SEROLOGICAL CROSS-REACTION BETWEEN RODENT MALARIA PARASITES
AS DETERMINED BY THE INDIRECT IMMUNOFLUORESCENT TECHNIQUE

by

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The work of Coons et al. (1941) in attaching a fluorescent label to serum globulin, and the application of the fluorescent antibody technique (FAT) by Goldman (1953, 1954) to protozoa, opened a wide field in the study of these organisms. In addition to its use in differentiating between different species of parasites and in following the antibody level after an infection, the FAT was found most useful in studies on antigenic relationships of related species of protozoa. Goldman (1953, 1954) used fluorescein-tagged antibody to identify cultures of Entamoeba histolytica and E. coli. Later, Goldman (1960) used microfluorimetry to detect small differences in fluorescence intensity of the Entamoebae. McIntegart et al. (1958) demonstrated wide antigenic differences between Trichomonas vaginalis and T. foetus using the direct technique of staining.

Fulton & Voller (1964) found that the FAT was specific for Toxoplasm and there was no cross-reaction with sera from cases of trypanosomiasis, malaria, kala-azar, sarcosporidiosis, leptospirosis, syphilis, schistosomiasis and filariasis.

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Corradetti et al. (1964) used sera from rabbits immune to *Plasmodium gallinaceum* sporozoites, to stain sporozoites of *P. gallinaceum* but not sporozoites of *P. givovonola*.

Sodeman & Jeffery (1964) stained sporozoites of *P. gallinaceum* using fluorescein-conjugated immune chicken serum (after recovery from infections with parasitized blood forms) but not with fluorescein-conjugated antiserum against *P. vivax*, *P. falciparum*, .... and *P. cynomolgi*.

In a study on the serological cross-reactions in human and simian malaria, Tobie et al. (1962, 1963) tested sera from volunteers infected with *P. cynomolgi* against the homologous parasite and Chesson and Venezuelan strains of *P. vivax*. Higher antibody titres were obtained when the sera were allowed to react with *P. cynomolgi* than when allowed to react with either strain of *P. vivax*. At the same time, sera from volunteers infected with either strain of *P. vivax* gave essentially the same titres whether *P. cynomolgi* or *P. vivax* parasites were used as antigens. It was impossible to differentiate between the two strains of *P. vivax*. Collins et al. (1965) found considerable cross-reactions when sera of infected monkeys were tested against several species of simian malaria parasites. Also when these sera were tested against *P. vivax*, *P. falciparum*, *P. ovale* and even *P. gallinaceum*, cross-reactions occurred. They concluded that cross-reactions occurred to some extent among all species of the genus and in many cases were unassociated with the morphological or life-pattern relationships. This is partly in contrast to Voller (1963) who found no cross-reactions between avian and mammalian malaria parasites, though finding strong cross-reactions between simian and human malaria parasites. The same result was obtained by Diggs & Sadun (1965) who concluded that both a species-specific and a group-specific component existed in *P. vivax* and *P. falciparum*.

The present work was undertaken in order to study the serological cross-reactions between rodent malaria parasites. Knowing the morphological similarity between the blood stages of *P. berghei berghei* and *P. berghei yoelii* on the one hand, and between *P. vinckei* and *P. chabaudi* on the other hand (Killick-Kendrick, Landau & Garnham, in press), it was hoped that the FAT might help to differentiate between these parasites.
MATERIALS AND METHODS

Strains of parasites. The following strains were used: P. berghei berghei (NK65 strain); P. berghei yoeli (17X strain); P. chabaudi (54 strain); P. vinckei (Adler strain).

P. chabaudi and P. vinckei were adapted to young white rats, three to four weeks old, before infecting the experimental animals.

Immune sera. The immune sera used were obtained from white rats recovered from and hypoinmunized against the four plasmodia, from rabbits immunized against the soluble antigens of the blood stages of the four parasites and from a rabbit immunized against the sporozoites of P. berghei yoelli.

1. Rat immune sera. To start with, four- to five-month-old rats were infected by the intraperitoneal inoculation of parasitized rat erythrocytes in the case of P. b. berghei and P. b. yoelli, and three- to four-week-old rats in the case of P. chabaudi and P. vinckei. One month later, the rats were challenged three times with the homologous strain, with a two-week interval. Ten days after the last challenge, the rats were bled and sera obtained from every five rats were pooled.

2. Rabbit immune sera to the soluble antigens of the blood stages. The technique used to prepare the soluble antigens was that of Spira & Zuckerman (1962), with some modifications. The blood was taken from rats at the peak of the primary parasitaemia, washed in phosphate-buffered saline (PBS) and the white cells removed by settling for one hour in 11% dextran in PBS (M.W. 60 000-90 000). The parasites were released by zaponin and washed four times in PBS. The deposit was resuspended in five times its volume of distilled water and lyophilized. The powder was ground in a mortar and the ground material was taken up again in 20-30 volumes distilled water and left overnight at 4°C to extract the soluble antigens. Following centrifugation at 11 000 rev./min., the supernatant was lyophilized, and the powder which contained the soluble antigens of the blood forms was used for immunizing rabbits. The rabbits were immunized according to the following schedule:
<table>
<thead>
<tr>
<th>Day</th>
<th>Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 mg antigen + 0.5 ml distilled water + 0.5 ml incomplete Freund's adjuvant, injected in the foot pads</td>
</tr>
<tr>
<td>7</td>
<td>5 mg antigen + 0.5 ml distilled water, injected subcutaneously</td>
</tr>
<tr>
<td>14</td>
<td>as day 1</td>
</tr>
<tr>
<td>21</td>
<td>as day 7</td>
</tr>
</tbody>
</table>

Two weeks after the last injection, the animals were bled and the sera separated. Two rabbits were immunized against each parasite, but one of those immunized against *P. vinckei* died.

3. **Rabbit immune serum to sporozoites of *P. b. yoelii***. Infected salivary glands of *Anopheles stephensi* were crushed in a tissue homogenizer with normal saline. A rabbit was immunized according to the following schedule:

<table>
<thead>
<tr>
<th>Day</th>
<th>Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 volume of sporozoite suspension (from 200 mosquitoes) + 1 volume incomplete Freund's adjuvant, injected into the hind foot pads</td>
</tr>
<tr>
<td>14</td>
<td>as day 1</td>
</tr>
<tr>
<td>28</td>
<td>sporozoite suspension of 150 mosquitoes + the same volume of incomplete Freund's adjuvant, injected intradermally</td>
</tr>
<tr>
<td>42</td>
<td>the same as day 28, but without Freund's adjuvant</td>
</tr>
</tbody>
</table>

Serum was separated two weeks after the last injection.

**Fluorescent antibody test**: Slide antigens were obtained from rats during the period of rising parasitaemia. The slides were fixed in N/3 HCl.

Every serum was titrated, in twofold dilutions starting at 1:5, against the homologous and the heterologous antigen. The technique used for staining was that described by El-Nahal & Bray (1966) in which the preparations, after staining with the indirect FAT, were stained with 0.1% Evans blue in PBS for 10 minutes and then given
two quick washings with acetone to remove excess of Evans blue. After washing for five minutes in PBS, the slides were examined. Normal sera always gave negative results, while the titre of the immune ones did not change. (Evans blue is known to decrease the intensity of non-specific fluorescence without affecting the specific fluorescence.)

It was observed that rabbit sera immune to the soluble antigens gave specific fluorescence of infected and non-infected rat erythrocytes. The whole surface of all the erythrocytes fluoresced. This result was attributed to one of two possibilities: either to the presence of remnants of red cells in the antigen or, more probably, to the release of undigested haemoglobin, found in the food vacuoles of the parasites, by grinding and lyophilization. It was essential to absorb these sera with normal rat erythrocytes in order to remove this fluorescence. Addition of complement during absorption, caused haemolysis of the rat erythrocytes (indicating the presence of haemolysins) and the release of haemoglobin which absorbed the unwanted antibodies.

RESULTS

The reciprocal titres of each serum, titrated against the homologous and heterologous antigens, are shown in Table 1. The results of rat sera are also illustrated in Figs. 1-4.

Fig. 1 shows the results of four rat sera immune to P. b. berghei titrated against the four parasites. Cross-reaction occurred in all four antigens. It is clear that higher titres were obtained when the sera were titrated against P. b. berghei and P. b. yoelii than when titrated against P. chabaudi and P. vinckei. It is also evident that the same titre was obtained for each serum when the two subspecies of P. berghei antigens were used; and very nearly the same titre was obtained for each serum when P. chabaudi and P. vinckei were used as antigens.

Fig. 2 shows the titres obtained with four rat sera immune to P. b. yoelii, with similar results.
The antibody levels of four rat sera immune to *P. vinckeii* are shown in Fig. 3. They behave similarly but this time higher titres were obtained when they were titrated against *P. vinckeii* and *P. chabaudi* than when they were titrated against *P. b. berghei* and *P. b. yoelii*. Similar results were obtained with two rat sera immune to *P. chabaudi* (Fig. 4).

Rabbit sera immune to the soluble antigens of the blood stages of the four parasites, and that immune to sporozoites of *P. b. yoelii*, gave exactly similar results, as described. Control normal rat and rabbit sera always gave negative reactions.

**DISCUSSION AND CONCLUSIONS**

It is interesting to note that rabbit sera immunized against sporozoites of *P. b. yoelii* stained the erythrocytic forms of the four rodent malaria parasites indicating common antigens between sporozoites and blood forms.

From the results shown in Table 1, we can conclude that there is a definite cross-reaction between the four rodent parasites indicating common antigens. Also using the FAT, no serological difference could be detected between *P. b. berghei* and *P. b. yoelii* both forming one group - Group 1 or *berghei* group. Similarly, there was no serological difference between *P. vinckeii* and *P. chabaudi*, which form another group - Group 2 or *vinckeii* group. On the other hand, there was a definite serological difference between Group 1 and Group 2 infections. Higher titre Group 1 antiserum gave lower titres with Group 2 antigens, and vice versa. It was thus impossible to differentiate by the FAT the morphologically similar parasites in each group.

The presence of a cross-reaction between rodent malaria parasites was expected from previous results obtained by other workers on rodent and other plasmodia. Voller (1965) found a cross-reaction between *P. berghei* and *P. vinckeii* using the indirect FAT, but none between *P. berghei* and the two avian malaria parasites, *P. gallinaceum* and *P. juxtanucleare*, and only a slight cross-reaction between *P. berghei* and several primate malaria parasites. In a comparison between plasmodial antigens by immune-electrophoresis, Zuckerman & Spira (1964) found four shared antigens between *P. berghei* and *P. vinckeii*, whilst each had, respectively, 1 and 2 specific bands, and none between *P. berghei* and *P. gallinaceum*. Banki & Bucci (1964) studied the antigenic structure
of *P. cynomolgi* and *P. berghei*, using the Ouchterlony and the immuno-electrophoresis techniques, and showed a band of "partial identity" and the presence of common fractions between the two plasmodia. Stein & Desowitz (1964) used *P. berghei* antigen to detect human malarial infections by the indirect haemagglutination technique, but at lower titres than those obtained with *P. cynomolgi*, *P. vivax* or *P. coatneyi* antigens.

The distinction between the two groups of rodent malaria parasites has been recently demonstrated by Bray & El-Nahal (1966) by the indirect haemagglutination test. Sera immune to *P. vinckei* and *P. chabaudi* failed to react with sensitized tanned sheep erythrocytes, whether or not the homologous or heterologous rodent plasmodial antigen was used, while sera immune to *P. b. berghei* and *P. b. yoelii* were always active in the test.

It is worth comparing the results of cross-immunity between rodent malaria parasites studied by Cox & Voller (1966) with the serological results reported here. Working on the same strains as in the present work, they found a cross-protection between *P. b. berghei* and *P. b. yoelii* but this did not extend to *P. chabaudi* and *P. vinckei* which similarly protected against each other. They confirmed the close relationships of *P. b. berghei* with *P. b. yoelii* and *P. chabaudi* with *P. vinckei* respectively and stressed the immunological differences between the two groups. Such close correspondence between the fluorescent antibody results in the present work and the actual functional cross-immunity in Cox & Voller's work is not always the case. Voller et al. (1966) have recently shown no cross-immunity between *P. cynomolgi bastlianelli* and *P. o. ceylonensis*, although they cross-react with identical titres in the FAT.

The antigenic structure of the four rodent malaria parasites, as detected by the FAT, is theoretically illustrated in Fig. 5. We can assume that the four parasites contain a common or "basic" antigen or antigens that could be detected by the FAT, using one of the heterologous group antisera. *P. b. berghei* and *P. b. yoelii* contain a "special" antigen or antigens not present in the other two parasites. Similarly, *P. vinckei* and *P. chabaudi* contain a "special" antigen or antigens shared only by them. These "special" antigens could be detected by the FAT using one of the homologous
group antisera. In addition, presumably, each parasite might contain its own "specific" antigen or antigens, which is probably a complex one containing species, subspecies, strains or even the variant specific components described by Brown & Brown (1965) in P. knowlesi. So far the "specific" antigen (or antigens) has not been detected by the FAT.

SUMMARY

Sera were obtained from three sources: (1) from rats hyperimmunized against P. berghei berghei, P. b. yoelii, P. chabaudi and P. vinckei; (2) from rabbits immunized against the soluble antigens of the blood stages of these parasites; and (3) from a rabbit immunized against the sporozoites of P. b. yoelii. The titre of each serum was determined by its reaction with the homologous and the heterologous antigen, using a modified indirect immunofluorescent technique. The results showed:

(1) a definite cross-reaction between the four parasites, indicating their possession of a common antigen;

(2) no serological difference between P. b. berghei and P. b. yoelii (these two parasites are referred to as Group 1 or berghei group);

(3) no serological difference between P. chabaudi and P. vinckei (these parasites are referred to as Group 2 or vinckei group).

(4) a definite serological difference between Group 1 infections and Group 2 infections. High-titre Group 1 antiserum gave much lower titres with Group 2 antigen, and vice versa.

The antigenic structure of rodent plasmodia is theoretically discussed on the basis of these observations.
ACKNOWLEDGEMENTS

I would like to express my deep gratitude to Professor P. C. C. Garnham, under whose supervision this work was done, for the keen interest he has shown and for his very valuable advice.

My sincere thanks are due to Dr R. S. Bray, Dr M. Wery and Dr J. R. Baker for all the help they offered. I am also grateful to Mr R. Killick-Kendrick and his staff for the extremely helpful technical assistance.

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<td>P. vinckeii</td>
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<tr>
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<tr>
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<td>40</td>
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<td>II 80</td>
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<tr>
<td>P. vinckeii s.a.*</td>
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<td>80</td>
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<td>P. b. yoelii sporoz.</td>
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<td>Control</td>
<td>II &lt;5</td>
<td>&lt;5</td>
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*s.a. - soluble antigen.*
RESUME

Les travaux qui font l'objet de la présente étude ont été entrepris en vue d'examiner les réactions sérologiques croisées entre les parasites du paludisme chez les rongeurs. Étant donné la ressemblance morphologique entre les stades érythrocytaires de *Plasmodium berghei berghei* et *P. berghei yoelii* d'une part, et entre *P. vinckei* et *P. chabaudi* d'autre part, on espérait que la technique des anticorps fluorescents (TAF) pourrait permettre de différencier ces parasites.

Les sérum ont été obtenus de trois sources :
- de rats hyperimmunisés contre *P. berghei berghei*, *P. berghei yoelii*, *P. chabaudi* et *P. vinckei*;
- de lapins immunisés contre les antigènes solubles des stades érythrocytaires de ces parasites;
- d'un lapin immunisé contre les sporozoïtes de *P. berghei yoelii*.

Le titre de chaque sérum a été déterminé par sa réaction avec l'antigène homologue et hétérologue au moyen d'une technique d'immunofluorescence indirecte modifiée.

Les résultats ont révélé une nette réaction croisée entre les quatre parasites, prouvant ainsi qu'ils possèdent un antigène commun. On n'a constaté aucune différence sérologique entre *P. b. berghei* et *P. b. yoelii* (groupe *berghei*) et entre *P. chabaudi* et *P. vinckei* (groupe *vinckei*). Toutefois, une nette différence sérologique a été observée entre les infections du groupe *berghei* et celles du groupe *vinckei*. L'antisérum à titre élevé du groupe *berghei* a donné des titres beaucoup plus faibles avec l'antigène du groupe *vinckei*, et réciproquement.

Théoriquement, on peut supposer que la structure antigénique des plasmodiums des rongeurs comprend un ou plusieurs antigènes communs ou fondamentaux, et chaque groupe un antigène spécial. En outre, on pense que chaque parasite possède son ou ses propres antigènes. Si les antigènes fondamentaux et spéciaux peuvent être décelés par la technique AF avec emploi d'antisérum de groupes hétérologues et homologues, l'antigène (ou les antigènes) spécifique n'a pu encore être décelé par cette technique.
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The reciprocal titres of Anti-berghel berghel (NK65) sera treated against the Homologous and the Heterologous Antigens, using the Indirect FAT.

- P. berghei berghel NK65
- P. berghei yoelli 17X RCA
- P. chabaudi 54X
- P. vinckei Adler st.
The reciprocal titres of Anti-berghei yoelli (17X RCA) Sera treated against the Homologous and the Heterologous Antigens, using the Indirect FAT.
The reciprocal titres of Anti-vinckei (Adler str.) Sera treated against the Homologous and the Heterologous Antigens using the Indirect FAT.

- P. berghei bergheri NK65
- P. berghei yoelli 17X RCA
- P. chabaudi 54X
- P. vinckei Adler str.
The reciprocal titres of Anti- *chabaudi* (54X) Sera treated against the Homologous and the Heterologous Antigens, using the Indirect FAT.

![Graph showing reciprocal titres of Anti-<i>chabaudi</i> (54X) Sera against homologous and heterologous antigens.]
Antigenic Structure of Rodent Malaria Parasites as determined by FAT:-

- "Basic Antigen or Antigens" Shared by the four parasites - detected by FAT
- "Special Antigen or Antigens" Shared only by *P. berghei* berghel and *P. berghei yoelli* - detected by FAT
- "Special Antigen or Antigens" Shared only by *P. chabaudi* and *P. vinckei* - detected by FAT
- "Specific Antigen or Antigens" Found only in the particular strain - NOT detected by FAT

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