further strains in studies of this kind may lead to the conclusion that antigenic variation among these viruses is a continuous process without sharp distinctions allowing the separation of subtypes.

However, for practical purposes, an arbitrary distinction of antigenic groupings may be of some use in the routine identification of isolates. Of particular value in this respect would be a system based on procedures kept as simple as possible. For this purpose a classification based exclusively on the results of haemagglutination inhibition, which is undoubtedly the test most commonly used in the study of influenza viruses, would divide the strains investigated so far into the following antigenic groupings:

(1) fowl plague and Turkey/England/63;

(2) virus N (and some isolates from Italy still under study);

(3) Duck/England/56;

(4) Duck/Czechoslovakia/56 and Duck/England/62;

(5) Chicken/Scotland/59, Tern/South Africa/61 (and some isolates from Canada still under study);

(6) Turkey/Canada/63, Turkey/Massachussetts/65 and Turkey/Wisconsin/65.

It must be pointed out, however, that this scheme applies only when post-infection avian sera are used. With hyperimmune sera prepared in rabbits or other mammals, a number of additional cross-reactions become apparent.

The isolation of the three turkey strains of group 6 from turkey flocks distant in time and location points to a wide dissemination of the infection by this particular serotype of influenza A viruses. The infection has been, so far, diagnosed only in turkeys, where it is responsible essentially for a mild upper respiratory disease. Information received (C.C. Wannop, personal communication) indicates that the infection is not restricted to the North American continent but exists also in Europe. The fact that none of the isolates was clearly identifiable with any of the other strains makes it unlikely that the outbreaks result from the direct transmission of the virus originating from a common enzootic focus. It seems rather that this is a newly recognized infection which has been, and still is, circulating in the turkey population.

Immunoelectrophoretic Analysis of Water-soluble Antigens Extracted from Parasitic Bodies of *Plasmodium berghei* Separated from the Blood

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The antigenic analysis of *Plasmodium berghei* was recently investigated by immunoelectrophoretic methods.[a-e] 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 with proteins 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 leucocyte contamination in the layers immediately below.

We have recently developed and fully described a method by which the parasitic bodies are obtained entirely free from any contaminating material.[f] The method consists of saponin haemolysis and filtration through Millipore SM membranes (5-μ mesh). It has

[a] Spira, D. & Zuckerman, A. (1962) *Science*, **137**, 536.

[b] Zuckerman, A. (1964) *Amer. J. trop. Med. Hyg.* **13**, Suppl., p. 209.

[c] Banki, G. & Bucci, A. (1964) *Parassitologia*, **6**, 251.

[d] Diggs, C. L. (1964) *J. Parasit.*, **50**, Suppl., p. 17.

[e] Zuckerman, A. & Spira, D. (1964) *Immunoelectrophoretic comparison of plasmodial antigens.* In: *Proceedings of the First International Congress of Parasitology, Rome*, London and Milan, Pergamon and Tamburini, vol. 1, p. 130.

[f] Corradetti, A., Verolini, F. & Ilardi, A. (1964) *Parassitologia*, **6**, 279.

also been very recently tested by Bray [g] and employed with slight modifications by Brown et al.[h]

Since this method gives an antigen containing pure parasitic proteins of *P. berghei*, it was natural to use the antigen to produce immune sera in rabbits, and to submit the antigen to immunoelectrophoretic analysis which could be interpreted with certainty as being due to proteins belonging to the parasite.

In the course of the present investigations we improved our old technique and tested its value by checking the immune sera with antigens of rat-blood components, and by the comparison of the immunoelectrophoretic results with those obtained with less highly purified antigens.

Technique

Preparation of antigen. In our previous investigations we had employed 47-mm Millipore SM filters. However, the filtering surface (9.6 cm^2) was too small, as blockage of the filter occurred too early, the aqueous extract had a protein density of only 2-3 mg/ml, and its injection into rabbits produced only a few weak antibodies.

With 142-mm Millipore SM filters with a filtering surface of 97 cm^2, filtration 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 mg/ml and 12 mg/ml.

The new method of preparation of the antigen in these experiments differs from that previously employed in certain respects.

Groups of 25-30 rats aged 3-4 months, infected with *P. berghei*, were killed when the parasites had invaded at least 60% of the red cells. The blood was taken aseptically from the heart. The total amount of blood obtained from each group varied between 60 ml and 90 ml.

The citrated blood was centrifuged at 2500 rev/min for 5 minutes. The plasma was removed and the sediment washed 3 times in phosphate-buffered physiological solution at pH 7.2. The sediment was then diluted 1/40 with a 0.01% solution of saponin and maintained at 37°C for 20 minutes. After haemolysis the liquid was filtered through 142-mm Millipore SM membranes. The filtrate was centrifuged at 9000 rev/min for 3 minutes and then washed twice in phosphate-buffered physiological solution at pH 7.2. The sediment, composed of parasites only, was mixed with quartz powder and homogenized in a Vir Tis 45 homogenizer 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 ml, and its protein content 8-12 mg/ml.

Control antigens. Citrated plasma was employed, after centrifugation and elimination of the sediment, as control antigen.

The haemoglobin was obtained from rat erythrocytes washed 3 times in physiological solution and haemolysed with 2 parts by volume of distilled water for 15 minutes. After haemolysis, NaCl was added to a concentration of 0.9%; the liquid was then centrifuged at 20 000 rev/min for 10 minutes and the supernatant fluid employed as antigen.

The erythrocytes, after having been washed in physiological solution 3 times, were haemolysed with saponin. The suspension was centrifuged at 3000 rev/min for 5 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 physiological solution at pH 7.2. After washing, the sediment was homogenized with quartz powder in a Vir Tis at 40 000 rev/min for 15 minutes and then centrifuged at 7000 rev/min for 5 minutes. The water-soluble extract obtained, the protein content of which was 6.5 mg/ml, was employed as antigen.

The citrated blood was centrifuged at 2000 rev/min for 10 minutes. The plasma was removed and the sediment washed 3 times with phosphate-buffered physiological solution at pH 7.2. The sediment was then haemolysed with 40 volumes of 0.01% saponin for 20 minutes. The liquid was centrifuged at 300 rev/min for 10 minutes. The sediment, composed of leucocytes, was washed 3 times in phosphate-buffered physiological solution at pH 7.2 and 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/ml, provided the antigen.

Preparation of immune sera in rabbits. The following method was used for immunizing rabbits: (*a*) subcutaneous inoculation of the rabbit in the nuchal area with 0.1 ml of *P. berghei* antigen mixed with an equal volume of Freund adjuvant; (*b*) subcutaneous inoculation, after 15 days, of 0.2 ml of antigen mixed with 0.2 ml of adjuvant; (*c*) subcutaneous

[g] Bray, R. S. (1965) *Ann. Soc. belge Méd. trop.*, **45**, 397.

[h] Brown, I. N., Brown, K. N. & Hillis, L. A. (1966) *Trans. roy. Soc. trop. Med. Hyg.*, **60**, 3.

inoculation, after another 15 days, of 0.35 ml of antigen mixed with 0.35 ml of adjuvant; (*d*) intravenous inoculation, after another 21 days, of 0.2 ml of antigen alone. The rabbits were bled a week after the last inoculation.

Immunoelectrophoresis. Immunoelectrophoresis was performed with the LKB apparatus for micro-immunoelectrophoresis 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 the procedure of Scheidegger.

Two microlitres of antigen, placed in the central well of the glass slide, were submitted to electrophoresis for 45 minutes at 6 V/ml. At the end of the electrophoresis the immune serum was placed in lateral troughs and allowed to diffuse for 48 hours. In each analysis the same amount of antigen (2μl) reacted with three different quantities of immune serum: 50 μl, 100 μl and 150 μl. The precipitation arcs were most evident in the reaction with 150 μl of immune serum.

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 0.9% Amido Black 10B in 10% acetic acid, 45% methyl alcohol and 45% distilled water.

Results

Results of immunoelectrophoretic experiments with pure water-soluble antigen. The inoculation of the water-soluble antigen prepared as described above produced in the immune sera 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 (see the accompanying figure).

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

The first area, near the anode, included the proteins with high migration speeds. Four precipitating systems, demonstrated by distinct precipitation arcs, were observed in this area: they constitute the *anodic fraction* of the proteins in the antigen.

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

The third area, near the well in the glass slide, contained three arcs of precipitation, belonging to the *intermediate fraction* of proteins in the antigen.

This immunoelectrophoretic picture remained uniform in all the tests employing the immune sera of the four rabbits. Naturally, some of the precipitating systems were more or less evident, or even absent, with the different immune sera, depending on the ability to produce antibodies shown by the rabbit in question.

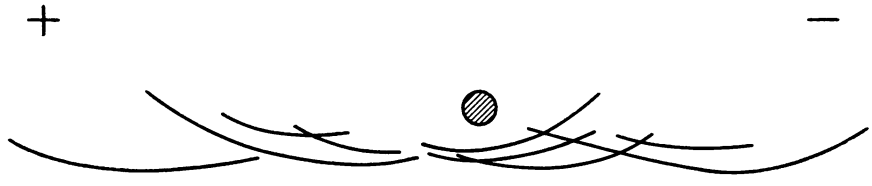
The precipitating antibodies coresponding to the various constituents of the antigen were produced at different times in the immune sera. The first to appear during the immunization process in the rabbits were one antibody of the anodic fraction and one of the cathodic. These two antibodies were detected from the twenty-fith day after inoculation, and were constantly present in the immune sera of all the immunized rabbits.

The other three antibodies corersponding to the anodic, and the second antibody corresponding to the cathodic, fraction of proteins appeared later in the immunization process.

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

The fact that the antigen was not contaminated with chemical or cellular components of rat blood was confirmed by testing the immune sera with the

IMMUNOELECTROPHORETIC PATTERN SHOWING PRECIPITATION ARCS OF PURE *P. BERGHEI* ANTIGEN SUBJECTED TO RABBIT ANTISERA



control antigens prepared with the various components of rat blood. Immunoelectrophoresis of the immune sera did not show any precipitation arc with these control antigens.

Comparison of preceding results with those obtained with less highly purified antigens. Experiments were also conducted with a less highly purified antigen. This antigen was extracted from parasites separated from the suspension in saponin through Millipore SC filters (8-μ mesh). The amount of parasites obtained was obviously higher than that obtained with 5-μ filters, and the protein content of the water-soluble extract varied from 12 mg/ml to 18 mg/ml.

This antigen was inoculated into four rabbits following the method of immunization described above. The immune sera obtained from these rabbits were tested with the antigen by the immunoelectrophoretic technique: the reaction revealed nine precipitating systems identical with those obtained with the antigen separated through Millipore 5-μ membranes, and one precipitation arc in the anodic area, corresponding to an antibody against the leucocytes of the rat.

Discussion and conclusions

In the present experiments on immunoelectrophoresis of mammalian plasmodia (*P. berghei*) a technique has been introduced for obtaining pure parasitic antigen by means of filtration through Millipore SM (5-μ) membranes.

This method has made it 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 immune sera.

The present experiments have shown that even when the saponin suspension of the blood is filtered through Millipore SC (8-μ) membranes, contamination with heterogeneous proteins from leucocytes is not eliminated, and that antileucocytic antibodies are produced in the rabbit's immune sera.

Nine precipitating systems were observed in the immunoelectrophoresis experiments described, corresponding to 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 highly purified antigens. Some of the differences observed between the results of Banki & Bucci [c] and those of Zuckerman & Spira [e] could be attributed not only to the factors quoted by Banki & Bucci [c] but also to differences in the purification of the antigens involved.

Studies on Transmission of Simian Malaria and on a Natural Infection of Man with *Plasmodium simium* in Brazil*

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For the past two years we have been studying simian malaria in Brazil. A station was established in a forest reservation—Horto Florestal da Cantareira—in the outskirts of the city of São Paulo, and observations have also been made in the State of Santa Catarina. Both places are in the coastal forested mountains of southern Brazil where howler monkeys (*Alouatta fusca*) are numerous while other primates, such as capuchin monkeys (*Cebus apella*) and marmosets (*Callithrix aurita*), are less common. During our studies, malaria parasites of at least two species, *Plasmodium simium* and *P. brasilianum*, have been frequently found in *A. fusca* and never in the other species.

Transmission studies were concentrated in the Horto Florestal station, where human malaria had never been detected. Since howlers are arboreal and

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