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Depletion of CD4+ or CD8+ T-cells prevents *Plasmodium berghei* induced cerebral malaria in end-stage disease

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SUMMARY

The role of T-cells in development of experimental cerebral malaria was analysed in C57Bl/6J and C57Bl/10 mice infected with *Plasmodium berghei* K173 or *Plasmodium berghei* ANKA by treatment with anti-CD4 or anti-CD8 mAbs. Mice were protected against cerebral malaria (CM) when anti-CD4 or anti-CD8 mAbs were injected before or during infection. Even in mice in end-stage disease, i.e. with a body temperature below 35·5 °C, treatment with anti-CD4 or anti-CD8 antibodies or the combination protected against CM, whereas chloroquine treatment was completely ineffective in inhibiting further development of the cerebral syndrome.

Key words: *Plasmodium berghei*, cerebral malaria, protection, CD4, CD8, blood–brain barrier.

INTRODUCTION

There are a number of rodent models available for the study of the pathogenesis of experimental cerebral malaria. In these studies mice (Rest, 1982; Grau *et al.* 1986; Thumwood *et al.* 1988; Curfs *et al.* 1989), rats (Wright, Masemble & Bazira, 1971) and hamsters (Rest & Wright, 1979) were used in combination with different strains of *P. berghei* parasites, e.g. ANKA (Grau *et al.* 1986) or K173 (Curfs *et al.* 1989).

In all these models an immunopathological reaction is considered to be involved in the development of the syndrome. Studies in rodent models showed sequestration and adherence of white blood cells to the endothelial lining of post-capillary venules in the brains in association with development of petechiae (Rest, 1982; Polder *et al.* 1992). In the model described by Grau *et al.* (1986) (*P. berghei* ANKA, CBA/Ca mice), CD4+ T-cells play an important role since depletion of CD4+ T-cells prevents, and transfer of CD4+ T-cells from mice developing cerebral malaria enhances, development of the cerebral syndrome. In addition, depletion of CD8+ T-cells did not prevent cerebral malaria. In mice with Murine Acquired Immunodeficiency Syndrome (MAIDS), which is characterized by abnormal functioning of CD4+ T-cells the level of protection against murine CM is significantly increased and is related to the duration of the viral infection and, hence, with the severity of CD4+ T-cell immunodeficiency (Eckwalanga *et al.* 1994). In the model using WM/Ms rats and *P. berghei* NK65 parasites (Imai & Kamiyama, 1994) cerebral malaria is prevented by CD8+ T-cell depletion, but not by CD4+ T-cell depletion. In our model (*P. berghei* K173, C57Bl mice) Curfs *et al.* (1989) confirmed a role for T-cells in the development of experimental cerebral malaria since nude mice, thymectomized mice and mice treated with an anti-T-cell serum were protected against cerebral malaria.

Here, we describe the results of treatment with anti-CD4 and anti-CD8 antibodies in *P. berghei* ANKA- and K173-infected C57Bl mice. The results show that treatment with anti-CD4 or anti-CD8 antibodies can prevent development of experimental cerebral malaria. Moreover, even when performed shortly before expected death, i.e. in end-stage disease when body temperature has decreased to 35·5 °C or lower, depletion of CD4 or CD8 T-cells effectively prevented further development of the cerebral syndrome.

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MATERIALS AND METHODS

Mice

C57Black/6J or C57Black/10 mice, aged 6–10 weeks, were obtained from specific pathogen-free colonies maintained at the Central Animal Facility of the University of Nijmegen. All mice were housed in plastic cages and received water and standard RMH food (Hope Farms, Woerden, The Netherlands) *ad libitum*.

Parasite

Plasmodium berghei K173 and *Plasmodium berghei* ANKA were maintained by weekly transfer of parasitized erythrocytes (PE) from infected into naive mice. Experimental mice were infected intra-

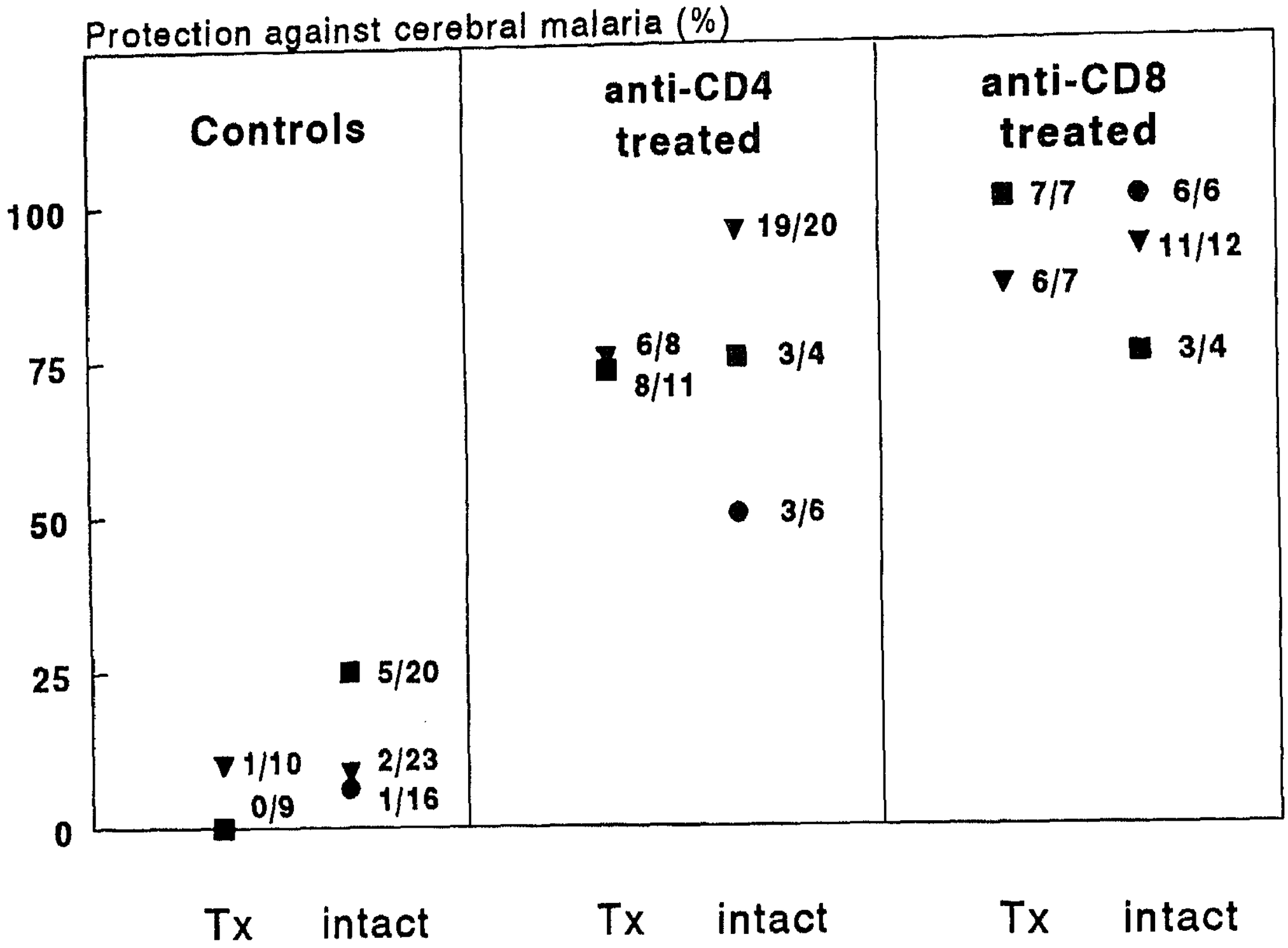

Fig. 1. Protection against cerebral malaria in thymectomized (Tx) versus intact mice and in relation to treatment with anti-CD4 or anti-CD8 mAbs 2 days before and 2 days after infection. C57Bl/6J infected with *Plasmodium berghei* K173 (▼) or with ANKA (●); C57Bl/10 infected with *P. berghei* K173 (■). The number of mice protected against CM versus the total number of infected mice is indicated for each experimental group.

peritoneally with 10^3 PE from blood of infected donor animals of the same strain. *P. berghei* ANKA (originally obtained from Dr B. Mons, Laboratory of Parasitology, University of Leiden, The Netherlands) is a gametocyte-producing strain which is passaged through *Anopheles gambiae* mosquitoes after 4 weekly blood passages. For this purpose mosquitoes are allowed to feed on mice 3 days after infection with 10^7 parasites. Blood-fed mosquitoes received an additional bloodmeal containing normal erythrocytes 5 days later and were allowed to infect mice 14 days later.

Parasitaemia

Thin blood films were made from tail-blood, stained with May-Grünwald and Giemsa's solutions, and the proportion of red blood cells infected with the parasite was determined.

Detection of cerebral malaria

Approximately 95 % of C57Bl mice infected with 10^3 *P. berghei* K173 (Curfs *et al.* 1992) or *P. berghei* ANKA parasites die early in the second week after infection. Approximately 1 day before death a progressive hypothermia develops which is strongly correlated with development of haemorrhages in the brains as observed by histology (Curfs *et al.* 1989). Mice that show a transient hypothermia (in the majority of cases > 32 °C) survive this critical period but die in the third week or later after infection without any noticeable cerebral pathology.

Body temperature

Body temperature was measured with a digital thermometer (Technoterm 1200) introduced into the rectum and read after 10 sec.

Histology

Mice were killed by an overdose of ether and their brains were collected or they were collected post-mortem. Brain tissue was fixed in Carnoy's fluid for 4 h. Paraffin sections (5 μm) were stained with PAS/Haematoxylin or Goldner's trichrome stain. Sections were scored for the presence of petechiae.

In vivo *$CD4^+$ or $CD8^+$ T-cell depletion*

Rat monoclonal anti-CD4 and anti-CD8 antibodies were produced by hybridoma YTS 191.1.2, ECACC no. 87072282 and YTS 169.4.2.1, ECACC no. 87072283 respectively. Both mAbs are of the IgG2b isotype. Pristane-primed nude mice were injected i.p. with 3×10^6 hybridoma cells and the ascites produced was collected. After ammonium sulfate

Table 1. Effect of treatment with anti-CD4 or anti-CD8 mAbs before and during infection with *Plasmodium berghei* K173 parasites on development of cerebral malaria in C57Bl mice

Treatment/ day(s) of treatment*	Mice protected against CM-treated mice	
	n	(%)
Control	9/78†	12
IgG2b-controls‡		
−2/2	0/5	0
9	1/7	14
Anti-CD4		
−2/2	39/49†	80
4	3/3	100
6	3/3	100
8	7/8	88
8+10	5/5	100
Anti-CD8		
−2/2	33/36†	92
4	3/3	100
6	2/3	66
8	5/8	63
8+10	5/5	100

* In relation to infection at day 0.
† Summarized data from *P. berghei* K173 or *P. berghei* ANKA-infected mice (see Fig. 1).
‡ Isotype-matched irrelevant mAb as a control of mAb anti-CD4 or anti-CD8 treatment.

(45 %) precipitation and dialysis against H_2O, the solution was freeze-dried and stored at 4 °C until used. Aliquots were solubilized in sterile, pyrogen-free saline and used immediately. Normal and thymectomized mice were treated once or twice (4 days apart) by i.p. injection of 0·3 mg of either anti-CD4 or anti-CD8 mAb. Treatment with an irrelevant isotype matched, ammonium sulfate precipitated rat mAb was used in comparison to treatment with normal rat serum as another control. Both control treatments did not prevent development of CM and did not affect parasitaemia. Therefore, both control treatments were used in the $CD4^+/CD8^+$ T-cell depletion experiments. The efficacy of T-cell depletion was determined in peripheral leucocytes isolated by water shock-treated peripheral blood, or in ACT-treated spleen cell suspensions from samples collected 3 days after treatment with the anti-T-cell mAbs (Hudson & Hay, 1989). Analysis of $CD4^+/CD8^+$ T-cell depletion immediately after injection of the mAbs was complicated by the presence of the injected mAb coating the $CD4^+/CD8^+$ T-cells in the cell suspensions, a problem that was absent when the analysis was performed 3 days after the last anti-T-cell treatment. Cell suspensions were incubated in PBS containing 10 % FCS, 0·05 % sodium azide and 0·05 mg/ml of the anti-CD4 or anti-CD8 mAbs for 30 min at 4 °C followed by a 30 min incubation of a 50 times diluted FITC-labelled rabbit anti-rat Ig (Dako) in PBS containing 40 % mouse serum and 0·05 % sodium azide. After washing with PBS + 0·05 % sodium azide fluorescence was read microscopically.

From the peripheral blood and the spleen respectively 80 % and 92 % of $CD4^+$ and 62 % and 79 % of $CD8^+$ T-cells were depleted. Treatment with either anti-CD4 or anti-CD8 mAbs did not change significantly the proportion of B-cells (data not shown).

Protection against cerebral malaria was independent of i.v. or i.p. injection of the anti-T-cell mAbs (data not shown), and i.p. injections were used for all experiments.

Thymectomy

Thymectomy (Tx) was performed on mice anaesthetized with chloralhydrate (4·5 % solution; 10 μl/g body weight). Mice were allowed to recover from surgery for a period of 2 weeks before they were used in experiments.

Statistical analysis

For statistical analysis the Student's *t*-test (comparison of 2 groups) and the Kruskal–Wallis test (comparison of more than 2 groups) were used. *P* values $< 0{\cdot}05$ were considered significant.

RESULTS

The effect of treatment with anti-CD4 or anti-CD8 mAbs on development of experimental cerebral malaria was analysed in thymectomized and intact C57Bl/6J and C57Bl/10 mice infected with *P. berghei* ANKA or *P. berghei* K173 parasites. Fig. 1 shows that treatment with anti-CD4 or anti-CD8 mAbs protected against CM, irrespective of thymectomy in both C57Bl/6J or C57Bl/10 mice infected with either *P. berghei* K173 or *P. berghei* ANKA parasites.

Parasitaemia in control mice that died of CM was approximately 5–10 % with a reduction of the haematocrit of approximately 20 %. Neither thymectomy, nor treatment with anti-CD4 or anti-CD8 mAbs significantly changed parasitaemia and haematocrit during infection as compared to controls. All treated mice that did not die of cerebral malaria developed the same severe anaemia in the course of the infection (data not shown). Treatment with either an isotype-matched rat mAb or normal rat serum as a control for depletion of $CD4^+$ or $CD8^+$ T-cells by specific rat mAbs had no effect on development of CM (Table 1) and did not change parasitaemia.

The data in Table 1 show that treatment with anti-CD4 or anti-CD8 mAbs 2 days before and 2 days

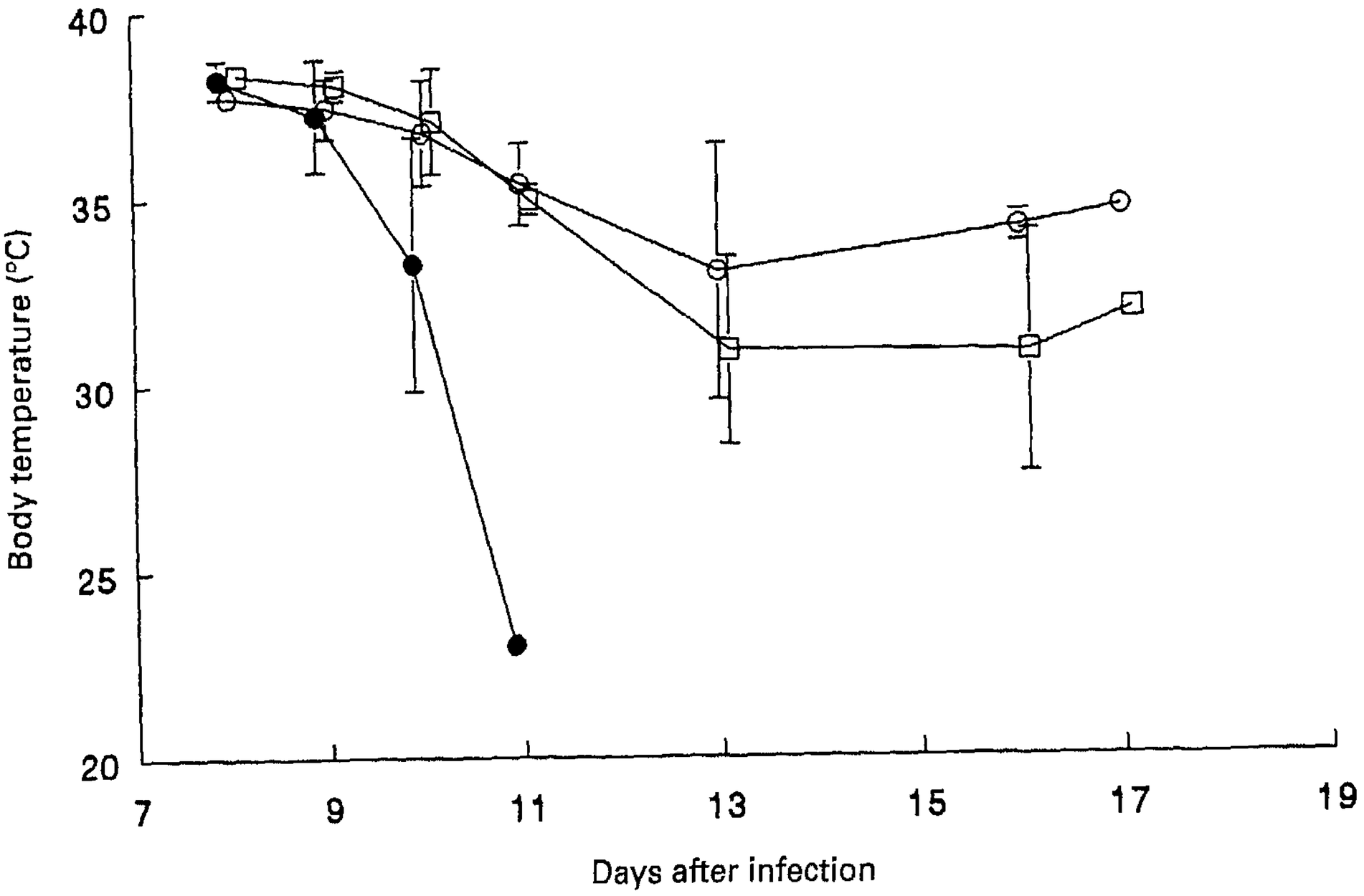

Fig. 2. The effect on body temperature of treatment with anti-CD4 or anti-CD8 mAbs 2 days before and 2 days after *Plasmodium berghei* K173 infection in C57Bl/6J mice. (●) Control mice; (○) anti-CD4 treated mice; (□) anti-CD8 treated mice ($n = 5$).

Table 2. Effect of treatment with anti-CD4 and/or anti-CD8 mAbs or chloroquine on development of cerebral malaria in C57Bl/6J mice infected with *Plasmodium berghei* K173 parasites

	Infected mice CM protected/total		CM protected mice increased T °C†/total	
Treatment*	*n*	(%)	*n*	(%)
Saline	2/52	4	1/2	50
Chloroquine‡	0/10	0	N.S.	
Anti-CD4	12/17	71	11/12	92
Anti-CD8	10/20	50	9/10	90
Anti-CD4+anti-CD8	7/8	88	7/7	100

* Treatment was given when the body temperature was between 35·5 and 30·0 °C.
† Mice with a statistically significant increase of their body temperature at 24 h after treatment.
‡ Chloroquine treatment: 0·8 mg i.p. mouse/day.
N.S., No survivors.

after infection protected 80% and 92% respectively of the mice, whereas 12% of the control mice were protected. Moreover, T-cell depletion during infection and even when performed on day 8, i.e. shortly before expected death as observed in non-depleted controls, effectively prevented development of the fatal syndrome (Table 1).

The effect on body temperature of treatment with anti-CD4 or anti-CD8 mAbs 2 days before and 2 days after infection with *P. berghei* K173 in C57Bl/6J mice was analysed in 8 independent experiments. The results of 1 representative experiment are depicted in Fig. 2. Body temperature of untreated infected mice dramatically decreased after 9 days of infection. Anti-CD4 or anti-CD8-treated mice showed a limited decrease of their body temperature which stabilized. The same effect on body temperature was found when treatment with anti-CD4 or anti-CD8 mAbs was given as a single treatment up to day 8 of infection (results not shown). Anti-CD8 treatment 2 days before and 2 days after infection prevented development of CM as effective as $CD4^+$ T-cell depletion (Table 1) and no significant difference in body temperature during infection of the anti-CD4 and anti-CD8 treated mice was found (Fig. 2).

To determine further the latest possible moment for effective anti-CD4 or anti-CD8 treatment in the

course of infection, mice were selected with a body temperature between 35·5 and 30 °C. If left untreated such infected mice died within 24 h as shown in Fig. 2. The results in Table 2 show that mice with such a hypothermia could be protected against fatal cerebral malaria by treatment with anti-CD4 and/or anti-CD8 antibodies, whereas chloroquine treatment could not save any of these mice. No relation could be found between body temperature at the moment of anti-T-cell treatment (30–35·5 °C) and protection against fatal cerebral malaria (data not shown). Anti-CD4 or anti-CD8 treatment of mice with a body temperature below 30 °C never prevented death. Treatment with anti-$CD4^+$ or anti-$CD8^+$ mAbs in mice with end-stage disease did not protect all mice from the cerebral syndrome (Table 2). These last two observations were probably due to petechiae that already developed before treatment (Polder *et al.* 1988).

Depletions that prevented further development of the cerebral syndrome also resulted in a subsequent increase in body temperature (Table 2). Overall in 93 % (27/29) of the anti-T-cell treated mice body temperature increased within 24 h and prevented further development of the cerebral syndrome.

DISCUSSION

These studies demonstrate the importance of $CD4^+$ and $CD8^+$ T-cells in the pathogenesis of murine cerebral malaria (CM), and this was independent of the use of C57Bl/6J or C57Bl/10 mice, or infection with *P. berghei* K173 or *P. berghei* ANKA parasites. Thymectomized mice were used to prevent the influx of new T-cells after treatment with anti-T-cell mAbs, but no significant difference was found when data were compared to intact mice. Treatment with anti-T-cell mAbs did not completely eliminate all $CD4^+$ or all $CD8^+$ T-cells. Together these observations suggest that complete absence of $CD4^+$ or $CD8^+$ T-cells is not necessary to prevent development of murine CM. Anti-$CD4^+$ or anti-$CD8^+$ T-cell treatment had no effect on parasitaemia and development of anaemia confirming the observations made by Grau *et al.* (1986) in another murine *P. berghei* CM model and by Imai & Kamiyama (1994) in a rat *P. berghei* NK 65 CM model.

The observation that both anti-$CD4^+$ and anti-$CD8^+$ T-cell depletion can prevent CM is in contrast to observations made previously by Grau *et al.* (1986), who described a role for $CD4^+$ but not $CD8^+$ T-cells in development of CM in *P. berghei* ANKA-infected CBA mice. In contrast, Waki *et al.* (1992) and Imai & Kamiyama (1994) found a role for $CD8^+$ but not $CD4^+$ T-cells in preventing either early mortality or CM, respectively, in *P. berghei* NK65-infected rodents. A role for both $CD4^+$ and $CD8^+$ T-cells in development of CM in *P. berghei* ANKA-infected mice was noted by Weidanz and collaborators using various types of knock-out mice (personal communication). Hancock *et al.* (1994) found that treatment with anti-CD4 antibodies not only depletes CD4-positive T-cells but also CD4-positive mononuclear cells; however, their possible role in CM remains to be determined.

In contrast to our results Grau *et al.* (1986) found that $CD8^+$ T-cell depletion did not prevent development of CM in *P. berghei* ANKA-infected CBA mice. This apparent discrepancy may relate to different mouse strains used and/or a difference in the strain of *P. berghei* ANKA.

Anti-CD4 or anti-CD8 T-cell treatment was effective even in mice with end-stage disease (body temperature between 35·5 and 30 °C). Histological analysis of brains of mice developing the cerebral syndrome showed that white blood cells adhere to the endothelial lining (Curfs *et al.* 1989; Polder *et al.* 1992) suggesting that these $CD4^+$ and $CD8^+$ T-cells are involved in disturbance of the endothelial lining and thereby in the functioning of the blood–brain barrier.

Disturbance of the blood–brain barrier in mice developing CM was also observed by Thumwood *et al.* (1988) and Neill & Hunt (1992). They described leakage of Evans blue from the vasculature into brain parenchyma. In addition, *P. berghei* K173-infected mice with end-stage disease are sensitive to development of folic acid-induced convulsions, while control mice are not. Treatment with anti-$CD4^+$ or anti-$CD8^+$ mAbs not only prevents CM but successfully treated mice also recover from their sensitivity to folic acid-induced convulsions within 4 h after mAb treatment (C. Hermsen *et al.*, unpublished observations). In human cerebral malaria convulsions are common (Waruiru *et al.* 1996), raising the question as to whether in human CM adherence of sequestered infected red blood cells and possibly the presence of leucocytes (Polder, 1989; Porta *et al.* 1993; Eling & Kremsner, 1994; Patnaik *et al.* 1994; Eling & Sauerwein, 1995) are involved in excessive activation of endothelial cells and subsequent disturbance of the function of the blood–brain barrier.

Chloroquine treatment could not save mice in end-stage disease while anti-CD4 or anti-CD8 treatment was still effective. This may be explained by the fact that chloroquine like most other antimalarial drugs needs more than 1 day to suppress parasitaemia effectively, whereas most of the mice in end-stage disease die within 1 day.

Our data are compatible with the hypothesis that a localized inflammatory reaction involving both $CD4^+$ and $CD8^+$ T-cells disturbs and damages the endothelial lining of the blood–brain barrier eventually leading to petechiae. Our data suggest that both $CD4^+$ and $CD8^+$ T-cells are involved in stimulation of a downstream process of the development of the petechiae.

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