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SUPPRESSION OF AUTOIMMUNE DISEASE IN NEW ZEALAND MICE ASSOCIATED WITH INFECTION WITH MALARIA

II. NZB MICE

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SUMMARY

The onset of a positive Coombs test was significantly delayed in NZB mice infected with *Plasmodium berghei* at the age of 1 month. At the age of 12 months, twelve out of fourteen malaria-infected NZB mice had become Coombs positive but their reticulocyte counts and mean spleen weight were significantly lower than those of control NZB mice of the same age.

Malaria infection of young NZB mice was associated with a significant increase in mean 24-hr urine protein excretion which fell gradually over the next 6 months. A secondary rise in urine protein excretion then occurred and at 12 months several malaria-infected NZB mice had severe renal disease.

No increased incidence of malignant disease was observed in NZB mice infected with malaria.

INTRODUCTION

Epidemiological surveys have established a relationship between the frequent occurrence of the nephrotic syndrome in some parts of the tropics and infection with *P. malariae* (Gilles & Hendrickse, 1963). Epidemiological studies have also suggested an association between malaria infection and Burkitt's lymphoma (Dalldorf *et al.*, 1964; Burkitt, 1969) and between parasitic infection and a low incidence of autoimmune disease (Greenwood, 1968). The spontaneous occurrence of complex nephritis, autoantibodies and lymphomas in NZB mice suggested that mice of this strain would be a good model in which to investigate the effects of experimental malaria infection on the development of this group of conditions. In a previous paper (Greenwood & Voller, 1970) we have described how infection with *P. berghei* at the age of 1 month old, protected (NZB × NZW) F_1 hybrid mice (B/W mice) from their spontaneous autoimmune disease. In this paper we describe the effects of malaria infection on the spontaneous disease of NZB mice.

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MATERIALS AND METHODS

Mice

Male and female NZB mice from the Taplow colony were studied, comparable numbers of mice of each sex being included in test and control groups. Control data have been derived from mice studied at Taplow over a number of years. A small group of four control mice were studied in parallel with the mice infected with malaria; the course of their disease closely followed that previously observed in larger control groups.

Malaria infection

Mice were infected at the age of 1 month with *P. berghei yoelii* and the course of their infection was followed as previously described (Greenwood & Voller, 1970).

Haematology

Haematocrit (PCV) and reticulocyte counts were measured in malaria-infected mice at monthly intervals and in groups of control mice of different ages. Direct Coombs tests were performed at monthly intervals using a rabbit anti-mouse globulin shown on immuno-electrophoresis to possess activity against mouse IgG and IgM. The antiserum was absorbed with washed mouse erythrocytes and diluted 1 : 5 before use. NZB cells were washed four times in phosphate buffered saline (PBS) and tested at a strength of 5–10%. The pattern of agglutination was read macroscopically.

Antinuclear factor

Sera from malaria-infected and control mice were tested at monthly intervals for the presence of antinuclear factor (ANF) using the technique previously described (Greenwood & Voller, 1970).

Renal disease

Proteinuria. 24-hr urine specimens were collected from malaria-infected and control mice at monthly intervals, the protein content of an aliquot measured by the method of Kingsbury *et al.* (1926) and 24-hr urine protein excretions determined. Cellulose acetate electrophoresis was performed on concentrated aliquots of 24-hr specimens found to contain a high content of protein on quantitative testing.

Renal pathology

Kidneys from sacrificed mice were examined by light, fluorescent and electron microscopy as previously described (Greenwood & Voller, 1970). Cryostat sections from the kidneys of malaria-infected NZB mice were also examined for the presence of malaria antigen using a conjugate prepared from sera of rats that had recovered from a heavy infection with *P. berghei.* This conjugate produced fluorescence of malaria parasites when applied to blood films prepared from mice with *P. berghei* parasitaemia.

Protein was eluted from the kidneys of five mice killed shortly after malaria infection by the method described by Lambert & Dixon (1968). A whole kidney was minced in saline and washed four times in PBS. The minced tissue was then incubated in 0·02 M citrate buffer, pH 3·2, at 37°C for 90 min. The suspension was then cooled to 4°C and the molarity of the solution re-adjusted to 0·15 M saline. After centrifugation at 3500 *g* for 15 min the

supernatant was removed and dialysed against PBS. Globulin was precipitated from the eluate with ammonium sulphate solution and the precipitate redissolved in PBS and dialysed against PBS overnight. The protein content of the eluate was estimated by the method of Lowry *et al.* (1951) and the nature of the protein present investigated by cellulose acetate electrophoresis. The eluate was tested for the presence of ANF and malaria antibody.

RESULTS

Malaria infection

All twenty-one NZB mice (ten male and eleven female) inoculated with 1×10^6 parasitized red cells, obtained from a heavily infected mouse of the same strain, developed malaria parasitaemia which persisted for a mean of 19 days (range 18–22 days). Most of the mice developed a heavy infection and mean maximal parasitaemia was 1570 parasites/10^4 red blood cells with a range of 600–2920 parasites/10^4 red blood cells. Pooled blood samples taken 3 months after infection did not induce a malaria parasitaemia when sub-inoculated into young BALB/c mice. Malaria antibody was still demonstrable on fluorescent testing many months after infection (Table 1).

TABLE 1. Malaria antibody levels in NZB mice infected with *P. berghei yoelii* when 1 month old

Age (months)	No. tested	Reciprocal of antibody titre			
		<50	50	100	200 or >
3	15	0	0	0	15
6	7	0	0	2	5
12	9	0	1	3	5

Haematological findings

Coombs test. The onset of a positive Coombs test in the malaria-infected mice was delayed in comparison with uninfected controls (Fig. 1). At 9 months only four out of sixteen malaria-infected NZB mice had become Coombs positive in contrast to thirty-two out of thirty-six ($\chi^2 = 20{\cdot}7$; $P<0{\cdot}001$). However, by 12 months, most of the malaria-infected NZB mice had become Coombs positive. The possibility that the results of the Coombs test might have been biased by the killing of five mice 2 or 3 months after malaria infection was considered. Even if these mice had all been Coombs positive at the age of 9 months the incidence of Coombs positivity in malaria-infected mice at this age would still have been significantly lower than in the controls.

Reticulocyte count. During and shortly after the period of malaria parasitaemia a high reticulocyte count was recorded in malaria infected NZB mice, but this had returned to normal 2 months after infection (Table 2). The mean reticulocyte count of the malaria infected NZB mice began to rise from the age of 8 months onwards but at a slower rate than that observed in control NZB mice. At the age of 12 months significantly fewer malaria infected mice had a reticulocyte count of over 10% (2/14) than did control of the same age (9/15) ($\chi^2 = 6{\cdot}5$; $P = 0{\cdot}01$) (Fig. 2).

Haematocrits. The mean PCV of malaria infected NZB mice fell sharply during the period of peak malaria parasitaemia but 1 week after parasites had disappeared from the peripheral blood the PCV had risen to a mean value of 40% (Table 2). A gradual increase in mean PCV

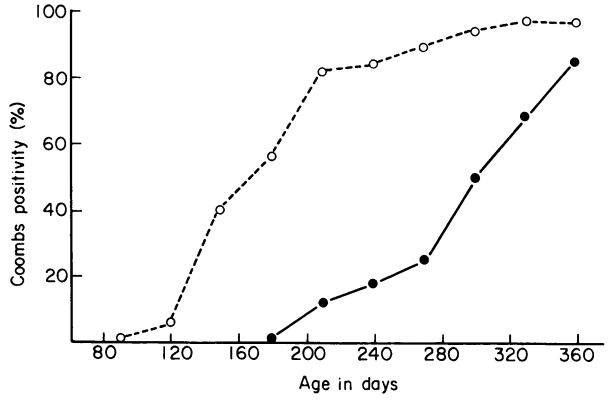

FIG. 1. Cumulative incidence of a positive Coombs test in sixteen malaria-infected NZB mice and in thirty-six NZB controls of the same sex distribution. ●, malaria infected; ○, controls.

TABLE 2. Mean PCV and reticulocyte count in malaria-infected NZB mice and in sex matched NZB controls

	Malaria-infected mice			Control Mice		
Age in months	No. tested	Mean PCV (%)	Mean reticulocyte count (%)	No. tested	Mean PCV (%)	Mean reticulocyte count (%)
1	21	46	2·7			
2	21	39	5·0			
3	18	40	1·7	15	48	1·2
4	16	40	2·4			
5	16	41	1·3			
6	16	43	2·8	15	49	1·4
7	16	43	1·8			
8	16	45	4·7			
9	16	42	5·0	15	41	10·4
10	16	40	8·5			
11	16	39	10·0			
12	14	39	9·4	15	37	18·7

then occurred but for some months the mean PCV remained below normal levels. At 8 months a further fall in PCV occurred in association with the development of Coombs positivity. At the age of 12 months the mean PCV of fourteen malaria infected NZB mice (39%) was only slightly higher than the mean PCV (37%) of fifteen age and sex matched controls.

Serum antinuclear factor

No significant difference was observed in the development of homogeneous staining ANF in malaria infected NZB mice and NZB controls. At the age of 10 months one serum was positive at a dilution of 1 : 10 in each group and at the age of 12 months three out of fourteen malaria infected NZB mice had become positive and two of the age and sex matched controls. Sera from eight malaria infected NZB mice showed speckled staining ANF at some stage after malaria infection, as previously noted in CBA mice (Greenwood, Herrick & Holborow, 1970).

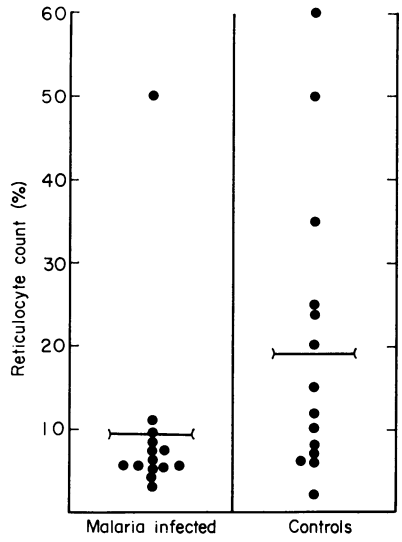

FIG 2. Reticulocyte count in fourteen malaria-infected NZB mice at the age of 12 months and in fourteen age and sex matched controls.

Early renal disease

Malaria infection was associated with a sharp rise in the mean 24-hr urine protein excretion of twenty-one young NZB mice (Fig. 3). The mean 24-hr urine excretion (3·6 mg/24 hr) 1 month after inoculation with malaria (1 week after disappearance of malaria parasites from the peripheral blood) was significantly higher than the mean 24-hr urine excretion of ten control mice of the same age (mean 1·4 mg/24 hr) ($t = 3{\cdot}0$; $P<0{\cdot}01$). Electrophoresis of urines with a high protein content showed that the urine protein was mainly albumin and pre-albumin, although small quantities of γ-globulin were present in some samples.

Five mice with elevated 24-hr urine protein excretions were killed 1 or 2 months after malaria inoculation and their kidneys examined for features of complex nephritis. Microscopic appearances were compared with those observed in the kidneys of five control NZB mice killed at the same age.

On light microscopy sections from the kidneys of the five control mice showed small mesangial deposits of PAS positive material but no basement membrane thickening or cellular infiltration with round cells. Sections from the five malaria-infected mice showed

similar small mesangiala accumulations of PAS positive material and in two kidneys these deposits were much more marked than those observed in any of the controls. Small aggregates of round cells were seen in three of the five kidneys. There were no histological features of pyelonephritis.

On fluorescent microscopy small deposits of fluorescent material were observed in the mesangia of some glomeruli of all kidneys from control NZB mice and young BALB/c mice as noted by Aarons (1964). These deposits were stained with both an anti-mouse whole immunoglobulin conjugate and an anti-mouse IgG conjugate but only occasional flecks of staining were observed with an anti-mouse β_1C conjugate. The degree of staining was greatly reduced by prior absorption of the anti-globulin conjugate with mouse γ-globulin. Similar deposits were seen in the kidneys of malaria-infected mice where they appeared to be

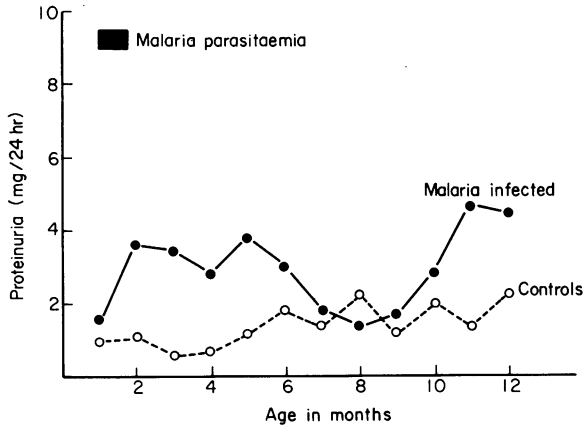

FIG. 3. Mean 24-hr urine protein excretion in fifteen to twenty-one malaria-infected NZB mice and in control NZB mice of different ages (six to ten mice in each control group). ●, malaria-infected; ○, controls.

more marked. In the kidneys of two of the malaria infected mice, some glomeruli contained large deposits staining with anti-mouse γ-globulin, anti-mouse IgG and anti-mouse β_1C conjugates. Considerable variation was observed in the extent of mesangial staining within different glomeruli from the same kidney. Representative glomeruli from control and malaria infected mice are illustrated in Fig. 4. Deposits were restricted to the mesangial region where they often had a granular appearance; discrete basement membrane staining was not observed. On staining with a rat anti-malarial conjugate no significant fluorescent staining was observed.

Electron micrographs prepared from four of the kidneys obtained from the malaria-infected mice showed areas of fusion of foot-processes, patchy thickening of the basement membrane and in one set of micrographs marked localized homogeneous expansions of the basement membrane. Electron dense deposits were not seen.

Eluates from kidneys of the five malaria-infected mice were found to contain 0·3–2·0 mg of protein and on electrophoresis the presence of γ-globulin and some denatured protein was demonstrated. None of the eluates contained ANF. Eluates from two kidneys gave bright fluorescent staining of *P. berghei* in blood films in an indirect immunofluorescent

test but the significance of this finding is uncertain owing to the tendency of the conjugate alone to produce some non-specific staining in the presence of solutions of low protein content.

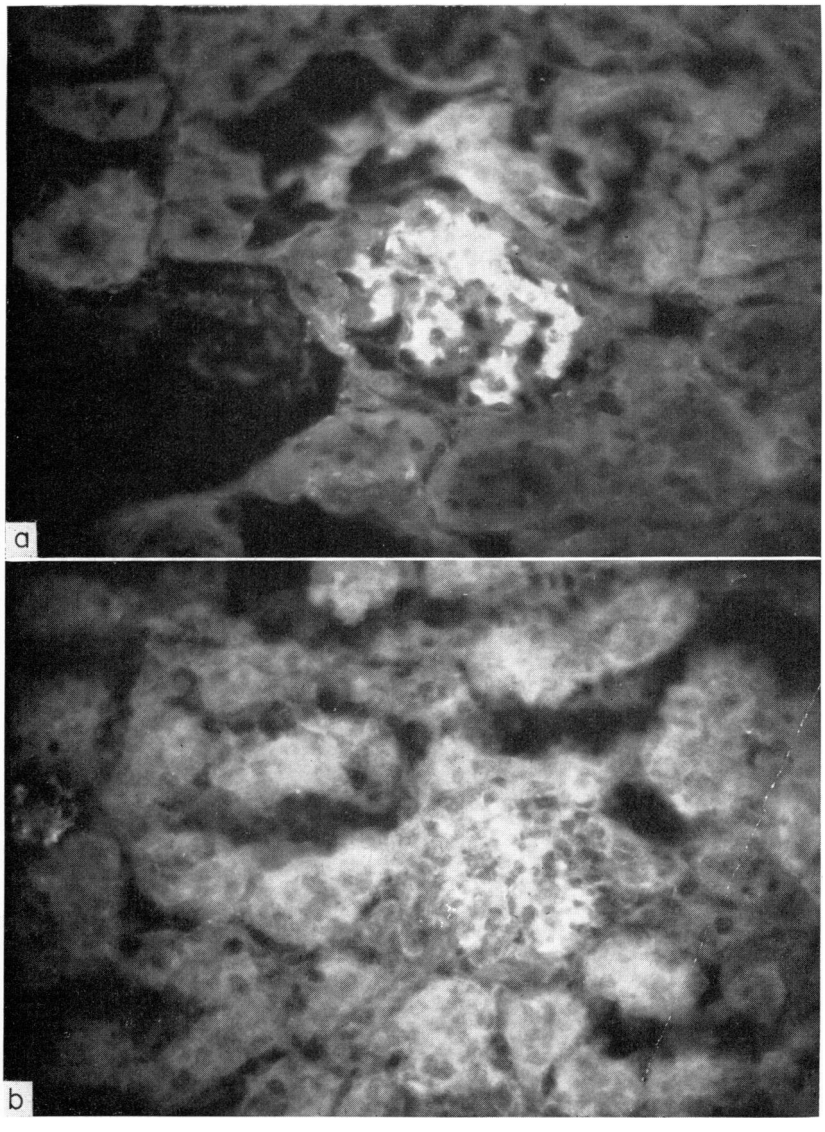

FIG. 4. Renal glomeruli stained for mouse γ-globulin by immunofluorescence from (a) malaria-infected NZB mouse aged 2 months (b) control NZB mouse aged 2 months.

Mortality

Five of the twenty-one malaria-infected mice were deliberately killed when 2 or 3 months old as described above. One apparently healthy mouse died suddenly when 11 months old. This mouse did not have severe anaemia or proteinuria and the cause of its death was not

apparent. The remaining fifteen malaria-infected mice were killed when 12 months old and the post-mortem findings in these mice have been compared with those found in control NZB mice killed at the same age.

Late renal disease

A gradual fall in the mean 24-hr urine protein excretion of the malaria-infected NZB mice occurred 3 months after the disappearance of malaria parasites from the peripheral blood and by 8 months the mean 24-hr urine protein excretion was similar to that found in control NZB mice of the same age (Fig. 3). However, a secondary rise in 24-hr urine protein excretion occurred from the age of 9 months and at 12 months malaria-infected NZB mice had a higher mean 24-hr urine protein excretion than NZB controls. The difference in means (4·5 mg/24 hr compared with 2·3 mg/24 hr) is, however, not statistically significant ($t = 1{\cdot}4$; $P = 0{\cdot}2$).

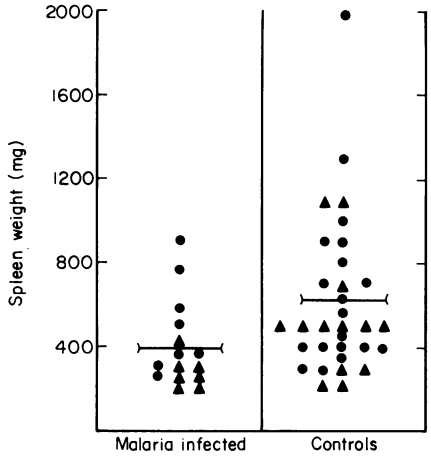

FIG. 5. Spleen weight of fifteen malaria-infected NZB mice killed at 12 months and of thirty-two control NZB mice of the same age and sex distribution. ▲, male; ●, female.

Kidneys from all fifteen malaria-infected NZB mice killed at 12 months old showed abnormalities on light microscopy and these were frequently marked. On PAS staining basement membrane thickening was seen in some glomeruli from each kidney and mesangial deposits of PAS positive material were also seen in some glomeruli of each kidney. In eleven of the fifteen kidneys these deposits were marked and in seven kidneys some glomeruli were completely replaced by amorphous material. Cellular infiltration with round cells was seen in eleven kidneys and was often marked. These changes were similar to those seen in kidneys from control NZB mice of the same age and were at least as severe and possibly more marked than in the controls.

Deposits of immunoglobulin and complement were seen in some glomeruli of the kidneys of all fifteen malaria-infected NZB mice killed at 1 yr. In nine cases the deposits of γ-globulin were marked and in seven kidneys heavy deposits of complement were also seen. In these cases fluorescent staining of both the basement membrane and the mesangium was seen, although the latter was usually more obvious and often had a granular appearance. Although the extent of the immunoglobulin deposits varied considerably between different glomeruli in the

same kidney, the overall impression obtained was that the deposits were more extensive in malaria-infected NZB mice than in control NZB mice of the same age.

Terminal pathology of lymphoid system

The mean weight of the spleen of fifteen malaria-infected mice killed at 12 months (390 mg ± 200 mg SD) was found to be significantly less than the mean spleen weight (625 mg ± 370 mg SD) of thirty-two control NZB mice of the same age and sex distribution ($t = 2{\cdot}8$; $P = 0{\cdot}01$) (Fig. 5). The difference between the mean spleen weight (270 mg ± 67 mg SD) of seven male malaria-infected NZB mice and the mean spleen weight (545 mg ± 126 mg SD) of thirteen male NZB controls of the same age was particularly striking ($t = 3{\cdot}6$; $P = 0{\cdot}005$–$0{\cdot}001$).

On histological examination some abnormality was found in all fifteen spleens from malaria-infected NZB mice. In each case some proliferation of the white pulp was seen with a tendency to form prominent germinal centres. Changes in the red pulp were less marked; an increase in the number of reticulum cells was observed in most spleens but sheets of pale reticulum cells were observed in only five spleens. Extensive hyperplasia of the red pulp was not observed. Spleens from malaria-infected mice contained much larger amounts of pigment than the spleens of control NZB mice and this was present mainly in the red pulp. Heavy deposits of pigment were also seen in the Kupffer cells of the liver.

Proliferative changes were observed in inguinal and mesenteric nodes from most malaria-infected mice but frankly malignant changes were seen in only one animal. At post-mortem this mouse was found to have a generalized lymphadenopathy and on histological examination many nodes were found to be totally replaced by primitive lymphoid cells showing frequent mitoses. Malignant tumours of the thymus were not found.

DISCUSSION

Complex nephritis has been described in malarial infections of man (Ward & Kibukamusoke, 1969; Allison *et al.*, 1969) and the monkey (Ward & Conran, 1966) but not in laboratory rodents although changes in renal function have been described in mice infected with *P. berghei* (Miller *et al.*, 1968). We were therefore interested to see whether infection of NZB mice with *P. berghei* might induce a malarial complex nephritis and provide a suitable laboratory model for investigations into the nature of the changes necessary for renal deposition of malaria antigen–antibody complexes to occur. Young NZB mice infected with *P. berghei* were found to develop a significant increase in mean 24-hr urine protein excretion which persisted for some months. Renal glomeruli of NZB mice killed shortly after malaria infection contained larger mesangial deposits of immunoglobulin and complement than glomeruli from control NZB mice of the same age, although the degree of deposition varied considerably from glomerulus to glomerulus within the same kidney making comparative studies difficult. Attempts to demonstrate malaria antigen within these immunoglobulin deposits were not successful. This may have been due to alteration of the antigen, for Ward & Kibukamusoke (1969) were only able to demonstrate malaria antigen in three of thirteen cases of *P. malariae* complex nephritis, even when 'unmasking' procedures were used. Electron-dense deposits within the basement membrane, seen in kidneys from Nigerian children with the malarial nephrotic syndrome, (Allison *et al.*, 1969) were not seen. The development of renal disease in NZB mice shortly after infection with *P. berghei* could be

explained either by premature induction of the spontaneous renal disease of these mice by the malarial infection or by the occurrence of a malarial antigen–antibody complex nephritis. The bimodal nature of their 24-hr urine protein excretion curve suggests that the latter possibility is most likely but we have been unable to establish this with certainty. The severe renal disease found in 1-year-old malaria-infected NZB mice may represent the development of spontaneous NZB renal disease in kidneys already damaged by a malarial complex nephritis.

The spleen and lymph nodes of old NZB mice frequently show disruption of the normal architecture by sheets of reticulum cells, and frankly malignant changes may occur (East, de Sousa & Parrott, 1965; East, de Sousa, Prosser & Jaquet, 1967). There is evidence that these malignant changes are related to a virus infection (Mellors & Huang, 1967; Prosser, 1968). The malignant disease of NZB mice thus bears some similarity to Burkitt's lymphoma of man which also appears to be related to a virus infection, possibly with the Epstein-Barr virus (Epstein, Achong & Barr, 1964). Burkitt (1969) has suggested that the striking geographical distribution of his tumour may indicate that the Epstein-Barr virus, or a similar virus, can only induce malignant change in a subject whose reticulo-endothelial system has been stimulated by repeated infection with malaria. Jerusalem (1968) has presented laboratory evidence to support this hypothesis. He reported that 6 months after infection with *P. berghei* sixteen out of twenty-five Swiss mice had changes in the thymus or spleen which he interpreted as indicating the presence of a malignant lymphoma. Virus particles were seen more frequently in the spleens of these malaria-infected mice than in controls. In view of this report, we were interested to see whether NZB mice infected with malaria would develop a premature onset of malignant disease. No histological features of malignant disease were found in five NZB mice killed 2 or 3 months after malaria infection and a malignant lymphoma was found in only one of fifteen mice killed when 12 months old. The incidence of malignant disease of the lymphoid system, based on histological criteria, was thus found to be no higher in a small number of malaria-infected NZB mice than the incidence in control NZB mice studies at Taplow (Denman, personal communication) and at other centres (Mellors, 1966; East *et al.*, 1965). However, in contrast to the single infection induced in our animal model, children living in an endemic area suffer repeated malaria infection and this may be a critical factor in predisposing to malignant change. Our negative findings cannot be taken to detract from the validity of the virus–malaria hypothesis of the aetiology of Burkitt's lymphoma in man.

Early infection of NZB mice with *P. berghei* was associated with a significant delay in the onset of Coombs positivity and reticulocytosis. The diminished spleen size of old malaria-infected NZB mice compared with controls were probably also related to the delayed onset of Coombs positivity as marked hyperplastic changes were not present in the red pulp of these spleens. In spite of the delay in the onset of Coombs positivity, the PCV of malaria-infected NZB mice fell in a similar way to that of the controls as noted in NZB mice treated with anti-lymphocyte serum (Denman, Denman & Holborow, 1967). We have thus found some evidence of suppression of autoimmune processes in NZB mice infected with *P. berghei* as well as in B/W mice and we have previously discussed the possibile mechanisms by which this may have been achieved (Greenwood & Voller, 1970). The results obtained with NZB mice have not been as striking as those observed in B/W mice, suggesting that the latter are probably a more suitable model for further studies into the mechanism of suppression of autoimmune processes by a parasitic infection.

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