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Resistance to superinfection with *Plasmodium berghei* in mice in which the original infection was suppressed by a milk diet

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Maegraith *et al.* (1952) first reported that the progress of *Plasmodium berghei* infection in rats fed exclusively on a milk diet was suppressed. Milk is known to be deficient in available p-aminobenzoic acid and Hawking (1953, 1954) found that when p-aminobenzoic acid was added to the milk given to mice infected with *P. berghei*, the progress of infection was almost like that in animals on a normal laboratory diet. The deficiency of p-aminobenzoic acid in the milk diet was thus a factor involved in suppressing the multiplication of the parasites.

Various groups of workers established the suppressive effect of milk on other types of malaria and in different hosts, and reported that there was an increase in parasitaemia when the animals were returned to their normal laboratory diets soon after parasites were absent in the peripheral blood (Bray and Garnham, 1953; Ramakrishnan *et al.*, 1953; Fabiani and Orfila, 1954, 1955). It was also found that even when infected animals were splenectomized and infected while on a milk diet, the diet was powerful enough to suppress the infection or prevent a breakthrough, but, within a few days of return to a normal diet a severe recrudescence took place (Bray and Garnham, 1953). This suggested that little or no premunition had developed during the first days of infection, when the animals were exposed to a low parasitaemia even for as long as a period of four weeks. Kretschmar (1966) reported that a milk diet given when an infection had become established in mice on a normal diet could halt *P. berghei* infection as actively as it could suppress the parasites when started at the same time as the infection. It was also shown that when mice which had survived a previous infection were reinfected after a suitable length of time on a milk diet, they developed only mild parasitaemias which they were often able to overcome when returned to a normal laboratory diet.

This suggested that during the first contact with *P. berghei* (acting as an antigen) the host might, under certain circumstances, develop resistance to superinfection.

Adler (1958) examined the possibility that during a primary infection protection might be developed in the host against subsequent challenge by the homologous strain of parasite. Using a strain of *P. vinckei*, which was very lethal to mice, he found that when infected mice were kept on a wholly meat diet (also deficient in p-aminobenzoic acid) before and after infection, there was suppression of the growth and numbers of parasites. Seventy-five per cent. of the animals recovered completely from the infection and the suppression was reversed by addition of PABA as in *P. berghei*-infected mice on a milk diet. When animals on the meat diet had recovered from the primary infection they were reinfected with the same strain of *P. vinckei* and placed on a normal laboratory diet. Only slight parasitaemias resulted, from which most animals recovered without any

treatment. Adler (1958) concluded that the first infection suppressed by the meat diet had led to the development in the host of protection against the superinfection.

This paper records experiments in the development of resistance to superinfection in mice in which the original infection with *P. berghei* was suppressed by placing the host on a milk diet.

MATERIALS AND METHODS

Plasmodium berghei

The strain used was the normal (N) strain, obtained from Professor P. C. C. Garnham in 1965 via Professor Peters (then in Ciba Ltd., Basel). This strain has been maintained in the Liverpool School by blood passage into mice (intravenous inoculation of citrated infected blood) every four to five days.

Mice. Females of the C.F.N. strain of albino mice, six to seven weeks old, weighing 20–25 gm. (Tuck and Son, Essex) were used in all experiments.

Diet. The normal diet was M.R.C. laboratory diet 41B (Oxoid), with water *ad. lib.*

Milk diet. Powdered whole cream Cow and Gate baby food (Cow and Gate Ltd., Guildford, Surrey) was mixed with warm water (100 gm. powder to 80 ml. water) to form a thick paste. This was rolled into pellets which were placed in the food compartment of the mouse cage. Water was given *ad. lib.* Milk food was made up freshly each day.

Additions to milk diet. Para-aminobenzoic acid or folic acid (B.D.H.). The weight needed to make up the concentration required for a particular experiment was dissolved in the water used for making the pellets, which contained a weighed amount of the milk powder.

Chloroquine administration. Chloroquine sulphate (Nivaquine: May and Baker) was dissolved in citrate saline (5.0 gm. trisodium citrate in 500 ml. 0·9 per cent. [w/v] sodium chloride solution) so that 1·0 ml. of the solution contained 0·2 mg. Individual mice were weighed and injected with the equivalent of 20 mgm. per kgm. body weight on each of five consecutive days.

Parasite counts. Thin smears were stained by Leishman's method and the number of parasitized cells in 500 erythrocytes was counted. The result was expressed as 'percentage parasitaemia'. Double infections in erythrocytes were counted as one parasitized cell. The total number of parasites in the 500 erythrocytes was also counted and expressed as a 'total parasite percentage'. When parasitaemia was below 5 per cent., thick films stained with Field's stain were prepared concurrently with thin films. Low parasitaemias were expressed as '+'.

Analysis of serum. Serum was obtained from a volume of 0·15 ml. blood collected from the tail of the mouse.

The total protein and albumin were measured by standard micro methods. Separation of serum proteins by electrophoresis was carried out on cellulose acetate strips using a Kohn Universal apparatus (Shandon) at pH 8·6.

Care. The animals were bedded in sterile sawdust in polypropylene cages, six mice to the cage. When on a milk diet, they were housed in cages fitted with wire grid bottoms through which the excreta fell to a level below the reach of the occupants, thus preventing them from obtaining PABA which might have been synthesized by the gut flora.

Cages were kept in a room in which the ambient temperature was maintained at between 65° and 70°F.

Method of infection. A standard inoculation of 30–40 million parasitized erythrocytes was given. The blood was taken into citrate saline in a syringe. The parasitaemia in the donor was measured and the relevant volume was injected intravenously into the recipient.

RESULTS

Controls

Mice on a normal diet, given the standard intravenous inoculation of 30–40 million infected erythrocytes, all died in six to nine days.

This result was consistent. In the experiments recorded here controls consisted of four mice on normal diet infected as above. All died.

Effect of Milk and of Meat Diets

Mice on the milk diet given as described above gained weight at the same rate as animals on a normal diet. Haematocrit levels and haemoglobin concentrations remained normal. Daily bleeding for blood examination caused a fall in haematocrit and haemoglobin concentration of the same order as in animals on a normal diet. Haematocrits fell from 50 to 30 per cent. and haemoglobin concentrations from 14 to about 10 gm. per cent. over a period of three weeks. Mice were successfully kept on the milk diet for as long as 12 months.

When placed on a meat diet (Adler, 1958) mice lost weight and over three weeks the haemoglobin concentration fell more rapidly than the haematocrit and considerable reticulocytosis developed. After six weeks, numbers of animals on the meat diet died without obvious cause. The suppression of the erythrocytic forms of *P. berghei* in animals on a meat diet as reported by Adler (1958) in *P. vinckei* was confirmed, after which the meat diet was given up in favour of milk, because of the long-term experiments planned.

Milk Diet Begun Seven Days Before Infection

Parasites could be detected on the first day after infection and parasitaemia persisted for six to 10 days, with a low maximum peak on the third or fourth day. Parasites appeared largely in the form of rings or trophozoites. During the peak of the infection ring forms predominated. Schizonts were uncommon and some were 'malnourished' with less nuclear material which was more widely dispersed and stained less densely than in normal schizonts. Similar changes were reported in *P. cynomolgi* infection. (Bray and Garnham, 1953).

So long as the mice were thereafter kept on the milk diet, parasites could not be detected in the peripheral blood.

The haemoglobin concentration fell between the third and sixth days and rose after the overt infection was terminated. The reticulocyte count rose after the first few days. It was occasionally as high as 20 per cent. and remained elevated for several weeks. It was noted that no differential infection of reticulocytes with *P. berghei* occurred.

We confirmed the earlier observation that return to normal diet soon after successful suppression on milk led to fatal recrudescence of the original infection. When mice were

returned to a normal diet 14 days after parasites had disappeared from the circulation subsequent to administration of the milk diet, parasites reappeared within 24 hours and death occurred in high parasitaemia 11–14 days later (Table I).

TABLE I

Showing mice infected with *P. berghei*

A

Infection and milk given at same time

No. of days after infection	Percentage parasitaemias of individual mice					
	1	2	3	4	5	6
1	+	+	+	0·3	0·4	0·5
2	0·2	1·0	2·5	1·3	1·5	0·8
3	0·5	4·0	2·5	2·2	0·4	2·0
4	1·0	2·5	4·0	1·0	0·4	0·6
5	1·0	5·0	2·5	0·5	0·8	0·1
6	0·5	1·2	0·6	0·4	0·2	0·3
7	+	0·2	+	0·1	+	+
8	+	+	+	+	+	−
9	−	−	−	−	−	−
10	−	−	−	−	−	−
11	−	−	−	−	−	−
12	−	−	−	−	−	−
13	−	−	−	−	−	−
14	−	−	−	−	−	−

B

Restored to normal diet after 14 days free of detectable parasites

No. of days after recrudescence	Percentage parasitaemias of individual mice					
	1	2	3	4	5	6
1	+	0·2	0·1	+	+	0·2
2	0·2	2·0	0·8	1·0	0·2	0·3
3	0·4	2·3	3·0	0·3	0·6	0·8
4	3·0	4·6	5·2	1·6	1·6	1·2
5	2·6	8·6	6·8	2·6	5·0	5·6
6	5·4	12·1	12·3	6·8	7·5	10·3
7	8·2	13·3	34·0	19·2	13·2	16·3
8	23·2	18·6	39·5	31·0	18·5	21·2
9	37·0	25·2	46·5	35·5	26·0	28·3
10	41·5	32·0	54·0	48·2	33·5	34·0
11	57·4	44·0	dead	dead	42·3	51·0
12	72·0	dead			48·0	60·3
13	dead				51·8	dead
14					dead	

This indicated that the parasites must still have been present in the blood during the period of suppression. The somewhat slower course of the relapse infection (controls died by the ninth day) also suggested the presence of some delaying mechanism.

Meat Diet

Similar results were recorded in experiments in which suppression was induced by a meat diet. Parasites were present for longer (12 days) and the peak parasitaemias (third to fourth day) were higher than in corresponding infection in animals on a milk diet. Recrudescence following return to a normal diet followed a similar pattern to that in animals which had been on a milk diet.

Effect of Addition of Para-Aminobenzoic Acid (PABA) and of Folic Acid on the Course of P. berghei *Infection in Mice on a Milk Diet*

When mice were infected and placed on a diet of milk containing 1:10,000 (w/w) PABA the course of infection was almost identical in terms of numbers of parasitized erythrocytes and total numbers of parasites per 100 erythrocytes to that of infections in animals on a normal diet.

In mice on a diet of milk containing either 1:20,000 (w/w) PABA or 1:10,000 (w/w) folic acid, infections were slowed, death occurring between the ninth and thirteenth or fifteenth days respectively and the peaks of parasitaemia (and total parasites per 100 erythrocytes) were lower, not exceeding 65 per cent.

These results confirmed the findings of Jerusalem (1966) and Jacob (1964).

Milk Diet and Infection Started Together

When the animals were placed on the standard milk diet on the day they were infected, the infection followed the same course as in animals placed on milk diet a week before infection, suggesting, as Hawking and Terry (1957) pointed out, that very little of the PABA from the normal diet could have been stored in the body. These results also agree with those of McKee and Geiman (1948) who demonstrated suppression of *P. cynomolgi* infection in monkeys during short periods of starvation, and restoration of normal infection on return to a normal diet. The depletion of PABA in the absence of alimentary replacement can thus apparently be attributed to the immediate needs of the parasites.

Once it had been established that the suppression of infection in animals started on the milk diet and given the infection simultaneously was similar to that in animals given the milk diet for a period before infection, it was decided to study the effects of a challenge infection with the same strain at various intervals after the last appearance of parasites in the blood during the original suppressed infection (7–11 days after infection).

In a series of experiments, mice were offered a challenge of a standard inoculation of infected erythrocytes 14 and 28 days after the last appearance of parasites in the suppressed original infection. From the time of the second infection the mice were placed on a normal diet.

The progress of the challenge infection was similar to that in previously uninfected animals on a normal diet, but was a little slower; all animals were dead by the eleventh day of the infection.

As there appeared to be little indication of any resistance to superinfection in the above experiments, a challenge infection was given 90 days after the original infection had been suppressed. It was thought that this extra time might allow a longer exposure to the latent infection known to exist and so possibly lead to some build-up of resistance.

TABLE II

Showing mice infected with *P. berghei*

A

Infection and milk given at same time

No. of days after infection	Percentage parasitaemias of individual mice						
	1	2	3	4	5	6	Controls
1	0·5	1·0	0·6	0·3	0·3	0·3	2·0
2	1·4	1·2	1·5	0·8	0·8	0·75	6·0
3	1·4	0·6	2·0	1·5	1·3	1·75	23·5
4	+	1·4	0·8	0·8	1·4	2·0	40·5
5	0·6	0·5	1·0	1·2	0·8	0·8	55·2
6	0·4	0·8	0·3	0·8	0·8	0·5	1D*68·0
7	0·2	0·4	0·8	+	0·5	0·5	1D 69·5
8	0·2	0·2	0·5	0·5	0·2	0·2	78·5
9	+	+	0·2	+	+	+	2D
10	+	+	+	+	+	−	
11	−	+	−	−	−	−	
12	−	−	−	−	−	−	
13	−	−	−	−	−	−	
14	−	−	−	−	−	−	

*D – indicates death of mice in control group

B

Challenge infection and normal diet given 28 days after blood free of parasites of original infection

No. of days after reinfection	Percentage parasitaemias of individual mice						
	1	2	3	4	5	6	Mean of 4 controls
1	0·4	1·2	0·6	0·2	+	0·8	0·6
2	0·8	3·2	2·0	0·8	0·5	1·6	2·8
3	1·0	11·3	9·6	3·0	1·0	8·0	9·5
4	1·5	25·0	15·5	15·0	1·5	23·2	21·5
5	2·6	37·8	23·0	39·3	3·6	30·3	37·3
6	5·8	54·5	26·0	42·8	5·3	49·4	1D*54·0
7	14·0	71·0	33·5	45·0	13·4	52·0	1D 65·5
8	46·8	80·3	50·6	51·5	27·5	66·0	1D 68·6
9	67·0	dead	68·5	58·5	52·8	dead	1D
10	dead		dead	dead	59·3		
11					dead		
12							
13							
14							

*D – indicates death of mice in control group

It will be seen that in this case the superinfection lasted longer than the infections in the control animals (not previously infected). Thus, the four fatal infections lasted 14, 14, 14 and 19 days respectively and the infection in the survivors lasted 17, 18, 18 days respectively. More significantly, the infections in all animals developed slowly over the first week and the parasitaemia reached before death ranged from 15·6 to 36·3 per cent., i.e. was lower than that reached by the tenth and final day in the controls (69·5 to 74·0 per cent.). In the survivors, the build up of parasitaemia was subdued and peaks were reached on the

TABLE III

Showing mice infected with *P. berghei*

A

Infection and milk given at same time

No. of days after infection	Percentage parasitaemias of individual mice							
	1	2	3	4	5	6	7	Mean of 4 controls
1	0·5	0·75	0·5	0·5	+	0·2	0·5	2·0
2	4·0	3·2	1·2	3·3	0·3	0·8	2·0	6·0
3	3·3	4·2	2·4	2·3	1·5	1·8	1·8	23·5
4	1·3	4·3	3·0	1·5	2·6	1·6	1·0	40·5
5	0·8	2·0	1·5	0·3	3·2	0·8	0·3	55·2
6	0·5	0·5	0·8	+	2·5	1·0	+	1D68·0
7	0·25	+	0·3	0·5	0·1	0·25	+	2D69·5
8	+	+	+	0·3	0·2	+	−	73·5
9	+	+	+	+	+	−	−	1D
10	−	−	−	+	−	−	−	
11	−	−	−	−	−	−	−	

D – indicates death of mouse in the control group

B

Challenge infection and normal diet given 90 days after blood free from parasites of original infection

No. of days after reinfection	Percentage parasitaemias of individual mice							
	1	2	3	4	5	6	7	Mean of 4 controls
1	0·5	2·3	1·0	1·5	0·6	3·3	1·0	2·8
2	1·3	1·5	0·25	1·3	1·0	2·6	0·3	9·3
3	0·5	4·5	3·0	0·3	1·0	3·3	2·5	21·5
4	0·3	2·5	5·5	0·3	5·2	5·3	4·8	42·3
5	1·5	6·5	9·5	2·5	6·3	6·3	6·3	60·3
6	4·2	5·2	11·2	4·25	3·3	7·3	10·2	68·5
7	8·2	4·7	14·6	1·5	1·5	4·6	5·8	71·0
8	10·5	4·0	19·5	0·3	0·8	5·6	11·4	2D69·5
9	4·3	1·6	12·6	0·5	0·5	4·3	18·3	74·0
10	0·8	5·0	10·4	1·0	0·8	9·5	24·5	2D
11	1·0	11·5	14·2	+	0·25	5·8	30·3	
12	+	10·8	22·5	0·25	+	12·3	36·5	
13	+	13·2	36·3	+	0·5	18·6	34·5	
14	0·3	11·5	dead	−	+	dead	dead	
15	0·5	14·6		−	+			
16	+	16·2		0·25	+			
17	+	12·5		0·25	0·25			
18	−	15·6		+	+			
19	−	dead		−	−			
20	−			−	−			
21	−			−	−			
22	−			−	−			
23	−			−	−			
24	−			−	−			

D – indicates death of mouse in the control group

fifth day (6·3 per cent.), the sixth day (4·25 per cent.) and the eighth day (10·5 per cent.), respectively.

The delayed course and relatively low parasitaemias reached in the survivors and the slow development in the fatal cases indicated that some resistance to the development of the superinfection had occurred in these animals.

There was thus some antiparasitic activity present, evidenced particularly in the first few days of the infection. In the fatal cases, after this delay, the parasitaemia rose steadily to a moderate level at death. In the survivors the parasitaemia rapidly declined after reaching a low peak. It appeared to us that in the fatal cases the antiparasitic activity was not accompanied by the antitoxic activity indicated in the course of the infection in the survivors.

In the light of these results it was decided to see whether the resistance to superinfection in terms of both antiparasitic and antitoxic elements could be increased by increasing the density of parasitaemia, i.e. by exposing the mice to greater parasitaemia in the early stages of the original infection.

Effect of Superinfection in Mice in which the Original Infection was Halted by a Milk Diet

A batch of 30 mice on a normal diet were infected and groups of six were kept on the normal diet for two, three, four and five days respectively before transferring to a milk diet. One group of six mice remained on the normal diet as a control. The infection developed in all groups in the same way up to the time of changing diets. In the groups placed on milk on the second and third days, four of the six survived. In the other groups all the animals died.

It was decided therefore that the infection could be allowed to run for three days before its effects became irreversible and in subsequent experiments infected mice remained on the normal diet for three days before the infection was halted by placing them on the milk diet. In this way, parasitaemias of 30–40 per cent. were commonly reached by the third day and in some animals peak parasitaemias of over 50 per cent. were subsequently reached before the infection declined.

As noted above, some infected animals died after administration of the milk diet, but in the survivors the parasitaemia continued for a further 11 to 16 days, sometimes for over a week at 20 per cent. The technique thus provided considerably more exposure to much greater numbers of parasites than when the milk diet and the infection were begun at the same time.

One group of mice treated in this way was returned to a normal diet 14 days after parasites were last seen in the peripheral blood. After two days there was a short wave of parasitaemia which did not exceed five per cent. and thereafter the mice recovered. In a second group placed on a normal diet 28 days after parasites were last seen, only one of eight showed parasites which persisted for eight days with a maximum parasitaemia of 2 per cent. All animals recovered.

These experiments showed that after 14 days some parasites remained in the blood which caused a mild transitory parasitaemia on a PABA-containing diet but not a fatal infection, as in animals in which milk and infection had been started together. In the animals given the normal diet after 28 days, only one in eight had latent parasites. Thus, a considerable degree of antiparasitic activity had been acquired in the animals which had

been exposed to a short period of much higher and slightly longer-lasting parasitaemia than in the animals in which milk and infection were started together. The failure of seven of the eight animals restored to a normal diet after 28 days to develop parasitaemia indicates, moreover, that in these mice the protection had persisted although the infection had been overcome. This was not the case in the former groups of animals in which milk and infection were started together.

There was thus indication that allowing the infection to develop for three days before placing the mice on the milk diet led to the development of considerable resistance to recrudescence.

In a further series of experiments mice in which the infection had been halted in this way were subsequently exposed to superinfection, in order to study the possible development of resistance.

Infection was halted in nine mice from the third day, by placing them on a milk diet. Seven survived and continued on the milk for 20 days after the parasites disappeared from the blood (13–19 days after infection).

The mice were then challenged with the standard inoculation of infected erythrocytes and were placed on a normal diet.

The results are shown in Table IV.

TABLE IV

Mice infected with *P. berghei*

A*

Primary infection halted after three days by milk diet

No. of days after infection	Percentage parasitaemias of individual mice								
	1	2	3	4	5	6	7	8	9
3	28·5	36·0	31·5	30·0	27·0	32·0	27·0	29·5	22·5
4	40·0	61·0	57·0	24·6	40·5	51·5	46·0	37·0	35·0
5	60·0	67·0	40·0	24·0	18·0	63·0	43·0	44·0	51·0
6	48·0	41·0	18·5	14·0	3·5	44·0	24·5	23·5	24·3
7	53·5	26·5	21·3	5·0	7·0	19·0	36·3	15·5	8·0
8	69·0	49·0	27·5	+	0·8	1·0	57·0	10·0	+
9	79·0	dead	21·5	+	1·0	+	37·0	5·5	+
10	82·0		19·5	+	+	−	34·0	5·0	+
11	81·0		3·0	−	−	+	27·0	+	1·0
12	69·0		13·5	−	+	−	8·2	+	+
13	dead		9·5	−	−	−	2·0	+	+
14			4·25	−	−	−	+	−	−
15			+	−	−	−	+	−	−
16			+	−	−	−	−	−	−
17			−	−	−	−	−	−	−

B: See page 506

Two died, one (no. 9) after 24 days, the other (no. 5) after 27 days and with parasitaemias of 71·8 and 72·3 per cent. respectively. The parasitaemia developed slowly in both, suddenly reaching 51 per cent. in one on the sixth day and 32 per cent. in the other on the ninth day, i.e. more slowly than in the original infection. The five survivors developed moderate parasitaemias which appeared in two or three waves with maximum

peaks of 15·8 to 30·5 per cent. Parasites finally disappeared from the blood in 25–32 days and did not reappear.

The survival of seven out of nine mice after the challenge is in striking contrast to the deaths of six out of six animals challenged 28 days after the disappearance of the original parasitaemia in animals in which the infection was suppressed by offering a milk diet from the time of infection. It is worth noting in this respect that the infections in these cases were not prolonged, as were the infections induced by the challenge in the halted infections, even in the fatal cases.

(TABLE IV cont.)

B

Challenge infection and normal diet given 20 days after blood free from parasites of original infection

No. of days after re-infection	Percentage parasitaemias of individual mice						
	3	4	5	6	7	8	9
1	1·5	1·8	1·5	1·3	+	0·8	1·0
2	3·5	6·3	3·5	4·0	+	3·0	2·5
3	1·5	28·0	12·5	10·5	+	19·0	14·5
4	3·0	19·4	7·3	8·3	−	11·3	9·3
5	5·0	14·0	5·6	15·8	−	6·0	12·5
6	+		51·0	14·0	+	6·5	11·0
7	+	+	52·0	8·8	+	+	11·0
8	4·0	+	52·0	7·2	+	+	16·5
9	+	+	42·0	6·5	+	+	32·0
10	−	−	56·0	+	+	+	38·0
11	+	+	35·0	−	19·0	−	41·0
12	+	+	35·0	+	17·3	−	39·0
13	+	+	63·0	+	16·5	+	51·0
14	+	+	50·0	+	12·5	+	54·0
15	0·5	2·5	61·0	0·5	8·5	+	49·0
16	+	1·0	68·0	3·3	7·0	−	53·5
17	2·0	5·0	52·0	9·7	1·0	+	60·0
18	2·0	6·5	79·0	4·3	4·0	−	64·5
19	16·3	8·0	61·0	+	+	−	58·0
20	30·5	18·5	47·0	+	+	+	63·0
21	4·3	19·0	58·0	+	+	16·0	65·0
22	+	20·0	74·0	−	+	12·0	69·0
23	+	22·0	72·0	−	+	6·3	71·8
24	−	25·0	70·0	−	+	+	dead
25	+	15·0	42·5	−	0·5	+	
26	−	6·0	72·3	+	+	+	
27	−	+	dead	+	+	+	
28	−	+		−	−	−	
29	−	−		−	−	−	
30	−	−		−	+	−	
31	−	+		−	−	−	
32	−	−		−	−	−	
33	−	−		−	−	−	
34	−	−				−	
35	−	−				−	

These results demonstrate considerable antiparasitic resistance to the challenge infection. The high prolonged terminal parasitaemias in the fatal cases suggest that this could eventually be overcome but that, under these circumstances, there was some degree of antitoxic protection.

A similar experiment was conducted in nine survivors from a group of 10 mice in which the original infection was halted after the third day by placing the animals on a milk diet. These mice were challenged 40 days after the final disappearance of parasites and were at the same time restored to a normal diet. Four died in 14–22 days, with parasitaemias of 86·0, 71·0, 49·0 and 14·8 per cent. respectively. Five survived, finally losing parasites from the blood in 44 to 55 days. All were parasite-negative at 65 days. The peak parasitaemias in two were much higher than in the survivors of the challenge at 20 days. Survival after parasitaemia of 73 per cent. and 46 per cent. respectively and particularly in no. 7 after a practically continuous high parasitaemia for over 40 days was remarkable and indicated some protection against toxicity in animals in which the antiparasitic protection was apparently inadequate.

TABLE V

Mice infected with *P. berghei*

A*

Primary infection halted after three days by a milk diet

No. of days after infection	Percentage parasitaemias of individual mice									
	1	2	3	4	5	6	7	8	9	10
3	33·0	36·0	41·5	50·5	36·5	16·5	25·5	38·0	38·5	43·5
4	24·0	29·0	31·0	42·5	19·5	24·3	12·3	20·0	22·0	22·3
5	5·0	10·0	7·5	30·3	9·0	9·3	9·0	6·0	10·3	10·0
6	0·5	0·5	0·3	20·0	4·5	1·5	4·8	2·25	4·8	2·0
7	+	4·5	+	12·0	2·5	0·8	1·8	1·3	6·0	1·3
8	0·5	+	+	1·5	1·3	+	2·3	1·5	0·3	1·0
9	0·3	+	+	+	0·8	+	0·5	0·5	+	0·5
10	+	+	−	+	+	+	+	+	−	+
11	+	+	−	+	+	+	+	−	−	+
12	−	dead	−	−	−	−	−	−	−	−
13	−		−	−	−	−	−	−	−	−
14	−		−	−	−	−	−	−	−	−

B: See page 508

The figures are too few for comparison, but there is a suggestion that the resistance at 20 days may have been stronger than at 40. Further work is needed on this point.

The results obtained following challenge 20 days after a halted first infection certainly showed the presence of much stronger resistance than that developed after 28 days in the first set of experiments, which was negligible. They are difficult to compare with the results after 90 days in animals in which the first infection had been checked from the beginning by a milk diet, since death occurred in fatal cases at about the same time but the parasitaemias were less prolonged in survivors.

Comparing the results shown in Tables II and IV it appeared that the low initial parasitaemia followed by latent infection which continued for at least 20 days produced no appreciable resistance, whereas the high initial parasitaemia and moderate parasitaemia lasting up to 16 days with roughly the same latent period produced considerable resistance.

The results in the experiments in which the challenge was given 40 days after the blood had cleared in the primary halted infection also show a high degree of resistance in circumstances in which the latent infection had almost certainly ceased before the challenge. The resistance thus must have persisted in the absence of parasites (Table V).

The indication is that the resistance was built up in the latter experiments more by the initial higher density of the infection than by its prolongation.

TABLE V (cont.)

B

Challenge infection and normal diet given 40 days after blood free from parasites of original infection

No. of days after infection	Percentage parasitaemias of individual mice								
	1	3	4	5	6	7	8	9	10
1	1·0	0·5	0·5	—	—	—	—	0·5	0·5
2	4·0	7·0	0·8	—	5·5	0·3	2·0	1·5	0·3
3	7·8	20·0	10·5	+	13·5	4·5	8·5	2·0	4·0
4	23·0	33·3	27·6	0·3	20·0	20·5	26·0	9·3	8·3
6	14·0	32·0	22·3	3·5	10·5	22·0	18·3	11·5	11·0
7	2·5	35·0	30·3	16·3	2·5	14·3	2·0	9·0	4·5
8	0·5	54·0	41·0	27·0	+	8·0	+	13·8	3·8
9	+	64·3	34·0	21·0	+	17·0	+	37·5	1·5
10	+	61·0	42·0	21·0	+	39·0	—	61·0	+
11	—	74·3	58·0	42·0	—	31·8	—	61·0	+
13	—	49·0	47·0	41·0	—	27·0	—	73·8	+
14	—	dead	81·0	31·5	—	29·5	—	48·0	—
15	—		61·0	41·0	—	50·0	—	59·8	—
16	—		71·0	21·0	—	61·3	+	52·5	—
17	—		dead	10·3	+	73·0	+	72·0	—
18	—			4·8	14·0	48·0	+	79·3	—
19	+			8·3	21·8	41·5	—	79·0	+
20	+			10·5	42·0	40·6	—	86·0	—
21	+			14·8	10·5	33·3	—	dead	+
22	5·0			dead	+	44·3	+		+
23	5·0				+	65·0	+		+
24	0·8				13·5	20·0	+		+
25	4·5				8·0	31·0	+		6·4
27	+				+	24·3	—		12·6
28	—				29·0	20·8	—		41·0
29	—				23·5	19·0	—		35·0
30	—				23·0	15·5	—		46·0
31	—				12·3	27·5	—		28·5
32	+				+	31·3	+		6·8
34	+				+	43·0	0·8		+
35	+				+	52·0	2·5		+
36	0·5				—	39·0	9·0		+
37	+				—	32·0	22·0		—
38	3·5				—	31·0	12·0		—
39	4·0				—	30·0	11·0		—
41	1·5				—	16·5	2·5		—
43	0·5				+	5·0	+		—
44	+				2·5	+	+		—
45	—				8·5	—	—		—
48	—				2·2	—	—		—
50	—				6·0		—		—
51	—				10·0		—		—
55	—				+		—		—
60	—				—		—		—
65	—				—		—		—

To test this hypothesis an experiment was carried out in a group of 10 infected mice which were placed on a milk diet on the third day of infection and, after a further three days were returned to normal diet for three days and then to the milk diet until the challenge

infection. The pattern produced in this way was a peak parasitaemia of about 30 per cent. reached on the fourth-fifth day and a fall to about 13 per cent. by the seventh day. The parasitaemia rose to over 15 per cent. for the next three days then fell slowly to disappear about the sixteenth day. Thereafter the blood remained negative until the challenge infection was given 20 days after the parasites were last seen, at which time also the normal diet was restored. The challenge did not kill any mice and the subsequent parasitaemia reached a mean maximum of 10 per cent. in five days and fell to zero by the 21st day.

Strong resistance could be produced by this technique but in the few experiments carried out it was not possible to decide whether this resistance was greater than that produced in the experiments detailed in Tables IV and V.

Further work on these lines would be rewarding.

Although it would appear that an initial high parasitaemia produced a powerful resistance to the effects of superinfection, and that the original density of infection was thus probably an important factor in the production of resistance, the results of the experiments in which challenge at 90 days followed simultaneous infection and milk suppression showed that a low initial parasitaemia followed by long exposure to a latent infection may have similar effects.

Control of Parasitaemia by Chloroquine

To examine the significance of short exposure to relatively high parasitaemia, experiments were carried out in which the initial infection was stopped by chloroquine.

For example, 10 mice were infected and kept on a normal diet. From the third day onwards each mouse received chloroquine sulphate 20 mgm. per kgm. once daily intramuscularly for five consecutive days.

By the second day of treatment, that is the fifth day of infection, parasites had disappeared from the peripheral blood. Over the next 20 days four mice developed recrudescences and died. The remainder were challenged 20 days after the parasites had disappeared from the blood. All developed characteristic infections and died within 14 days, with parasitaemias ranging from 50 to 68 per cent.

There was thus no evidence of the acquisition of resistance, indicating that a short high parasitaemia was not enough to promote measurable resistance. It seems that the duration of the first infection is important as well as the density.

The existence of resistance after 90 days in the first set of experiments and after 40 days after the halted infections support Kretschmar's observations (1964) that the 'immunity' produced persists for some time. The latter experiment indicates, moreover, that once stimulated by the parasites it persists after the termination of the latent infection (Kretschmar 1962, 1963).

Measurements of total protein and gamma globulin were made in groups of mice (i) on a normal diet, (ii) on a milk diet started at the same time as the infection, (iii) on a normal diet for the first three days of infection followed by a milk diet (halted infection) and (iv) in mice challenged after a halted infection.

The changes in the infected animals on a normal diet corresponded to those described by Sadun *et al.* (1965) and Tella and Maegraith (1965).

Very similar changes were observed in animals put on milk at the time of infection. In animals in which the infection was halted by a milk diet after the third day, there was a

more pronounced rise in gamma globulin by the sixth day, which persisted to the fourteenth day.

In the mice in which infection had been halted, the high levels of gamma globulin (about three times the normal level) reached by the fourteenth day persisted until the challenge infection, During the challenge the gamma globulin reached a level by the sixth day of about twice that in other groups; this level persisted to the fourteenth day after the infection.

DISCUSSION

Previous work on the suppression of *P. berghei* infection which follows placing the host on a milk diet was confirmed.

It was found that suppression was not accelerated by placing the animals on the milk diet a week before infection. The control of parasitaemia was the same when the milk diet was started at the time of infection. This indicated that in the normal body tissues there was little free PABA available for the parasites and that in the absence of an external source of PABA the developing parasites rapidly use up the available supplies.

Infections which had been suppressed by a milk diet reappeared and killed the host on restoration of a normal diet after 14 days from the disappearance of the original parasite infection from the blood. This indicated that no measurable resistance to recrudescence had been acquired in that time.

The experiments in which infection and suppression were commenced simultaneously and in which a challenge infection by the same strain was offered at 28 days after disappearance of the original parasitaemia showed that there was no resistance to superinfection at this stage. After 90 days, however, there was clear evidence of some resistance.

We concluded that this resistance had been built up as a result of exposure to the original brief parasitaemia followed by long exposure to a latent infection.

It was found that the original infection could be suppressed successfully in most animals by transferring the mice to the milk diet not later than the third day of the overt infection. At this point a considerable peak of parasitaemia had developed and moderate parasitaemia continued until the infection was controlled. On return to a normal diet up to 20 days following such halted infections, there were no deaths although there was some mild recrudescence in one of eight animals after 28 days. This indicated that allowing the original parasitaemia to develop before suppression by milk led to the removal of the infection in most animals after three to four weeks. Furthermore, it built up a greater resistance to the effects of subsequent recrudescence than was established when the original parasitaemia was suppressed from the beginning by the milk diet, since in the latter circumstances the recrudescences at the same point after clearance from the blood of the original infection were all fatal.

The presence of this resistance was demonstrated when the animals were challenged with the same strain of *P. berghei* at 20 and 40 days after the original halted infection. The delayed deaths in the fatal cases and the recovery of some animals indicated the presence of strong resistance. This appeared to be mainly antiparasitic although there was some evidence in the few animals which survived very high parasitaemia that there was also an antitoxic element.

Further experiments in which halted infection was allowed to recrudesce for a few

days before finally being suppressed showed that the high parasitaemia attained before suppression was followed by notable resistance on challenge 20 days later without fatal issue.

Challenge after infection which was controlled in the first five days by chloroquine showed no evidence of resistance, indicating that a short exposure to moderate parasitaemia was not followed by detectable resistance to superinfection. The fatal recrudescences following chloroquine therapy likewise showed absence of resistance to the parasites, in contrast to the resistance demonstrable to recrudescence in the halted infections in which the original parasitaemia had persisted for 10 – 16 days.

We conclude from these experiments that the protection against superinfection with *P. berghei* developed in a primary infection is dependent on both the density of the original parasitaemia and on its duration. This view is somewhat contrary to that expressed by other authors who have stressed the importance of the duration of the original patent infection without reference to its density (Kretschmar, 1962; Jerusalem, 1966).

Immunity against *P. berghei* infection in mice has been produced by Weiss and De Giustis (1966) using 'vaccination' with a strain of parasite which was active in rats but had lost its infectivity for mice. On infection with a virulent strain which killed normal mice, the 'vaccinated' animals exhibited high degrees of both antiparasitic and antitoxic immunity. Jerusalem (1968) has recently shown that antitoxic immunity may be established without the antiparasitic equivalent. Some of our results point to the existence of both forms of immunity in our animals with halted infections, for instance, the development in some mice of very high parasitaemia without fatality and in others, death at a relatively low parasitaemia.

We consider that the manoeuvres described in this paper amount to using a highly virulent strain of *P. berghei* as a 'living vaccine' in the susceptible host. This effect can be produced solely by providing the animal with a PABA deficient diet, in this case milk. There are completely opposite views on whether such a phenomenon can occur in nature in man (McGregor *et al.*, 1956; Gilles, 1957; Kretschmar, 1964) by the suppression of *P. falciparum* infection by breast feeding. The demonstration of antibodies in milk (Bruce-Chwatt, 1954) provides one explanation of the control of malaria in infants. It does not exclude the possibility that other factors such as milk-suppression which has such striking effects experimentally, are also at work. It seems about time that this problem was resolved.

SUMMARY

1. Infection with *Plasmodium berghei* in mice was suppressed by placing the host on a milk diet (a) as from the time of infection and (b) three days after the overt infection had been established (halted infection.)

2. Challenge infections with the same strain of *P. berghei* were given at various times after the parasites of the original infection had disappeared from the blood.

3. Resistance to the challenge was observed at 90 days after infection controlled simultaneously by the milk diet, but not earlier.

Resistance to the challenge was observed 20 days and 40 days after the halted infection. Strong resistance was also demonstrated in animals in which the original infection was allowed to persist longer by alternating normal and milk diets.

4. It was concluded that resistance to superinfection, in which there were both

antiparasitic and antitoxic elements, was developed in animals in which the infection was suppressed by placing the host on a milk diet and that the resultant resistance was dependent on both the density of the original infection and its duration.

REFERENCES

ADLER, S. (1958). The effect of a meat diet on the course of infections with *Plasmodium vinckei* in mice. *Bull. Res. Coun. Israel*, Vol. 7E, 9.

BRAY, R. S., and GARNHAM, P. C. C. (1953). Effect of milk diet on *P. cynomologi* infections in monkeys. *Br. med. J.*, **1**, 1200.

BRUCE-CHWATT, L. J. (1954). *Plasmodium berghei* in the placenta of mice and rats: Transmission of specific immunity from mother rats to litters. *Nature, Lond.*, **173**, 353.

FABIANI, G., and ORFILA, J. (1954). Action du régime lacté sur le paludisme expérimental de la souris blanche. *C. r. Séanc. Soc. Biol.*, **148**, 1239.

——— ——— (1955). Apparition de l'immunité contre *Plasmodium berghei* chez les souris soumises au régime lacté ou a la sulfamidothérapic. *Annls. Inst. Pasteur Paris*, **88**, 108.

GILLES, H. M. (1957). The development of malarial infection in breast-fed Gambian infants. *Ann. trop. Med. Parasit.*, **51**, 58.

HAWKING, F. (1953). Milk diet, p-aminobenzoic acid and malaria (*Plasmodium berghei*). Preliminary communication.

——— (1954). Milk, p-aminobenzoate, and malaria of rats and monkeys. *Br. med. J.*, **1**, 425.

——— and TERRY, R. J. (1957). *Plasmodium berghei*, milk diet and p-hydroxybenzoate. *Z. Tropenmed. Parasit.*, **8**, 151.

JACOB, R. L. (1964). Role of p-aminobenzoic acid in *Plasmodium berghei* infection in the mouse. *Expl Parasit.*, **15**, 213.

JERUSALEM, C. (1966). Dauer der Parasitämie (*Plasmodium berghei*) und grad der Immunität bei Swiss-Mausen. *Z. Tropenmed, Parasit.*, **17**, 210.

——— (1968). Active immunization against malaria (*Plasmodium berghei*). I: Definition of antimalarial immunity. *Z. Tropenmed. Parasit.*, **19**, 171.

KRETSCHMAR, W. (1962). Resistenz und immunität bei mit *Plasmodium berghei* infizierten mausen. *Z. Tropenmed. Parasit.*, 13, 159.

——— (1963). Wietere untersuchungen über die Immunität bei der Nagetiemalaria. *Z. Tropenmed. Parasit.*, **14**, 41.

——— (1964). The effect of stress and diet on resistance to *Plasmodium berghei* and malarial immunity in the mouse. Int. Colloq. on *Plasmodium berghei*. Antwerpen (1964).

——— (1966). Die bedeutung der p-aminobenzœsäure fur den krankheitsverlauf und die immunität bei der malaria im tier (*Plasmodium berghei*) und im menschen (*Pl. falciparum*). II: Untersuchungen ein natürlich ernährten kleinkindern in Nigeria. *Z. Tropenmed. Parasit.*, **17**, 369.

——— (1967). The significance of p-aminobenzoic acid for the course of infection and immunity in animal malaria (*Plasmodium berghei*) and that of man (*Plasmodium falciparum*). Investigations with NMRI mice. *Z. Tropen med. Parasit.*, **17**, 301.

MAEGRAITH, B. G., DEEGAN, T., and JONES, E. S. (1952). Suppression of malaria (*P. berghei*) by milk. *Br. med. J.*, **2**, 1382.

MCGREGOR, I. A., GILLES, H. M., and FULLER, A. T. (1956). Human malaria, milk and p-aminobenzoate. *Proc. Conf. Com. Tech. Af.* Sehara Luanda (1956).

MCKEE, R. W., and GEIMAN, Q. M. (1948). Methionine in the growth of the malaria parasite *Plasmodium knowlesi*. *Fedn Proc. Fedn Am. Socs exp. Biol.*, **7**, 172.

RAMAKRISHNAN, S. P., PRAKASH, S., KRISHNASWAMI, A. K., and SINGH, C. (1953). Studies on *Plasmodium berghei* N. sp. Vincke and Lips 1948. IX: Effect of milk diet on the course of blood-induced infection in albino rats. *Indian J. Malar.*, **7**, 61.

SADUN, E. H., WILLIAMS, J. S., MERONEY, F. C., and HUTT, G. (1965). Pathophysiology of *Plasmodium berghei* infection in mice. *Expl Parasit.*, **17**, 277.

TELLA, A., and MAEGRAITH, B. G. (1965). Physiopathological changes in primary acute blood-transmitted malaria and *Babesia* infections. II: A comparative study of serum protein levels in infected rhesus monkeys, mice and puppies. *Ann. trop. Med. Parasit.*, **59**, 153.

WEISS, M. L. and DE GIUSTIS, D. L. (1966). Active immunization against *Plasmodium berghei*. Malaria in mice. *Am. J. trop. Med. Hyg.*, **15**, 472.