IMMUNOELECTROPHORETIC ANALYSIS OF WATER-SOLUBLE ANTIGENS EXTRACTED FROM PARASITIC BODIES OF Plasmodium berghei SEPARATED FROM THE BLOOD

by

A. Corradetti, F. Verolini, A. Ilardi and A. Bucci
Istituto Superiore di Sanità, Rome, Italy

INTRODUCTION

The antigenic analysis of Plasmodium berghei was recently investigated by immunoelectrophoretic methods (Spira & Zuckerman, 1962; Zuckerman, 1964; Banki & Bucci, 1964; Diggs, 1964; Zuckerman & Spira, 1964).

The extracts of parasites employed in all these experiments were obtained by saponin haemolysis, centrifugation and removal of the layer containing the leucocytes.

This method does not eliminate contamination from proteins coming from the host cells. The centrifugation alone does not produce complete separation of the leucocytes in the supernatant layer: a certain percentage of white cells are found also in the lower layers after centrifugation. Moreover the mechanical removal of the supernatant layer, containing the leucocytes, very often produces movements in the liquid which give rise to contamination with leucocytes of the layers placed immediately below.

We have recently developed and fully described a method by which the parasitic bodies are obtained entirely free from any contaminating material (Corradetti et al., 1964). The method consists of saponin haemolysis and

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filtering through millipore membranes SM 5 micron. It has been also very recently
tested by Bray (1965) and employed with slight modifications by Brown et al. (1966).

Having obtained by this method an antigen containing pure parasitic proteins of
*P. berghei*, it was natural to get from it immunesera in rabbits and to submit the
antigen to immunoelectrophoretic analysis which could be interpreted with certainty as
derived from proteins belonging to the parasite.

In the course of the present investigations we improved our old technique and
tested its value by controlling the immunesera with antigens of the rat’s blood
components, and by the comparison of the immunoelectrophoretic results with those
obtained with less purified antigens.

**TECHNIQUE**

**Preparation of the antigen**

In our previous investigations we had employed millipore filters SM 5 micron
47 mm. However, this filtering surface (9.6 cm²) was too small, as blockage of the
filter occurred too early, the aqueous extract had a density in proteins of only
2-3 mg per cc, and its injection into rabbits produced only a few weak antibodies.

With filters millipore SM 5 micron 142 mm with a filtering surface of 97 cm², the
filtrage was much easier, and we could obtain an aqueous extract of parasites, the
protein content of which, determined by the method of Folin-Ciocalteau-Lawry, varied
between 8 and 12 mg per cc.

The new method of preparation of the antigen in these experiments shows some
modifications from that previously employed.

Groups of 25-30 rats, three to four months old, infected with *P. berghei*, were
killed when the parasites had invaded at least 60 per cent. of the red cells. The
blood was taken aseptically from the heart. The total amount of blood obtained from
each group varied between 60 and 90 cc.

The citrated blood was centrifuged at 2500 rev/min for five minutes. The plasma
was removed and the sediment washed three times in phosphate buffered physiological
solution at pH 7.2. The sediment was then diluted in the proportion of 1/40 with a
solution of saponin at 1/10 000 and maintained at 37°C for 20 minutes. After
haemolysis the liquid was filtered through millipore membranes SM 5 micron 142 mm.
The filtrate was centrifuged at 9000 rev/min for three minutes and then washed twice
in phosphate buffered physiological solution at pH 7.2. The sediment, composed of
parasites only, was introduced in a container of a homogenizer Vir Tis 45, mixed with
quartz powder. The material was homogenized at 40 000 rev/min for 15 minutes; and
then centrifuged at 5000 rev/min for 10 minutes. The supernatant liquid was the
water-soluble antigen of P. berghei: its volume was about 0.2-0.3 cc, and its protein
content 8-12 mg per cc.

Control antigens

(1) Citrated plasma was employed, after centrifugation and elimination of the
sediment, as control antigen.

(2) The haemoglobin was obtained from rat erythrocytes washed three times in
physiological solution and haemolized with two volumes of distilled water for 15
minutes. After the haemolysis NaCl was added up to the concentration of 0.9 per
cent.; then the liquid was centrifuged at 20 000 rev/min for 10 minutes and the
supernatant fluid was employed as antigen.

(3) The erythrocytes, after having been washed in physiological solution three
times, were haemolized with saponin. The suspension was centrifuged at 3000
rev/min for five minutes to sediment and remove the leucocytes. The supernatant
fluid was centrifuged at 20 000 rev/min for 20 minutes. The sediment, containing
the stromata of the erythrocytes, was washed twice in phosphate buffered physio-
logical solution at pH 7.2. After washing, the sediment was homogenized with
quartz powder in Vir Tis at 40 000 rev/min for 15 minutes and then centrifuged at
7000 rev/min for five minutes. The water-soluble extract obtained, the protein
content of which was 6.5 mg per cc, was employed as antigen.

(4) The citrated blood was centrifuged at 2000 rev/min for 10 minutes. The
plasma was removed and the sediment washed three times with phosphate buffered
physiological solution at pH 7.2. The sediment was then haemolized with 40
volumes of saponin 1/10 000 for 20 minutes. The liquid was centrifuged at 300
rev/min for 10 minutes. The sediment, constituted by leucocytes, was washed
three times in phosphate buffered physiological solution at pH 7.2, and then homogenized with a small amount of quartz powder at 40 000 rev/min for 15 minutes. The homogenate was centrifuged at 5000 rev/min for 10 minutes. The supernatant liquid, the protein content of which was 9.34 mg per cc, provided the antigen.

**Preparation of the immunesera in rabbits**

The following method was used for immunizing rabbits:

1. subcutaneous inoculation in the rabbit nuchal area of 0.1 cc of *P. berghei* antigen mixed with an equal volume of Freund adjuvant;
2. subcutaneous inoculation, after 15 days, of 0.2 cc of antigen mixed with 0.2 cc of adjuvant;
3. subcutaneous inoculation, after another 15 days, of 0.35 cc of antigen mixed with 0.35 cc of adjuvant;
4. intravenous inoculation after another 21 days, of 0.2 cc of antigen alone.

Bleeding of the rabbits was made a week after the last inoculation.

**Immunoelectrophoresis**

Immunoelectrophoresis was performed with the apparatus for micro-immunoelectrophoresis LKB on agar gel (Difco Special Noble Agar) in veronal buffer salts (0.05 M sodium diethyl-barbiturate, 0.01 M diethyl-barbituric acid, 0.05 M sodium acetate) at pH 8.6 and ionic strength 0.1, by following the procedure of Scheidegger.

Two microlitres of antigen, placed in the central well of the glass slide, were submitted to electrophoretic migration for 45 minutes at 6 V/cc. At the end of the electrophoretic migration the immuneserum was placed in lateral throughs and diffusion was allowed for 48 hours. In each analysis the same amount of antigen (two microlitres) reacted with three different quantities of immuneserum: 50, 100 and 150 microlitres respectively. The precipitation arcs were most evident in the reaction with 150 microlitres of immuneserum.

After the reaction the glass slides were washed with physiological solution for 48 hours, then dried at room temperature for 24 hours. The slides were finally stained with black amid at 9/1000 in 10 per cent. acetic acid, 45 per cent. methyl alcohol and 45 per cent. distilled water.
RESULTS

1. Results of immunoelectrophoretic experiments with the pure water-soluble antigen

The inoculation of the water-soluble antigen, prepared as described above, produced in the immunesera of four rabbits a maximum of nine precipitating systems (each one corresponding to a parasite protein), which were revealed through the immunoelectrophoretic procedure on agar gel (Fig. 1).

The population of heterogeneous proteins which constituted the parasite aqueous extract, placed on agar gel for 45 minutes at 6 V/cc, demonstrated various speeds of migration which gave rise, at the end of the electrophoresis, to three distinct areas of precipitation.

The first area was placed near the anode and included the proteins with higher speed of migration. Four precipitating systems, demonstrated by distinct arcs of precipitation, were observed in this area: they revealed the anodic fraction of the proteins in the antigen.

Two precipitating systems appeared in the second area near the cathode, revealing the proteins with lower speed of migration: they constitute the cathodic fraction of proteins in the antigen.

The third area was situated in proximity of the well of the glass slide. Three arcs of precipitation, belonging to the intermediate fraction of proteins in the antigen, appeared in this area.

This immunoelectrophoretic picture remained uniform in all the tests performed by employing the immunesera of the four rabbits. Obviously some of the precipitating systems appeared more or less evident or even absent with the different immunesera, depending on the ability to produce antibodies shown by the respective rabbit.

The precipitating antibodies, corresponding to the various constituents of the antigen, were produced at different times in the immunesera.

The first to appear during the process of immunization in the rabbits were one antibody of the anodic fraction and one of the cathodic. These two antibodies were detected from the 25th day after the beginning of the immunization, and were constantly present in the immunesera of all the immunized rabbits.
During the subsequent course of the process of immunization there appeared the other three antibodies corresponding to the anodic, and the second antibody corresponding to the cathodic fraction of proteins.

The last three antibodies corresponding to proteins of the intermediate fraction appeared late in the serum and in some rabbits were very weak or absent.

The absence of contamination of the antigen with chemical or cellular components of the blood of the rat was confirmed by testing the immunesera with the control antigens prepared with the various components of the rat's blood. Immunelectrophoresis of the immunesera placed in contact with these control antigens did not show any arc of precipitation.

2. Comparison of the preceding results with those obtained with less purified antigens

Experiments were also conducted with a less purified antigen. This antigen was extracted from parasites separated from the suspension in saponin through filters millipore SC 8 micron. The amount of parasites obtained was obviously higher in comparison to that obtained with millipore filters SM 5 micron, and the content in proteins of water-soluble extract varied from 12 to 18 mg.

This antigen was inoculated in four rabbits following the method of immunization described above. The immunesera obtained from these rabbits were tested with the antigen through the immunelectrophoretic technique: the reaction revealed nine precipitating systems identical to those obtained with the antigen separated through millipore membranes SM 5 micron. But the reactions of the immunesera with the control antigens demonstrated an arc of precipitation corresponding to an antibody against the leucocytes of the rat: this arc was localized in the anodic area of migration of the extract of leucocytes.

DISCUSSION AND CONCLUSIONS

In the present experiments with immunelectrophoresis on mammal plasmodia (P. berghei) a technique has been introduced for obtaining pure parasitic antigen by the means of filtration through millipore SM 5 micron membranes.
By this method it has been possible, for the first time, to immunize rabbits with proteins belonging only to the parasites, with the consequent production of strictly specific antibodies in the immunesera.

The present experiments have shown that even when the saponin suspension of the blood is filtered through millipore membranes SC 8 micron, contamination with heterogeneous proteins coming from leucocytes is not eliminated, and that antileuocytic antibodies in the rabbit's immunesera are produced.

Nine precipitating systems were observed in the immunoelectrophoresis experiments described: these revealed four species of proteins of P. berghei in the anodic, two in the cathodic, and three in the intermediate fraction of the antigen.

The immunoelectrophoretic pattern of P. berghei obtained in the present investigations, being the result of the reactions between purely parasitic antigen and strictly specific antibodies, appears to be more reliable than those previously obtained with less purified antigens. Some of the differences observed between the results of Banki & Bucci (1964) and those of Zuckerman & Spira (1964) could be attributed not only to the factors quoted by Banki & Bucci (1964) but also to differences in the purification of the antigens involved.
RESUME

Cette étude décrit une technique perfectionnée pour l'extraction d'antigènes contenant des protéines parasitaires purées de *Plasmodium berghei* totalement dépourvues de toute impureté provenant du sang de l'hôte.

 Après filtration des parasites à travers des membranes Millipores SM 5 microns, les auteurs ont comparé la qualité de l'antigène ainsi préparé et la constance de son degré de pureté en procédant à l'immunisation de lapins et au contrôle des sérum par immuno-électrophorèse avec des antigènes préparés à partir de composants sanguins de rats.

 En comparant les résultats obtenus au cours de ces contrôles avec ceux résultant de l'utilisation d'antigènes d'un moindre degré de pureté, il apparaît que le recours à l'antigène pur s'impose pour les expériences d'immuno-électrophorèse et qu'il fournit des résultats d'une haute fidélité.

 Les expériences d'immuno-électrophorèse ont révélé neuf systèmes précipitants dans la réaction de cet antigène pur extrait de *P. berghei* avec les anticorps dont il suscite la formation dans le sérum du lapin. Quatre systèmes précipitants se trouvaient dans la fraction anodique, deux dans la fraction cathodique et trois dans la fraction intermédiaire de l'antigène.
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FIG. 1

IMMUNOELECTOPHORETIC PATTERN SHOWING PRECIPITATION ARCS OF PURE P. BERGHEI ANTIGEN SUBJECTED TO RABBIT ANTI SERA
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