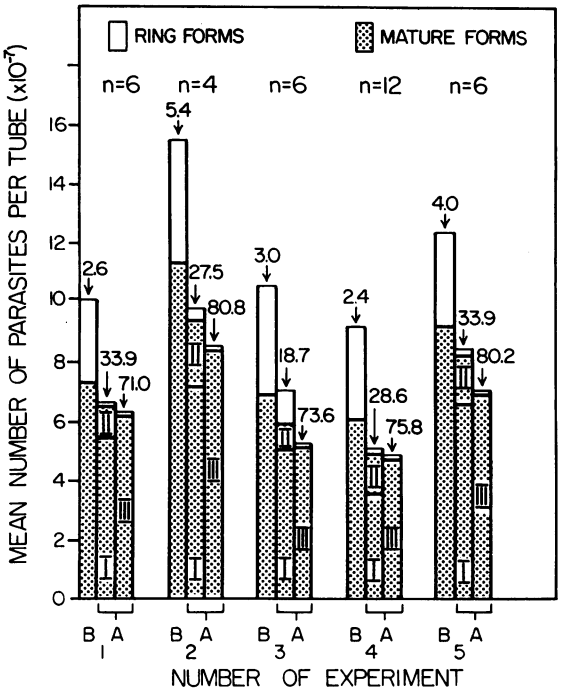

Fig. 3. The yield and concentration of mature forms of *P. falciparum* in the gradient column after centrifugation (at 33 *g*) of erythrocyte suspensions through 5% Ficoll solutions. The bars represent the mean number of ring and mature forms per tube before (B) and after (A) centrifugation. Stippled bars with Roman numerals denote the number of mature forms collected from the top fractions after centrifugation I, II, or III (see Fig. 2). Numerals above bars represent the percentages of erythrocytes that contain mature forms. n = number of samples.

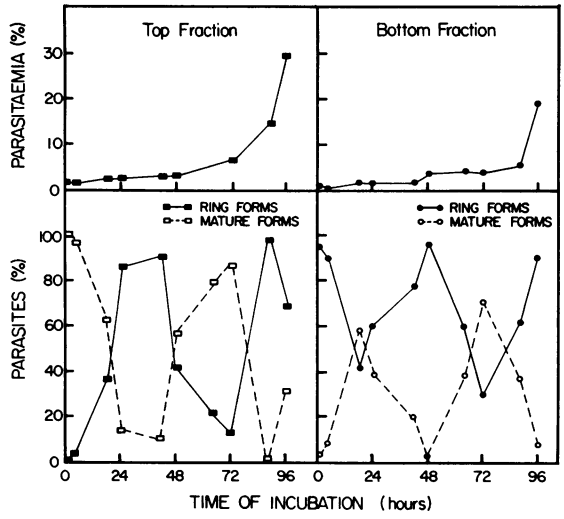

Fig. 4. Growth of parasites of *P. falciparum* from the final top and bottom fractions after gradient centrifugation. Points represent mean values (from duplicate samples) of the parasitaemia and the relative percentages of ring and mature forms obtained at different times during 96 hours of incubation.

varied between 3.6% and 7.4%, with MFs constituting about two thirds of the total number of parasites. After centrifugation I, 58–75% of the MFs were recovered from the top fraction. The bottom fraction yielded another 12–23% of the MFs after centrifugation II. By pooling the top fractions from centrifugations I and II, 80–90% of the MFs were recovered from the original sample. The concentration of MFs in the pooled top fractions was 19–34%, representing a 5- to 13-fold increase in the level of parasitaemia. Although the yield of MFs dropped slightly after further centrifugation (III) of the pooled top fractions, a marked increase was observed in the concentration of MFs. The percentage of erythrocytes containing MFs in the final top fraction was 71–81%, representing a 15- to 31-fold increase in the concentration of parasites after completion of the procedure. Most ring forms were not trapped in the gradient and they constituted at least 90% of the parasitized cells found in the bottom fraction after the second centrifugation.

Fig. 4 illustrates graphically the maturation and multiplication of parasites exposed to Ficoll gradient centrifugation. Parasites obtained from either the top or bottom fraction of cells were viable, as shown by a 15- to 18-fold increase in the number of infected cells during 4 days of incubation. The multiplication rate during *in vitro* cultivation was similar to that of parasites that had not been exposed to Ficoll gradient centrifugation. By starting these cultures with either predominantly ring or mature forms, fairly synchronous development of different parasite stages was maintained during the period of incubation.

DISCUSSION

The findings presented in this report indicate that low-speed centrifugation of parasitized erythrocytes through a low-density Ficoll gradient selectively concentrates mature asexual erythrocytic forms of *P. falciparum*. The mature forms (MFs), trapped

in the gradient column, are concentrated in the top fraction of cells. The bottom fraction of cells, located beneath the gradient column at the bottom of the tube, contained most of the erythrocytes that were either not infected or were infected with the less mature parasite stages. In addition, the bottom fraction contained the MFs that were not trapped by the gradient during the first centrifugation. More than half of these remaining MFs could usually be retrieved through gradient centrifugation of the bottom fraction of cells. Further centrifugation of the two top cell fractions containing MFs slightly lowered the total yield of trophozoites and schizonts collected in the gradient. On the other hand, many uninfected erythrocytes were not retained in the gradient, thereby greatly increasing the proportion of parasitized cells in the erythrocyte suspension obtained from the final top fraction.

Cultivation of parasites collected from both the top and bottom fractions showed that parasites retained their viability after gradient centrifugation. In addition to a 15- to 18-fold increase in parasitaemia, parasites from both cell fractions followed a synchronous pattern of development during 4 days of incubation.

Ficoll has often been preferred to other gradients, such as sucrose, which might affect the metabolism and, possibly, the viability of parasites. The cost of Ficoll, a deterrent when high-density gradients are required, was reduced considerably in our studies by using low concentrations of the material. Agglutination of erythrocytes (*14*) was not observed during our investigations with cultured parasitized cells, possibly because the cells were exposed to relatively low concentrations of gradient solutions and serum and because they were centrifuged gently for short periods of time. Satisfactory results could only be obtained by allowing freshly prepared Ficoll solutions to stand overnight before they were used, in order to stabilize the gradient and permit the escape of trapped air bubbles. Although low-speed centrifugation of specimens was usually carried out at 4°C, results were equally satisfactory at room temperature (about 20°C).

The method described in this report usually extracted more than 75% of the mature forms of *P. falciparum* from continuous culture and increased the percentage of infected cells from an average of 3.5% to 76.2%. After retention of most mature forms in the gradient, ring forms were found predominantly in the lower cell fraction. The yield of specific parasite stages from both cell fractions was greatly increased following their dilution with uninfected erythrocytes and synchronous development in culture for a few days.

The availability of concentrated preparations of mature forms of *P. falciparum* will facilitate immunization studies that require antigen preparations with as little erythrocyte contamination as possible. It will also promote the development of *in vitro* assays to measure protective immunity and the study of merozoite release and reinvasion. Cell preparations containing predominantly ring forms will be particularly useful in determining the antimalarial activity of different compounds and in establishing synchronous cultures of *P. falciparum*.

RÉSUMÉ

CONCENTRATION ET SÉPARATION DES ÉRYTHROCYTES INFECTÉS PROVENANT DE CULTURES DE *PLASMODIUM FALCIPARUM* PAR CENTRIFUGATION AVEC UNE SOLUTION DE DENSITÉ DÉTERMINÉE

La méthode décrite dans cet article permet d'obtenir des préparations à forte concentration de parasites à partir de cultures asynchrones à long terme de *Plasmodium falciparum* dans des érythrocytes humains. Le procédé fait appel à la centrifugation à faible vitesse d'une suspension d'érythrocytes à travers une solution de Ficoll à 5%.

Une couche formée de 1 ml d'érythrocytes en suspension à 18-22% est déposée dans un tube conique contenant 9 ml de la solution Ficoll. Après centrifugation à 33 *g*, les érythrocytes infectés par des formes mûres (*mature forms*) des stades asexués du parasite se trouvent en suspension dans la solution Ficoll, ou fraction supérieure (TF), et la plupart des érythrocytes non infectés ou contenant des parasites au stade annulaire (*ring forms*) se déposent à la base du tube, ou fraction inférieure (BF), mais cette dernière contient aussi des formes mûres qui n'ont pas été retenues dans la solution au cours de la première centrifugation. On peut normalement récupérer celles-ci en centrifugeant la fraction inférieure avec la solution Ficoll. Après cette deuxième opération, les deux fractions supérieures successivement obtenues contiennent ensemble 80 à 90% des formes mûres qui se trouvaient dans l'échantillon initial. Bien que, dans les

expériences faites, le nombre des formes mûres recueillies ait diminué à l'issue d'une troisième centrifugation effectuée avec ces deux fractions additionnées, le pourcentage d'érythrocytes contenant des formes mûres avait encore augmenté et était de 15 à 31 fois plus élevé dans la fraction supérieure finale que dans l'échantillon original, la parasitémie passant d'une valeur médiane de 3,5 à 76,2%. Comme la plupart des formes annulaires ne sont pas retenues dans la solution, elles constituent au moins 90% des érythrocytes parasités se trouvant dans la fraction inférieure après la deuxième centrifugation.

La viabilité des parasites après centrifugation dans la solution Ficoll a été confirmée par l'augmentation de la proportion d'érythrocytes infectés, qui était 15 à 18 fois plus forte après 4 jours d'incubation. Quelle que soit la forme de parasite prédominant au début des cultures — formes annulaires ou formes mûres — le développement synchrone des différents stades s'est poursuivi pendant la période d'incubation.

La possibilité de disposer de préparations concentrées de formes mûres de *P. falciparum* facilitera les travaux visant à la mise au point d'un vaccin, lesquels requièrent des préparations d'antigènes aussi peu contaminées que possible par les érythrocytes. Elle permettra aussi le développement des titrages *in vitro* pour mesurer la protection immunitaire, ainsi que l'étude de la libération des mérozoïtes et de la réinvasion des érythrocytes. Les préparations cellulaires dans lesquelles prédominent les formes annulaires seront particulièrement utiles pour déterminer l'activité antipaludique de divers composés et pour entreprendre des cultures synchrones de *P. falciparum*.

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