Active Immunization against *Plasmodium berghei*
Malaria in Mice, Using Different Preparations of Plasmodial Antigen and Different Pathways of Administration*

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With regard to the effectiveness of the antigens in inducing clinical immunity against malaria parasites, the minimum amount of living antigen developed in mice during controlled low parasitaemia with *Plasmodium berghei* has been estimated and compared with the amount of non-living antigen obtained by various methods of freeing parasites from their erythrocyte hosts.

Whereas about 100 mg of living antigen per kg of body-weight are sufficient to induce a degree of hyperimmunity, 1240 mg/kg of a freshly prepared crude antigen are necessary to enable the mice to survive a challenge infection while 3500 mg–7000 mg/kg of a vaccine prepared from freshly isolated plasmodia are necessary to produce a degree of immunity comparable with hyperimmunity. It appears, therefore, that every manipulation of the parasitized erythrocyte or the isolated plasmodium outside the host organism, as well as a storage time in excess of 36 hours, causes a reduction in antigenicity, up to a factor of $10^{-2}$. However, this decrease in antigenicity is disproportionate compared with the reduced rate of infectivity of stored, parasitized erythrocytes and isolated parasites. After an incubation period of 18 hours, the ID₁₀₀ increases from $2 \times 10^2$ to $5 \times 10^3$ parasites. Therefore, the differences between the essential amount of living plasmodia and non-living antigen may be due to other, hitherto unknown, factors and not exclusively to degradation of the most important antigen.

The saponin method of freeing parasites from their erythrocyte hosts was found to yield the purest antigen. Preparations of parasites obtained by treating parasitized erythrocytes with anti-erythrocyte serum or with formalin were highly contaminated with remnants of the host cells and showed no better antigenic qualities than the parasites isolated by means of saponin.

Since the decrease of antigenicity associated with harvesting and isolation procedures is constant, vaccination with a fractionated antigen pool should be possible.

In a previous paper (Jerusalem, 1969), attempts to immunize Swiss mice actively against *Plasmodium berghei* using a non-living vaccine were described. From the results it was concluded that an unexpectedly large amount of non-living antigen would be necessary to induce a satisfactory degree of clinical immunity. Although as much as 10 000 mg/kg of body-weight had been administered, non-living antigen was not able to induce the highest degree of antiparasitic immunity which is generally seen after extended contact with living parasites. The point in question was whether the antigen that gives rise to the protective immunity would be degraded by the procedures of isolation and harvesting.

According to Zuckerman, Hamburger & Spira (1967) and Zuckerman & Ristic (1968), the antigen should be isolated in the native form and methods for harvesting and preserving the infected cells, as well as the isolation procedures, should do no harm

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to the parasite itself or, finally, to its antigens. The method usually recommended for freeing plasmodia from their erythrocyte host is the saponin method (Christophers & Fulton, 1939; Sherman & Hull, 1960; Spira & Zuckerman, 1962; for further literature see also Zuckerman & Ristic, 1968). This procedure is assumed to do no harm to the plasmodial antigens because parasites freed by this technique remain infective; however, they show a reduced rate of infectivity. The most physiologically based process of separation seems to be a technique employing haemolytic antiserum (Bowman, Grant & Kermack, 1960).

Up to the present time, no investigations have been carried out concerning a possible relationship between the viability and rate of infectivity of the plasmodia and the native state of those antigens which give rise to protective immunity in the clinical sense. (Jerusalem, 1969). Furthermore, the problem of whether an antigen can be degraded by macrophages of the host has not been thoroughly studied because intramuscularly administered antigen seems not to induce the same degree of clinical immunity as intraperitoneally injected homogenates of plasmodia (Jerusalem, 1968b). Therefore, the aim of this study was to determine the minimum amount of viable antigen that could induce full protection against P. berghei and to compare the antigenicity of plasmodial antigens obtained by different methods of separation, as well as to investigate the influence of the different routes of inoculation.

MATERIAL AND METHODS

For all experiments young adult Swiss mice (TNÖ-Zeist) weighing about 20 g at the beginning of each experiment and the K 173 strain of P. berghei were used. The strains of both the mice and the malaria parasites were the same as described in a previous paper (Jerusalem, 1969).

Five basic experiments were carried out (A–E) and these were divided into groups (numbered 1–14) and subgroups.

The degrees of acquired immunity have been classified (Jerusalem, 1968a) as follows: hyperimmunity (HYPI), high antiparasitic immunity (HAPI), low antiparasitic immunity (LAPI) and antitoxic immunity (ATI).

(A) Amount of viable antigen necessary to induce clinical immunity

Group 1. A total of 390 mice was divided into 3 lots of 130 each (a–c) and immunized by inducing controlled low parasitaemia. The mice were kept on a para-aminobenzoic acid (PABA)-free diet as described by Jerusalem (1965, 1966) and Jerusalem & Kretschmar (1967). Mice in subgroup 1a were inoculated once, those in subgroup 1b twice and those in subgroup 1c 3 times at intervals of 10 days with $2.5 \times 10^4$ living parasites. The PABA-free diet was replaced by a standard laboratory diet 15 days after the last injection.

Group 2. A total of 250 mice on a standard diet were inoculated with $2.5 \times 10^4$ parasites and 5 days later the standard diet was replaced by a PABA-free diet for a period of 30 days.

Group 3. A total of 125 mice were immunized with serum of infected mice as described by Jerusalem & Bruchhausen (1966) and Jerusalem (1968a). The mice were challenged with $2.5 \times 10^4$ parasites.

To avoid uncontrolled blood-loss, alternating groups of 10 mice of each subgroup (1a–1c) and from each of groups 2 and 3 were used to determine the number of parasites and red cells, the packed cell volume and the total amount of plasmodial protein. Thin blood smears were made 3 times a week and the parasite numbers were determined and expressed in terms of number of parasites per 2000 erythrocytes. Haematocrit estimations as well as red cell counts were carried out twice a week.

The total volume of plasmodia (expressed as percentage of packed cell volume) and the amount of plasmodial proteins present during different periods of a patent infection were determined in thin blood smears according to the method of Hennig (1957), using a modified surface integration method (Glagoleff, 1933; Chalkley, 1943; Haug, 1955). As a control for the calculated values, 20 mice of subgroups 1a–1c showing a mean parasitaemia of 0.5% and mice of group 2 which exhibited a mean parasitaemia of 6%, as well as 10 animals of a donor group (see experiments 6–8) showing a mean parasitaemia of 25%, were selected at random and bled from the orbital plexus, as described by Reitsma (1967). The blood of each group was pooled, the erythrocyte volume determined and the plasmodia isolated as described by Spira & Zuckerman (1962). Finally, the volume of isolated plasmodia was determined and the protein content of isolated plasmodia was measured by the Kjeldahl micro-method. The degree of purity was tested by means of electron microscopy. From subgroups 1a, 1b, 1c, and group 2, each 100 surviving mice were challenged 60 days after they had received the first inoculum and 25 surviving mice
of group 3 were once more reinfected with $2.5 \times 10^4$ parasites 90 days after the first challenge infection.

**(B) Rate of infectivity (ID$_{100}$) in relation to the incubation medium, the duration of storage and the incubation temperature**

**Group 4.** Parasitized blood was withdrawn from mice by orbital puncture on the fourteenth day of infection. The citrated blood was pooled and the erythrocytes were washed free from plasma with citrated saline. Serial dilutions were prepared containing 50–50 000 000 infected erythrocytes per 0.1 ml. Citrated saline, phosphate-buffered saline (PBS) and mouse serum were used as the dilution media. The suspensions were stored in roll tubes at 4°C, 22°C, 30°C and 37°C. All procedures were carried out under aseptic conditions. After incubation periods of 2, 5, 10 and 18 hours, 0.1 ml of each suspension was injected into Swiss mice in groups of 10; 3, 7 and 14 days after the inoculation blood smears were made and parasite numbers were determined.

**Group 5.** Parasitized erythrocytes were obtained as described in experiment 4. The parasites were liberated according to the method described by Spira & Zuckerman (1962). To avoid conglomerates, the isolated plasmodia were filtered through cheesecloth and suspended in citrated saline and isologous serum. Serial dilutions, incubation and injections were carried out as described above. In one series, a standardized human serum (from Europeans) was used as an incubation medium. The incubation temperatures in this series were 4°C and 22°C. The ID$_{100}$ was regarded as the number of parasitized erythrocytes per inoculum being able to infect 100% of the mice.

**(C) Antigenicity of parasite preparations in relation to the duration of storage**

Twice a week, lots of about 200 mice were inoculated with *P. berghei*. Between 10 and 14 days later the surviving mice of these lots were bled from the orbital plexus. The blood was collected in citrated saline at 4°C.

To investigate the antigenicity of a freshly prepared antigen it was necessary to kill all living parasites. Since previous investigations had shown that homogenization at 20 000 rev/min with an Ultraturrax mixer was not sufficient to suppress completely the infectivity of the parasitized blood (Jerusalem, 1968b), ultrasonic and formol treatments were preferred.

**Group 6.** The whole-blood pool (including the plasma) was subjected to ultrasonic treatment (20 000 c/s) for 15 s and during the treatment the preparation was cooled with ice-cold methanol. The homogenized whole blood was then immediately injected in different amounts into 4 lots of 15 mice (subgroups 6a–6c). By calculating the total blood volume, the hematocrit reading and the number of parasitized erythrocytes, the amount of plasmodial protein was estimated (see experiments 1–3). Each mouse in subgroup 6a received a total of 100 mg of plasmodial antigen per kg of body-weight, those in subgroup 6b received 500 mg/kg, subgroup 6c, 1240 mg/kg and subgroup 6d, 2500 mg/kg. The period between withdrawal of the infected blood from donor animals and the injection of the treated blood into test animals was not longer than 45 min.

**Group 7.** Parasites were freed from their host cells according to the method of Spira & Zuckerman (1962). The isolated plasmodia were suspended in buffered saline containing 0.1% formol for 20 min at 4°C and were successively washed and gently homogenized with a Potter homogenizer. The preparation was administered intraperitoneally to 4 lots of 15 mice: subgroup 7a received 500 mg/kg of body-weight, subgroup 7b 1250 mg/kg, subgroup 7c 3500 mg/kg and subgroup 7d 7500 mg/kg. The injections were administered about 4 hours after the withdrawal of the infected blood.

**Group 8.** Isolated plasmodia were treated as in experiment 7 and the preparation was stored at $-18°C$ for about 6 weeks. The vaccine was then inoculated into 4 lots of 15 mice; subgroup 8a received 1250 mg/kg of body-weight, subgroup 8b, 3500 mg/kg, subgroup 8c 7500 mg/kg and subgroup 8d 10 000 mg/kg.

Having regard to the toxicity of all the preparations (Jerusalem, 1969), the vaccine was administered during a period of 6 weeks in 15–20 injections. Nevertheless, some mice in subgroups 8b and 8c died during the immunization period. Four weeks after the last injection, from 9 to 14 surviving mice in each subgroup were challenged with $2.5 \times 10^4$ parasites and the parasite number in these animals was determined in blood smears. Red cell counts and haemoglobin estimations were carried out.

**(D) Antigenicity of parasite preparations after different freeing methods**

For experiments of groups 9–11, donor groups were used to obtain the antigen as described for experiments of groups 4–8.
Group 9. Parasites were isolated from their erythrocyte host cell according to the method of Spira & Zuckerman (1962), using a 0.01% saponin solution in 0.9% saline.

Group 10. Parasitized erythrocytes were suspended twice for 30 min at 37°C in a 10-fold larger volume of anti-mouse-erythrocyte serum (AMES). The preparation was centrifuged and then washed with buffered saline. The AMES was obtained from rabbits which were sensitized by weekly intravenous injections of about 10⁸ freshly prepared and washed mouse erythrocytes per injection. Altogether 8 injections were administered; no adjuvant was used.

Group 11. Parasitized erythrocytes were treated with a 100-fold larger volume of 0.1% formol in PBS for 6 hours at 4°C. Thereafter, the preparation was washed twice with PBS.

Small samples of all preparations in groups 9–11 were pre-fixed in glutaraldehyde, post-fixed in OsO₄, embedded in Epon and examined under the electron microscope. The protein content of all preparations was determined by the Kjeldahl micro-method. The real amount of plasmodial protein in preparations of groups 10 and 11 was calculated after the degree of contamination had been determined.

Preparations of groups 9–11 were stored for 36 hours at 4°C according to the method of Jerusalem (1969) and then administered to 4 lots of mice. Subgroup 11a (25 mice) received 110 mg/kg of body-weight, subgroup 11b (15 mice), 330 mg/kg, subgroup 11c (15 mice), 1000 mg/kg and subgroup 11d (15 mice), 3000 mg/kg (Table 2). The injection schedule was the same as used for experiments of groups 6–8 (see also Jerusalem, 1969). Four weeks after the last injection, the mice in subgroups 11a–11d were challenged with 2.5 × 10⁸ parasites, and the developing parasitaemia was followed in blood smears. Red cell counts and haemoglobin estimations were carried out.

(E) Degree of immunity dependent on different routes of antigen inoculation

Isolated plasmodia were obtained and treated with 0.1% formol according to the methods described for experiment 7. Instead of a Potter mixer, an Ultraturrax mixer (20 000 rev/min) was used for homogenization. During homogenization the preparation was cooled in ice-water.

The preparation was administered to 3 lots of 30 mice each mouse receiving 22.3 mg or 900 mg/kg of body-weight. Inoculations were intravenous (group 12), intraperitoneal (group 13) and intramuscular (group 14). The total amount of antigen was administered over a period of 7 weeks and it was necessary to start with small amounts (0.1 mg) because the preparation was highly toxic, particularly on intravenous administration. After the first week (3 injections of 0.1 mg) increasing doses were administered: 0.5 mg, 1.0 mg and 1.5 mg (each dose being given 3 times) and 6 inoculations of 2.0 mg. No adjuvant was used.

RESULTS

Amount of viable antigen necessary to induce clinical immunity (A)

The quantity of parasites metabolized by the host during the period of controlled low parasitaemia was determined by the following calculation: the total blood volume of a mouse weighing 22 g is about 1.75 ml and the haematocrit reading in mice with controlled low parasitaemia was 40% ± 0.75%. Consequently, the total volume of erythrocytes is about 700 μl. The proportion of the parasite volume in the total blood volume is strongly dependent on the number of parasitized erythrocytes but there is no direct correlation between total parasite volume and parasitaemia because, with the increase in number and immaturity of polychromatophilic erythrocytes, the size of the parasite increases as well as the number of parasites per erythrocyte (Fig. 1). When about 50% of the erythrocytes are parasitized, the total parasite volume is about 22.5% ± 3.6% of the haematocrit reading. During a low parasitaemia of about 5%, the parasite volume is only 0.8% ± 0.1%, and in about 1% parasitaemia, only 0.1% ± 0.04% (Fig. 1).

The result of this calculation was confirmed by the direct determination of the parasite volume in infected blood (Fig. 1). In mice showing a mean parasitaemia of 0.5%, 0.13 μl of parasites per animal was calculated but this volume must be corrected by the factor 2.16 because, of the assumed total blood volume (1.75 ml), only 0.81 ml was withdrawn. Thus, a parasite volume of 0.28 μl (0.04%) of the packed cell volume per animal can be calculated (compare Table 1, subgroups 1a–1c). In mice showing 6% parasitized erythrocytes, after correction by the factor 2.04 (0.86 ml withdrawn blood), 5.62 μl of parasites (1.15% of the packed cell volume) were calculated and in highly parasitized blood (23%) 13.2 μl per animal (about 8% of the packed cell volume). The correction factor for the
CORRELATION BETWEEN TOTAL PARASITE CONTENT \(a\) OF PACKED CELL VOLUME AND PERCENTAGE PARASITAEMIA IN MICE

![Diagram](image)

\(a\) As log percentage.

The total amount of plasmodial protein metabolized by the host during a controlled or uncontrollable patent parasitaemia is dependent on both the degree and the duration of parasitaemia. It can be calculated by multiplying the plasmodial volume by the factor 0.18, because the protein content of isolated plasmodia was 18 \(\pm\) 2.8% of the parasite volume.

The results from investigations on metabolized plasmodial protein are summarized in Table 1. During the period of controlled low parasitaemia, an average of 0.5% parasitized erythrocytes was observed in most of the animals; thus, the parasite volume was about 0.05% of the packed cell volume and not greater than 0.35 \(\mu\)l at the day of investigation. The patent parasitaemia lasted about 13 days in mice of subgroup 1a, 24 days in mice of subgroup 1b and about 40 days in the animals of subgroup 1c. Thus, assuming a 24-hour cycle for \(P.\) berghei, a total amount of about 33 mg/kg of body-weight (subgroup 1a), 60 mg/kg (subgroup 1b) and about 100 mg/kg (subgroup 1c) were metabolized during the different extended periods of patent parasitaemia.

In some animals (subgroup 1c) the mean parasitaemia rose to 2.1% during controlled low parasitaemia. In these animals the haematocrit reading was low, averaging 33% \(\pm\) 1.35%. During a parasitaemia of 40 days in mice weighing 25 g, about 550 mg/kg of body-weight of plasmodial protein were metabolized.

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Parasitaemia</th>
<th>Haematocrit (a)</th>
<th>Quantity of parasites</th>
<th>Quantity of plasmodial protein (b)</th>
<th>Survival after challenge infection (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>%</td>
<td>Duration (days)</td>
<td>%</td>
<td>Volume ((\mu)l)</td>
<td>Percentage of haematocrit</td>
</tr>
<tr>
<td>1a</td>
<td>0.5 (\pm) 0.01</td>
<td>13</td>
<td>40 (\pm) 0.75</td>
<td>700</td>
<td>0.05 (\pm) 0.01</td>
</tr>
<tr>
<td>1b</td>
<td>6.3 (\pm) 2.3</td>
<td>14</td>
<td>28 (\pm) 3.9</td>
<td>490</td>
<td>1.3 (\pm) 0.32</td>
</tr>
<tr>
<td>1c2</td>
<td>21 (\pm) 0.6</td>
<td>40</td>
<td>33 (\pm) 1.35</td>
<td>577</td>
<td>0.27 (\pm) 0.06</td>
</tr>
<tr>
<td>2</td>
<td>25 (\pm) 6.32</td>
<td>56</td>
<td>10.65 (\pm) 2.6</td>
<td>186</td>
<td>13.2 (\pm) 1.7</td>
</tr>
</tbody>
</table>

\(a\) The calculation is based on the assumption that the total blood volume in mice weighing 22 g is about 1.75 ml.

\(b\) The protein content of isolated plasmodia is 18% \(\pm\) 2.8% of the parasite volume.

\(c\) In mice showing a mean body weight of 18 g.

\(d\) After second challenge infection.
In mice given a PABA-free diet (group 2), a mean parasitaemia of 6.3% lasting about 14 days was observed. In animals weighing about 20 g, 800 mg/kg of body-weight of plasmodial antigen were metabolized.

In challenged mice that were immunized exclusively against toxic products of plasmodial origin (Jerusalem & Bruchhausen, 1966), enormous amounts of antigen were metabolized because of the intense and long-lasting parasitaemia (group 3). Taking into account the loss in body-weight of the animals (the mean body-weight during the period of 56 days of patent parasitaemia was 18 g) it can be calculated that nearly 14 g of antigen per kg of body-weight were metabolized.

The results concerning the degree of acquired immunity, as judged from mortality after challenge infection, show the importance of both the amount of antigen and the duration of parasitaemia. Using living parasites, it can be computed that a minimum of about 100 mg/kg should be metabolized during a period of 5-6 weeks. The number of surviving animals does not increase strikingly when the amount of antigen increases by a factor of 4 (Table 1, 1c) or even by a factor of 137 (group 3). On the other hand, 642 mg of antigen per kg forming during a period of 14 days are not able to induce the same degree of protective immunity as 100 mg/kg metabolized during a period of 40 days (group 2 and subgroup 1c).

$ID_{100}$ in relation to the incubation medium and the duration of storage at different temperatures (B)

The number of parasitized erythrocytes necessary to infect 100% of the animals ($ID_{100}$) depended largely on the incubation medium and the storage time as well as on the incubation temperature. However, no significant differences were observed in the decrease of infectivity when parasitized erythrocytes were incubated in salt-containing media (either citrated saline or PBS) at room temperature (22°C) and at 30°C and 37°C (Fig. 2, left side, A). The decrease of infectivity was less striking after an incubation in either citrated saline or PBS at 4°C (Fig. 2, left side, B). In this series of experiments, mouse serum caused the lowest decrease in infectivity of parasitized erythrocytes when the incubation temperature was 22°C (Fig. 2, left side, C). Compared with the number of freshly withdrawn parasites necessary to infect an animal (2×10) after an incubation period of 10 hours, between 225 times (incubation in mouse serum at 22°C) and 350 000 times (incubation in salt-containing media at 22°C, 30°C and 37°C) more parasitized erythrocytes are required to infect 100% of the animals, and 10 000 times and 2.5 million

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**FIG. 2**

$ID_{100}$ OF PARASITIZED ERYTHROCYTES (LEFT SIDE) $^a$ AND ISOLATED PARASITES (RIGHT SIDE) $^b$ AFTER DIFFERENT INCUBATION PERIODS IN DIFFERENT MEDIA AT VARIOUS TEMPERATURES

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$^a$ Parasitized erythrocytes incubated (A) in citrated saline and in PBS at 22°C, 30°C and 37°C; (B) in citrated saline and in PBS at 4°C; (C) in mouse serum at 22°C.

$^b$ Isolated parasites Incubated (a) in citrated saline at 22°C; in citrated saline at 4°C; (c) in mouse serum at 22°C; (d) in standardized human serum at 22°C.
times more, respectively, after an incubation period of 18 hours.

The influence of the incubation temperature on stored isolated plasmodia was not as marked as on parasitized erythrocytes. Moreover, whereas after incubation in citrated saline at 22°C for periods of 2–10 hours, the infectivity did not decrease by the same factor (Fig. 2, right side, a) as stated for parasitized erythrocytes (Fig. 2, left side, A), the ID_{100} increased after incubation in citrated saline at 4°C (Fig. 2, right side, b). After incubation in mouse serum between 6–7 times (5–10 hours storage time) and 35 times (18 hours storage time) more isolated parasites were necessary to infect 100% of the animals (Fig. 2, right side, c) than parasitized erythrocytes which were stored in the same incubation medium for the same periods (Fig. 2, left side, C). The most striking inhibition of the infectivity of isolated plasmodia was seen after incubation in a standardized human serum (Fig. 2, right side, d).

These results hold true for aseptic preparations. A bacterial superinfection of the incubation medium causes a rapid decrease of the viability of the incubated plasmodia and parasitized erythrocytes, particularly at higher incubation temperatures (22°C or above).

Antigenicity of parasite preparation in relation to the duration of storage (C)

The effect of the preparations used as antigens was judged by both the proportion of mice surviving the challenge infection and the course of the parasitaemia in challenged animals. Since the K 173 strain of *P. berghei* produces a uniformly fatal infection in Swiss mice, 70%–80% of surviving mice after active vaccination was regarded as a satisfactory result.

This level of protection was obtained after the administration of 1240 mg of preparation 6 per kg (injected 45 min after the withdrawal of infected blood), 3500 mg of preparation 7 per kg (injected 4 hours later) and 5000 mg of preparation 8 per kg (injected after a storage time of about 6 weeks) as shown in Fig. 3. The same amount as needed for experiments of group 8 was necessary after a storage time of 36 hours in groups 9, 10 and 11 (experiment D) (Jerusalem, 1969).

In previous papers (Jerusalem, 1968a, 1969) it was pointed out that a decrease in mortality does not necessarily indicate the establishment of antiparasitic immunity. Also, an experimentally provoked non-specific resistance or an acquired specific antitoxic immunity causes a decrease in mortality.

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**Fig. 3**

**Comparison between decrease of infectivity of living plasmodia (ID_{100}) and decrease of antigenicity of non-living antigen compared with the storage time (experiments 6 and 7)**

<table>
<thead>
<tr>
<th>Duration of storage (hours)</th>
<th>Number of parasites (ID_{100}) and mg antigen per kg</th>
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<tbody>
<tr>
<td>18</td>
<td>10^8</td>
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<tr>
<td>35</td>
<td>10^7</td>
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<tr>
<td>6</td>
<td>10^6</td>
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</tbody>
</table>

A = minimum amount of living antigen necessary to induce the highest level of antiparasitic immunity (HYPI).

B = estimated maximum amount of living antigen metabolized during chronic infection.

C = minimum amount of non-living antigen necessary to induce a low level of antiparasitic immunity (LAPI) or antitoxic immunity (ATI).

D = minimum amount of non-living antigen necessary to induce a high level of antiparasitic immunity (HAPI).

E = formol-treated isolated parasites.

However, when the change in degree of the parasitaemia indicates that there is an established antiparasitic immunity, it is necessary to administer about twice the amount of the above-mentioned total dose (Fig. 3).

Compared with the minimum amount of living antigen able to induce the highest level of antiparasitic immunity (HYPI) during controlled low parasitaemia (subgroup 1c), the amount of "non-living" antigen necessary to induce a high level of antiparasitic immunity (HAPI) increased by a factor of 10–35, depending on the length of time the preparation was stored. Long-term storage reduces the antigenicity of a preparation considerably. It should be recalled, however, that during a chronic malarial infection in Swiss mice as much as 14 000 mg/kg of body-weight may be metabolized (Table 1).
There is, however, no direct correlation between the rate of infectivity (ID₉₀) and the antigenicity as demonstrated in experiments of groups 4 and 5. Every manipulation involving the parasitized erythrocyte or the isolated parasite caused a more rapid decrease in antigenicity than in infectivity during the first 4 hours of treatment. Between 4 and 18 hours the number of parasites necessary to infect 100% of the mice increased once again by about a factor of 10⁴, whereas the antigenicity of an antigen preparation decreased by no more than the same factor as during the first 4 hours even after a storage time of 6 weeks (Fig. 3).

It was surprising, however, that mice vaccinated with small amounts (100 mg/kg) of preparation 6 showed a significantly reduced survival (see also experiments of groups 10 and 11).

Antigenicity of parasite preparations after different freeing methods (D)

Electron microscopy. Parasites freed from their erythrocyte host by the saponin method (group 9) showed a slightly altered structure (e.g., no distinct cell organelles were seen; Fig. 4A) though this preparation was still highly infectious (Fig. 2). There was no contamination by remnants of the host cells. The electron photomicrographs of preparation 10 showed that after a treatment with haemolytic antibodies the parasites were not really freed from their host cells (Fig. 4B). Although nearly all unparasitized erythrocytes were empty, the erythrocyte membranes were still present and appeared to be thicker than normal. Perhaps the erythrocyte membrane forms a complex with the antibody. Polychromatophilic erythrocytes, the host cells most likely to be infected, contained some electron-dense granules and remnants of a smooth tubular system. The parasites showed numerous vacuoles. Preparation 11 was also highly contaminated, particularly with erythrocyte membranes. The membranes were ruptured but did not look disintegrated (Fig. 4C).

Result of vaccination. As summarized in Table 2, there were no significant differences in either survival rate or degrees of acquired immunity in groups that were vaccinated with the same amount of antigen per kg of body-weight. This indicates that the method of freeing parasites from their host cells is of secondary importance. Corresponding to group 9, the number of survivals increased in the groups 10 and 11 when the amount of antigen was increased from 330 to 3000 mg/kg (subgroups b-d). However, mice in experiments of subgroups 10a as well as 11a and 11b showed a remarkably reduced mean survival time due to an increase in mortality during the first 9 days of the challenge infection. This applies also to subgroup 10b, though 1 animal survived the

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Vaccination</th>
<th>Challenge infection</th>
<th>Amount of antigen (mg/kg)</th>
<th>Group 9 experiments (saponin treatment)</th>
<th>Group 10 experiments (haemolytic antiserum treatment)</th>
<th>Group 11 experiments (formol haemolysis)</th>
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<tr>
<td></td>
<td>No. of animals used for:</td>
<td>No of survivals</td>
<td>Established immunity</td>
<td>No of survivals</td>
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<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

* HAPI = high antiparasitic immunity; LAPI = low antiparasitic immunity; ATI = antitoxic immunity.
FIG. 4
ELECTRON-PHOTOMICROGRAPHS OF (A) PARASITES FREED BY THE SAPONIN METHOD;
(B) TREATMENT OF PARASITIZED ERYTHROCYTES WITH HAEMOLYTIC ANTISERUM;
(C) TREATMENT OF PARASITIZED ERYTHROCYTES WITH 0.1 % FORMOL

*Preparations B and C are contaminated with remnants of the erythrocyte host.*
challenge infection. Whereas 51% of the control animals did not survive the ninth day of the infection, an average of 80.6% of the mice that were vaccinated with 110 and 330 mg/kg of contaminated antigen (subgroups 10a, 10b, 11a, 11b) succumbed during this period (Fig. 5). Surprisingly, most of the animals of the groups 10 and 11 which died during the first 9 days showed a lower degree of parasitaemia but a more rapid loss of red cells than unvaccinated controls.

**Degree of immunity dependent on different pathways of antigen administration (E)**

The route of antigen inoculation was of an unexpectedly great importance (Table 3). Intravenous injection of the antigen was found to show the best results not only in the percentage of surviving mice (38.1%) but also in the degree of antiparasitic immunity (HAPI; 53.8%). However, intravenous application resulted in a high rate of mortality during the vaccination period, although having

**TABLE 3**

**DEGREE OF IMMUNITY DEPENDENT ON DIFFERENT ROUTES OF INOCULATION**

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>No. of animals</th>
<th>Mortality (%)</th>
<th>Challenge infection</th>
<th>No. of animals</th>
<th>Survival (%)</th>
<th>Type of acquired immunity</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>Intravenous</td>
<td>30</td>
<td>30</td>
<td></td>
<td>21</td>
<td>38.1</td>
<td>HAPI 53.8, LAPI 38.5, ATI 7.7</td>
</tr>
<tr>
<td>13</td>
<td>Intraperitoneal</td>
<td>30</td>
<td>3.3</td>
<td></td>
<td>26</td>
<td>17.3</td>
<td>HAPI 37.5, LAPI 33.3, ATI 29.2</td>
</tr>
<tr>
<td>14</td>
<td>Intramuscular</td>
<td>30</td>
<td>0</td>
<td></td>
<td>30</td>
<td>13.4</td>
<td>HAPI 50.0, LAPI 50.0, ATI 50.0</td>
</tr>
</tbody>
</table>

*a HAPI = high antiparasitic immunity; LAPI = low antiparasitic immunity; ATI = antitoxic immunity.*
regard to the toxicity of the vaccine a special injection schedule was used. Disappointing results were recorded after intramuscular application (group 14) though the animals received the same amount of antigen as administered to the groups 12 and 13. Only 13.4% of the mice survived the challenge infection showing a low degree of antiparasitic immunity (LAPI) or at best antitoxic immunity (ATI). In some mice the parasitaemia did not rise to high values, but lasted for 40–55 days and the animals died of fatal relapses as shown in a previous paper (Jerusalem, 1969).

DISCUSSION

A critical examination of the literature reporting investigations on active immunization against malaria parasites using non-living plasmodial preparations as antigens reveals that only partial successes have been achieved. The use of Freund’s adjuvants was either optional or essential (Freund et al., 1945, 1948; Thomson et al., 1947; Targett & Fulton, 1965; Targett & Voller, 1965; Zuckerman et al., 1967; Voller & Richards, 1968) or the amount of antigen necessary to induce clinical immunity was too large to be able to put into practice the recommended methods of vaccination against human malaria (Zuckerman & Ristic, 1968; Jerusalem, 1969). Furthermore, in no instance did the vaccination with a non-living antigen give rise to the establishment of a degree of immunity that would commonly be acquired after contact with living plasmodia.

Jerusalem (1969) pointed out that the ratio of amounts of living to non-living antigen necessary to induce a high degree of protective immunity is about 1:50 and the author suggested that the antigen that gives rise to the protective immunity is a labile one and that every manipulation of the parasitized erythrocyte and the parasite outside the host organism results in a rapid decrease of antigenicity.

Results reported in this paper have led us to question whether this assumption can be sustained in its entirety. It is known that about 100 mg of living plasmodia per kg of body-weight are sufficient to induce the observed degree of hyperimmunity when this quantity is metabolized during a period of about 40 days (1c1). On the other hand, a 12-fold larger amount of freshly prepared antigen is essential for the establishment of the lowest degree of immunity which enables the mice to survive the challenge infection (group 6). It should, however, be remembered that the amount of antigen metabolized in the course of a chronic malarial infection in mice can be as much as 140 times that quantity which generally induces a protective immunity.

After ultrasonic treatment (group 6) and formol treatment (groups 7 and 8), as well as after a storage time of 36 hours (groups 9, 10 and 11), a certain decrease of antigenicity was noticed. Compared with the activity of a freshly prepared antigen (group 6), however, the decrease of antigenicity after a storage period of about 6 weeks (group 8) was only one-fourth of the decrease observed during the first 45 min. Furthermore, the assumed degradation of the antigens was disproportionate to the decrease in viability and infectivity of parasites which were stored outside the host organism. The significant influence of the storage temperature and the incubation medium is of secondary importance because, after a storage time of 18 hours, generally 200 000–2 500 000 times more parasites were necessary to infect 100% of the mice. Human serum used as an incubation medium is an exception; that is, it kills the plasmodia rapidly because, in particular, it extracts RNA from the cells (see Mayersbach, 1967). Also of secondary importance seems to be the method by which the parasites were freed from their erythrocyte host. No significant differences in the antigenicity of plasmodial preparations were observed after the use of saponin (Spira & Zuckerman, 1962), haemolysis with anti-erythrocyte serum (Bowman, Grant & Kermack, 1960)—a method which Zuckerman & Ristic (1968) regarded as the most “physiological”—and after treatment of parasitized erythrocytes with formol (Targett & Fulton, 1965; Voller & Richards, 1968).

Moreover, parasites freed from their erythrocyte host by the saponin method generally show the same reduced rate of infectivity as parasitized erythrocytes when both preparations have been exposed to the same incubation medium and to the same storage temperature (groups 4 and 5). This indicates that the erythrocytic membrane does not protect the parasite against degradation phenomena. Another fact also supports the use of saponin. According to the electron-photomicrographs, only the parasites were entirely isolated by the saponin method. Parasite preparations obtained by incubation in haemolytic antiserum (group 10) and by treatment of parasitized erythrocytes with formol (group 11) were found to be highly contaminated by remnants of the host cell. There is every reason to believe that this contamination may give rise to an immunopathological side-effect because it can
can stimulate an allergic response to allogeneic or even autologous red cell antigens. This state of altered reactivity might be harmful to the host. Perhaps the fulminating course of the challenge infection in mice vaccinated with small amounts of preparations 6, 10 and 11 was caused by reactions of clinical hypersensitivity. However, why this phenomenon was only induced after the administration of relatively small amounts of the contaminated vaccine remains open to question. This problem should be studied in detail with regard to tropical anaemias.

Surprisingly, the route of inoculation of the vaccinated material was very important. After intravenous injection, not only the largest number of surviving mice but also the greatest degree of anti-parasitic immunity were seen. Presumably, repeated intramuscular injections lead to a degradation of the antigen by macrophages in the train of some Arthus phenomenon. This might account for the assumption that the mechanisms of protective malaria immunity are of a complex nature and not restricted to the mode A allergic reaction described by Coombs (1968) by which a serum antibody acts (with or without other molecular cofactors) as an antitoxin, or the antibody alone might block a membrane enzyme system or might activate a complement to lyse a cell and kill the parasite. It is possible that other modes of allergic response, e.g., actively or passively allergized macrophages, play important roles.

The present work indicates that the amount of antigen which should be administered in order to induce a satisfactory degree of protective immunity competes with the toxicity of the material used for vaccination. Since it can be expected that only a small portion of the whole plasmodium is the antigen that gives rise to clinical immunity (Curtain et al., 1964; Targett & Voller, 1965; Weiss & Degiusti, 1966), it seems to be a logical step to fractionate the pool of antigens in order to eliminate the unwanted material and to avoid, as much as possible, secondary path-histological and pathophysiological effects.

From the present study it can be concluded that the recommended methods for harvesting plasmodia and freeing them from their erythrocyte host do result in a certain decrease of antigenicity, but that this decrease is not too serious. Therefore, vaccination with pooled fractionated antigen should be possible.

RÉSUMÉ

IMMUNISATION ACTIVE DE LA SOURIS CONTRE LE PALUDISME À PLASMODIUM BERGHEI PAR L'UTILISATION DE DIFFÉRENTES PRÉPARATIONS D'ANTIGÈNE PLASMODIQUE ET DE DIVERSES VOIES D'ADMINISTRATION

Le modèle expérimental constitué par l'infection de la souris par Plasmodium berghei a servi à étudier les possibilités d'immunisation active contre le paludisme par un antigène non vivant. Les recherches, comportant 14 expériences et la manipulation de plus de 9000 souris, ont porté sur cinq points principaux: a) la quantité d'antigène vivant nécessaire pour susciter une immunité clinique; b) la diminution du pouvoir infectant — exprimé par la dose entraînant une mortalité de 100% (D150) — des érythrocytes parasités et des plasmodies libres en fonction de la durée de conservation, du milieu et de la température d'incubation; c) la perte d'antigénicité du matériel non vivant en fonction de la durée de conservation; d) l'influence des divers procédés de libération des parasites sur l'antigénicité de ce matériel; et e) l'influence de la voie choisie pour administrer l'antigène. L'efficacité de la vaccination a été appréciée d'une part par la mortalité quotidienne chez la souris après injection d'épreuve et d'autre part par la qualité de l'immunité antiparasitaire.

Il faut environ 100 mg/kg d'antigène vivant pour produire une immunité protectrice à la condition que la transformation métabolique de cet antigène se poursuive sans interruption pendant 5-6 semaines. Si la durée du contact avec l'antigène vivant n'est que de deux semaines, la protection est de moindre de valeur, même si la quantité de matériel parasitaire transformé par l'hôte est de 640 mg/kg. La quantité d'antigène et la durée de la parasitémie ont donc toutes deux de l'importance.

Tous les traitements appliqués aux érythrocytes parasités en vue de libérer l'antigène ou aux parasites libres en dehors de l'organisme de l'hôte, de même que la conservation du vaccin, ont pour conséquence une perte d'antigénicité. L'administration de 1240 mg/kg d'antigène 45 minutes après le prélèvement du sang infecté chez le donneur animal permet à la souris de survivre à l'incubation. Par contre, il faut injecter 3500 mg/kg et 7000 mg/kg pour obtenir une protection équivalente si l'antigène a été stocké respectivement pendant 4 et 36 heures.

Cette perte d'antigénicité est toutefois minime en comparaison de la perte de pouvoir infectant qu'entraîne la conservation des érythrocytes parasités et des parasites libres. Avec des érythrocytes parasités fraîchement préle-
vés, la \( D_{190} \) est de 2 x 10 parasites. Elle s’élève à 5 x 10^7 parasites après 18 heures de conservation.

La différence d’activité entre l’antigène plasmodique vivant et l’antigène non vivant paraît due à des facteurs encore inconnus et n’a pas uniquement pour origine l’altération du matériel antigénique au cours de la récolte, du traitement et de la conservation. La nature des méthodes utilisées pour libérer le parasite n’a apparemment qu’une importance secondaire. L’emploi de la saponine permet d’obtenir des préparations très purifiées. Après traitement des érythrocytes parasités par un sérum anti-érythrocytes de souris ou par la formaline, le matériel antigénique est fortement contaminé par des résidus cellulaires.

La voie choisie pour l’administration du vaccin joue un grand rôle. Après injection intraveineuse, le nombre d’animaux survivants et la valeur de l’immunité antiparasitaire sont plus élevés qu’après injection intramusculaire.

Les présentes recherches indiquent que la quantité d’antigène à administrer pour obtenir une immunité satisfaisante doit être limitée en fonction du risque de toxicité du matériel vaccinal. D’autre part, les opérations de récolte et de libération des plasmodes ont pour effet d’atténuer dans une certaine mesure l’antigénicité. Les auteurs estiment néanmoins possible une vaccination effectuée à l’aide d’un antigène obtenu après élimination des fractions non directement immunogènes.

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