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A Procedure for the Harvesting of Mammalian Plasmodia*

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Immunochemical research into the antigenic structure of a given disease agent presupposes the availability of undegraded antigen. Some types of immunochemical studies of plasmodia can be carried out with the intracellular parasites in situ *in the host cell (for example, studies using the fluorescent antibody technique). In other techniques (such as double diffusion in gel, disc electrophoresis) the presence of host cell contaminants is undesirable, and these require to be reduced to a minimum.*

A method is described for harvesting mammalian (rodent, simian, and human) plasmodia. Plasmodia in the product are significantly concentrated as compared with the original samples. This point is particularly important in harvesting human plasmodia, in which parasitaemias tend to be very low. Significant reduction of red- and white-cell contaminants is achieved. Antigens in the cell-free plasmodial products obtained are apparently in their native state, and give replicable results in studies of double diffusion in gel, immunoelectrophoresis and disc electrophoresis, passive haemagglutination and vaccination.

Current techniques for the harvesting of infectious agents for studies in antigenic analysis and immunochemistry must meet exacting requirements. Since antigens are either proteins or closely associated with them, protein degradation must be avoided in the harvesting procedure if the antigens are to be recovered in their native state. Antigenic binding sites should remain unchanged. Finally, the antigenic product should be capable of being preserved indefinitely in the undegraded state for convenience in stockpiling, storage and transfer to central laboratories for analysis.

Where infectious agents are obligate intracellular parasites, as in the case of the erythrocytic stages of plasmodia, the parasites must first be freed from their host cells; and contamination of the parasitic product with host cell components must be reduced to a minimum.

The present study describes one such procedure for the harvesting of mammalian plasmodia from infected blood. Our approach was first to work out the technical details using rodent plasmodia, then to apply them to simian plasmodia maintained in the laboratory, and finally to adapt them to the collection of human plasmodia in the field. Procedures for the harvesting of human plasmodia should be applicable under field conditions in endemic areas.

We have employed our procedure in collecting the rodent plasmodia *Plasmodium berghei, P. vinckei* and *P. chabaudi*; the simian plasmodia *P. cynomolgi, P. cynomolgi bastianellii, P. knowlesi, P. inui* and *P. coatneyi*; and the human plasmodia, chiefly *P. falciparum.*

The rodent plasmodia were collected and processed at the Departments of Parasitology of the Hebrew University and of the London School of Hygiene and Tropical Medicine; the simian plasmodia at the London School and at the National Center for Primate Biology at the Davis campus of the University of California; and the human plasmodia at the Medical Research Council Laboratories at Fajara, near Bathurst, Gambia, and at the SEATO Laboratories in Bangkok, Thailand. We offer our grateful thanks to all the host laboratories, without

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HARVESTING PROCEDURE

General information

To avoid protein degradation, particularly when collections are being made in the field under tropical conditions, infected blood, and the parasitic material harvested from it, is maintained either in an ice-bath or in the refrigerator at 4°C wherever possible throughout the procedure. Since products prepared in tropical climates are readily contaminated with bacteria developing during the harvesting process, extreme care was taken to keep glassware as clean as possible, while not maintaining sterile conditions. Thus, in harvesting human plasmodia, all washed glassware was immersed overnight in 70% alcohol, and was rinsed first in tap water and then in distilled water immediately prior to use.

Films of the product stained in Giemsa's stain are required to check the effect of various stages of the procedure on the parasites. Since such films prepared from blood cells or liberated parasites suspended in the aqueous washing solutions stain poorly, all films were drawn on slides previously coated with a film of normal homologous citrated plasma, then dried in air and stored in the refrigerator.

The procedure, and particularly that relating to the human plasmodia, was controlled throughout by routine red and white cell counts of all fractions at all stages of the process. These were done in a Thoma counter.

Parasite numbers in the various fractions were roughly assessed as follows. A measured bacteriological loopful (=1/200 ml) was spread as evenly as possible over a 1-cm² area of a slide. When such thick drops contained erythrocytes they were dehaemoglobinized by immersion in water, then dried in air, fixed in methanol and stained with Giemsa's stain. Fractions examined at stages of the procedure after lysis of the erythrocytes were fixed in methanol and stained with Giemsa's stain. If the number of parasites in a standard area of average thickness be counted, rough but surprisingly replicable comparisons can be made among the thick films.

Collecting infected blood

Rodent blood. Parasites were harvested from infected mouse blood at peak parasitaemia according to the technique of Spira & Zuckerman (1962). It was convenient to bleed about 20 infected mice at the same time. The thorax of an anaesthetized mouse was opened and a few drops of 3.8% Na citrate solution were placed in the thoracic basket. The aorta was severed and the cardiac muscle incised, so that the blood flowed freely into the thorax. The citrated blood was aspirated with a Pasteur pipette.

Simian blood. Infected *Macaca mulatta* or *Macaca irus* monkeys were exsanguinated on or near the day of peak parasitaemia. They were deeply anaesthetized before bleeding, either by the intramuscular injection of phencyclidine hydrochloride (Sernylan), 3 mg to 5 mg per kg of body-weight (in London), or by the intravenous inoculation of pentobarbital sodium (Nembutal) until pupillary and finger-nail reflexes were negative (at Davis). The chest was shaved, and the heart was exposed by reflecting the chest wall following a V-shaped incision from the angle of the sternum through the ribs. A 16-gauge needle attached to a polyethylene tube was inserted into the left ventricle, and the freely flowing blood was collected into a flask containing chilled 3.8% Na citrate solution. When the flow began to slacken, chilled Na citrate solution was placed in the thoracic basket and the aorta was severed. The citrated blood collecting in the thorax was aspirated. An average yield from a single monkey was 100 ml to 150 ml of blood in 75 ml citrate solution.

Infected blood was stored overnight at 4°C before processing. When 2 or more monkeys of the same species and with the same species of *Plasmodium* were bled at the same time, the bloods were pooled. The citrated plasma was first removed by centrifugation, and was replaced by phosphate-buffered saline (PBS). This was done to avoid isoagglutination.

Human blood. Since human patients must not be harmed by the collecting procedure, harvesting human plasmodia is feasible only if small samples of infected blood can be stockpiled and pooled. It is assumed that the antigenic composition of parasites of the same species of *Plasmodium* collected at a single site is identical. This assumption will eventually require rigorous control.

Infected patients at out-patients' clinics with at least 0.5% to 1% of their erythrocytes parasitized with a single species of *Plasmodium* were bled by venepuncture into Na citrate solution either with conventional syringes or with Vacutainer tubes; 5 ml to 10 ml of blood were taken from a child,

depending on its age and physical state, and 20 ml to 30 ml from an adult.

Collected samples, totalling up to 150 ml of infected citrated blood, were transported to a central laboratory at 4°C and were stored in the refrigerator overnight before being processed. Since bleedings were generally completed during the forenoon in the Gambia and in Thailand, and since the processing of infected blood takes about 10 hours and should be completed from start to finish without a break, we found it convenient to begin to process the bloods collected on the previous day early on the following morning. Parasites in Giemsa-stained films from blood samples refrigerated overnight regularly appeared quite normal. Indeed, we repeatedly had the impression that *P. falciparum* ring forms had grown somewhat in the interval, but this point would obviously require rigorous checking.

Before further processing, the citrated plasma was removed from a given sample following centrifugation, and was replaced by PBS. Samples taken at the same time and containing the same species of *Plasmodium* were then pooled.

Separating erythrocytes from leucocytes

Since leucocytes are not dissolved by haemolytic agents, it is necessary to remove as many leucocytes and other blood-formed elements as possible from the infected blood before parasites are liberated from their host erythrocytes, to minimize later contamination of the parasitic product with leucocytic and thrombocytic components.

Rodent blood. At peak infection, mice of our strain develop very high parasitaemias (70% or more in the case of *P. berghei*). At the same time leucopenia is marked. When the citrated mouse blood is centrifuged at 3000 rev/min, the buffy coat of leucocytes is easily removed from the underlying erythrocytes, using a Pasteur pipette with a flat tip. Some of the underlying erythrocytes are perforce aspirated with the leucocytes. Since the parasitized erythrocytes are lighter than the uninfected ones, the discarded erythrocytes tend to have a relatively high parasite content. However, where parasitaemias are so high and infected animals are so readily available, it is permissible to disregard this fact, and to remove the buffy coat together with as many of the underlying erythrocytes as will ensure radical reduction of the leucocyte population.

Where parasitized blood is less readily available, and where peak parasitaemias are low, this procedure would result in discarding most of the parasitic harvest. In such a situation it is therefore inadmissible.

Simian blood. In many cases of simian malaria parasitaemias were high and large volumes of infected blood were available. Leucocytes were removed from such samples by the method described above for the rodent plasmodia. Where parasites were less abundant, simian leucocytes were removed with the aid of dextran, as described below for human blood.

Human blood. Human plasmodial parasitaemias are generally very low, and a 0.5% or 1% infection was usually the highest available for bleeding; 2% infections were very rare. In view of the extreme paucity of parasites and the relative non-availability of donors, we separated the leucocytes and thrombocytes from human blood by the more arduous but less wasteful method of treatment with dextran according to the technique of Nelken et al. (1960). This method was also applied to monkey bloods with low parasitaemias.

Chilled, infected blood was centrifuged at 3000 rev/min for 5 minutes. The citrated plasma was removed, and the sedimented cells were washed by centrifuging in PBS. The first washings were discarded, and the sedimented cells were suspended in 6 volumes of 3.6% dextran of average molecular weight 115 000. The suspension was refrigerated for 30 minutes at 4°C in tall glass cylinders. Erythrocytes in the dextran solution form rouleaux and sediment, leaving most of the leucocytes and thrombocytes in the supernatant fluid. The latter was decanted and replaced by a similar volume of fresh dextran solution. The cells were resuspended and replaced in the refrigerator for an additional 30 minutes.

The discarded supernatant fluids together contained about 99% of the leucocytes and thrombocytes in the original sample, and about 2% of the erythrocytes. Since the proportion of infected to normal erythrocytes in the supernatant fluids was the same as in the original sample, the procedure involves no selective removal of infected erythrocytes. The residual 1% of leucocytes posed a technical problem following lysis of the erythrocytes with saponin, as discussed below in the following section.

Following the second dextran treatment, the sedimented cells were redispersed in PBS. The staining properties of erythrocytes and parasites were unimpaired by treatment with dextran.

Freeing parasites from the host erythrocytes

This stage of the procedure was identical for all the mammalian plasmodia studied.

Erythrocytes were lysed with saponin according to a modification of a method originally described by Christophers & Fulton (1939). This method, employing a non-protein lytic agent, yielded parasitic products with undegraded antigenic binding sites as demonstrated by gel diffusion, passive haemagglutination and vaccination. Parasites have repeatedly been shown still to be viable following treatment with saponin.

Saponin 1 : 7500 in saline was heated to 37°C. The washed, packed erythrocytes from 1 volume of the original blood sample were suspended in 5 volumes of the saponin solution; and the suspension was incubated, with occasional stirring, in a water-bath at 37°C for exactly 15 minutes. Following rapid chilling in an ice-bath, it was centrifuged at 10 000 rev/min in a refrigerated centrifuge for half a minute. The suspension was resuspended in half the volume of fresh saponin solution used in the first treatment, and incubated at 37°C for exactly 10 minutes. Again it was rapidly chilled in an ice-bath, and spun in a refrigerated centrifuge for half a minute at 10 000 rev/min. The supernatant fluid was discarded, and the liberated parasites were washed 3 times in large volumes of chilled PBS by centrifugation at high speed.

At this stage fibrils of greyish gummy substance frequently appeared in the previously fluid product. Teased apart on a slide and stained with Giemsa's stain, they proved to consist of felted pink threads in the meshes of which numerous parasites were trapped. Spira & Zuckerman (1966) have shown that this contaminant consists of nucleic acids, probably freed from residual leucocytes injured but not lysed by the saponin treatment. The injured leucocytes break up under the stress of high-speed centrifugation and release nucleic acids which form the gummy aggregates. The latter dissolve in DNA-ase and in 1 M NaCl, and fluoresce in acridine orange, all indicating that they consist of DNA. The contaminating fibrils were flushed in PBS to release as many parasites as possible from their meshes, and were removed with an applicator from the suspension of parasites.

Following centrifugation at 10 000 rev/min, the parasite sediment was concentrated into a few millilitres of PBS. Two layers (fractions A and B) can be distinguished in the sediment:

Fraction A, the lower layer, is a densely packed, brown button. It consists chiefly of the larger pigmented parasites, together with more than half of the leucocytes remaining in the product. Few stromata occur in this fraction, which contains roughly half the parasites in the harvested product.

Fraction B, the upper, cloudy layer, is of a milky opacity. Numerous smaller and less pigmented parasites occur in this fraction, which contains relatively few leucocytes but many stromata.

Fraction A is less voluminous than Fraction B, but contains more parasites per unit volume. The two are easily separable from one another, since Fraction B can be decanted, whereas Fraction A adheres firmly to the tube wall, and requires an effort to dislodge it. Fraction A may contain residual fibres of the DNA discussed above.

Fig. 1-6 illustrate steps in the harvesting procedure.

Fractions A and B were collected separately in glass ampoules and lyophilized under vacuum. The ampoules were sealed under vacuum and stored at −20°C. It is convenient to stockpile and to transport plasmodial antigen in this lyophilized form.

When antigen was reconstituted to its original volume by the addition of distilled water, the parasites were broken up by passing them through a Hughes (1951) press. This disintegrates the product as a frozen mass without diluting it any further. Such ground plasmodia have served as antigens in studies on gel diffusion (Spira & Zuckerman, 1962, 1966; Banki & Bucci, 1964; Zuckerman et al., 1965; Guberman & Zuckerman, 1966; McGregor et al., 1966); haemagglutination (Bray, 1965); disc electrophoresis (Sodeman & Meuwissen, 1966; Spira & Zuckerman, 1966); and vaccination (Zuckerman, Hamburger & Spira, 1967).

DISCUSSION

Each method devised for the harvesting of the mammalian plasmodia should be adapted to the objective for which it is to be employed. Different objectives will obviously lead to procedural differences in harvesting. Thus, Trager (1950), in tissue culture studies in which viability of the liberated parasites was the essential concern, lysed the host erythrocytes with haemolytic antiserum. While such treatment is most probably less traumatic than saponin lysis, we did not adopt it since we wished to avoid contaminating our product with a foreign protein.

No parasitic product yet described is completely free of homologous contaminants, such as erythro-

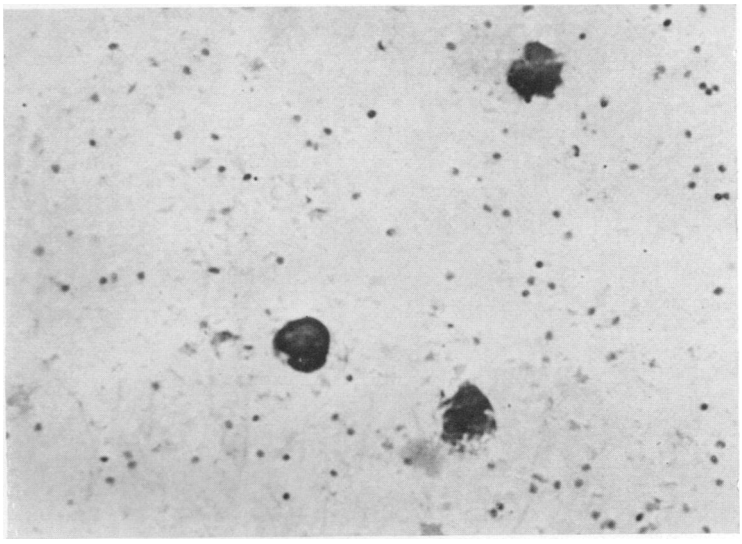

FIG. 1

Thick drop of pooled human blood containing *P. falciparum* ring forms. The dark globular dots are nuclei, and the lighter grey areas are wisps of cytoplasm. Several leucocytes appear in the field (×1300).

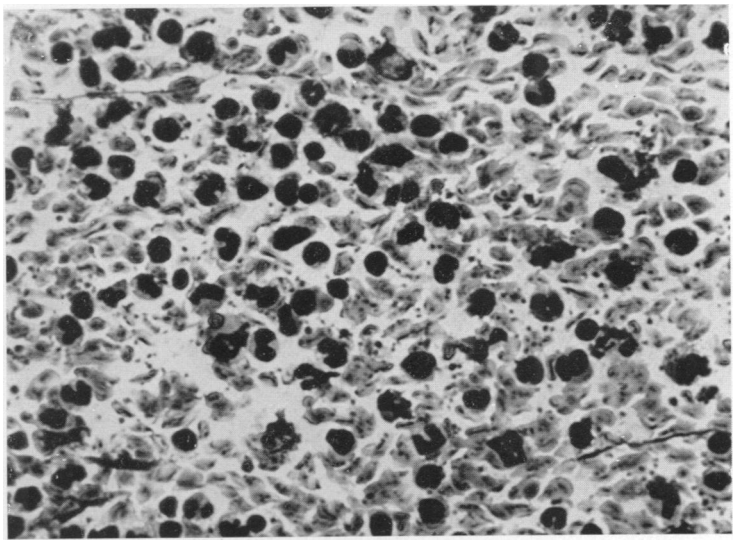

FIG. 2

Film of the cell sediment from the first dextran supernatant fluid (×420). Note the numerous leucocytes occurring in this supernatant fluid. Note also that erythrocytes are distorted in shape when in dextran, but they regain their normal shape in PBS.

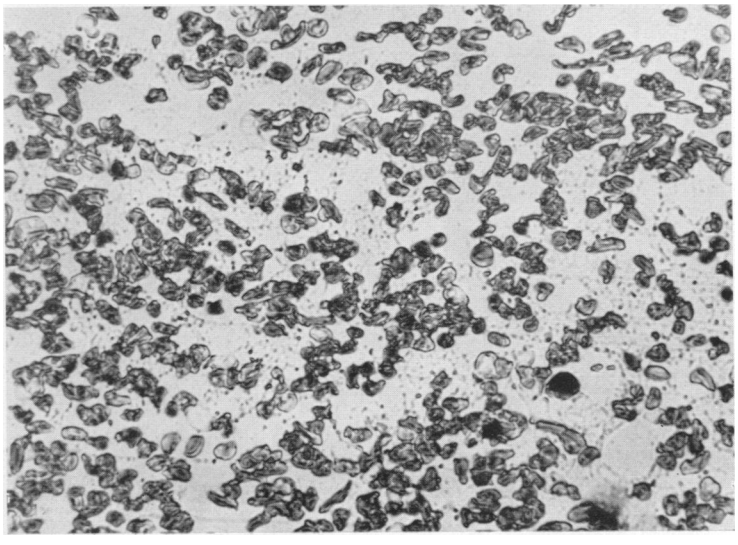

FIG. 3

Film of the cell sediment from the second dextran supernatant fluid (×420). Note that most of the leucocytes have already been removed by the first treatment with dextran.

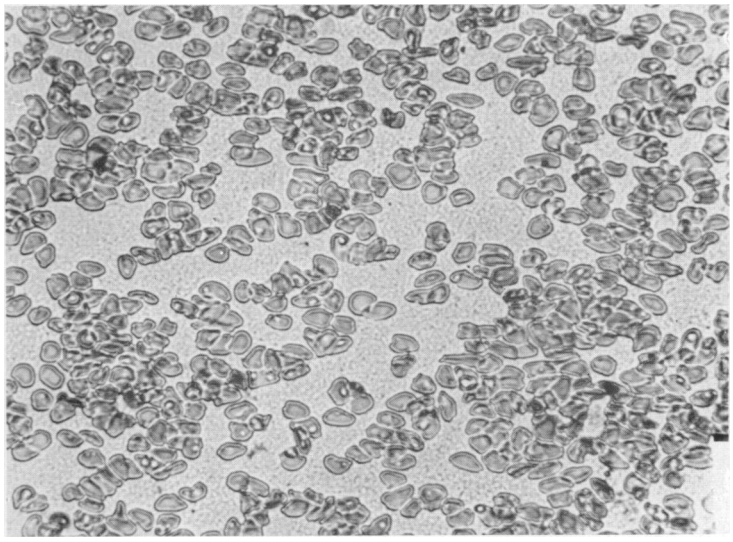

FIG. 4

Film of infected human blood following the two treatments with dextran (×420). Note the virtual absence of leucocytes.

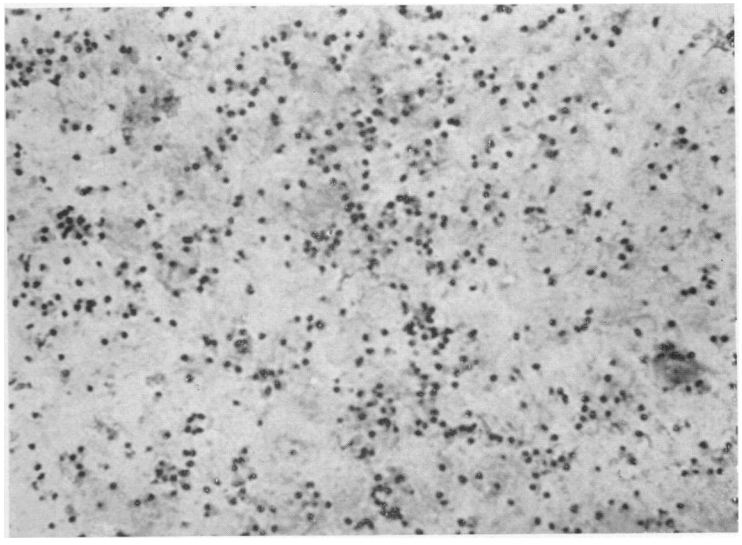

FIG. 5

Parasite product following treatment with saponin, Fraction A (×1300). Parasites are highly concentrated, stain well, and the product is practically free of leucocytes.

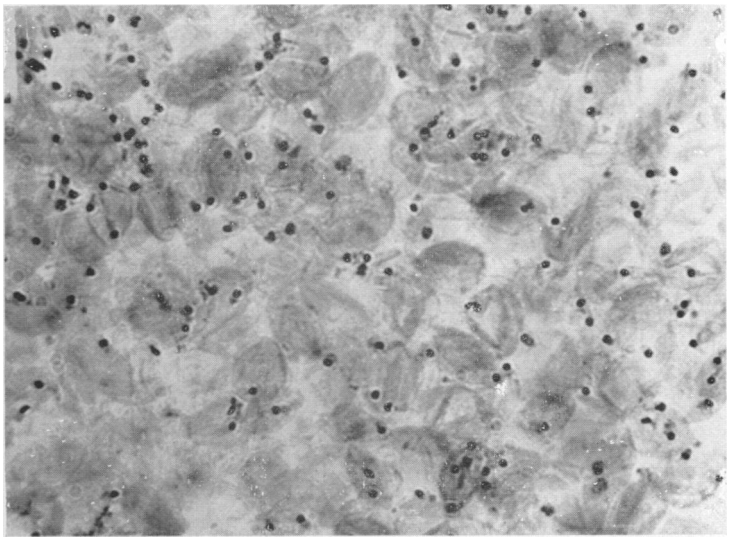

FIG. 6

Parasite product following treatment with saponin, Fraction B (×1300). Parasites are less concentrated than in Fraction A, but stain equally well. This fraction contains numerous stromata.

cytic stromata and leucocytes. However, when such contaminants are reduced to a minimum, the relatively crude parasitic products already available enable us today to carry out a variety of immunochemical and immunological studies. The fact that our product did not react with antibody against homologous blood cells in immunoelectrophoresis (Spira & Zuckerman, 1962) probably reflects the relatively high titre of antibody required to yield positive results in gel-diffusion studies.

Mammalian plasmodial antigen harvested by our procedure or modifications thereof is capable of detecting the presence of antiplasmodial precipitin in animals recovering from infection. This has been demonstrated in rodents (Zuckerman et al., 1965; Guberman & Zuckerman, 1966), in monkeys (Banki & Bucci, 1964; Spira & Zuckerman, 1966), and in man (McGregor et al., 1966). Indeed, McGregor (personal communication, 1966), in discussing his study on human antiplasmodial precipitins in hundreds of sera from the Gambia, has said that he believes " it would be very difficult to miss positive reactions in this country ". Bray (1955) has used antigen harvested by our method to demonstrate antibody by passive haemagglutination. Disc electrophoretic studies have been done on simian and rodent plasmodia (Spira & Zuckerman, 1966); and their results for *P. berghei* tally well with similar results of Sodeman & Meuwissen (1966), if it be borne in mind that possible host cell contaminants still require to be identified and subtracted from the total patterns. Finally, non-living rodent plasmodial antigen harvested by our method is capable of producing a measure of protection against viable *P. berghei* in young rats (Zuckerman, Hamburger & Spira, 1967).

RÉSUMÉ

La récolte du matériel antigénique destiné aux études immunochimiques requiert l'emploi de techniques minutieuses garantissant le maintien de la structure chimique de l'antigène, l'intégrité des sites de combinaison et la stabilité du produit final aux fins de conservation et d'expédition. Lorsque l'agent infectieux est un organisme intracellulaire, on doit veiller en outre à limiter au maximum la souillure de l'antigène par des résidus de cellules de l'hôte. Le présent travail décrit un procédé d'obtention de plasmodiums de mammifères.

Le sang infecté de plasmodiums est recueilli chez le rongeur (souris) ou chez le singe (*Macaca mulatta* ou *Macaca irus*) par exsanguination en période de forte parasitémie. Chez l'homme, le sang est prélevé par ponction veineuse. Les échantillons, citratés, renfermant une même souche de plasmodium sont réunis en pool.

On procède ensuite à la séparation des érythrocytes et des leucocytes. Lorsqu'il s'agit de sang de rongeurs ou de singes, chez lesquels la parasitémie est généralement élevée et qui fournissent des échantillons volumineux, l'élimination des leucocytes est effectuée après centrifugation du sang. En cas de faible parasitémie (sang humain), la technique est différente: centrifugation, élimination du plasma citraté, mise en suspension dans une solution de dextran à 3,6% de l'ensemble des éléments cellulaires, réfrigération à 4°C pendant 30 minutes. Les érythrocytes se mettent en rouleaux et forment un sédiment, cependant que les leucocytes et les thrombocytes restent en suspension. Après élimination du surnageant et nouveau traitement par le dextran, le culot ne comprend plus que les érythrocytes et une faible proportion (1%) des leucocytes présents à l'origine.

Le 3e stade, libération des parasites des érythrocytes, est identique dans tous les cas. Deux traitements successifs (d'une durée de 10 et 15 minutes) par une solution diluée de saponine détruisent les érythrocytes, libèrent l'hémoglobine et séparent plasmodiums et résidus cellulaires. Les parasites sont recueillis par centrifugation à grande vitesse, après lavages répétés dans le soluté salin tamponné. Le produit final, composé de deux fractions différant par leur contenu en parasites, est lyophilisé sous vide et conservé à −20°C.

Après reconstitution par addition d'eau distillée, cette préparation a fait la preuve de ses qualités antigéniques. On l'a utilisée au cours d'études de diffusion sur gel, d'hémagglutination passive, d'électrophorèse, ainsi que pour l'immunisation de rongeurs.

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