

Cloned Lines of *Plasmodium berghei* ANKA Differ in Their Abilities To Induce Experimental Cerebral Malaria

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Infection with *Plasmodium berghei* ANKA is usually lethal. The parasite causes in some mouse strains a neurovascular syndrome, experimental cerebral malaria (ECM), involving immunopathological reactions. The effects on the development of ECM of the mouse genetic background have been clearly demonstrated, but nothing is known about the effects of the clonal diversity of the parasite. We showed that various cloned lines derived from a polyclonal line of *P. berghei* ANKA caused ECM but that the extent of ECM induction was dependent on the amount of inoculum. Subtle differences in ECM characteristics (survival time and hypothermia) were also observed. We also confirmed, using the 1.49L cloned line, that the mouse genetic background strongly affects ECM.

The infection of some mouse strains with the ANKA or K173 strain of *Plasmodium berghei* leads to the development of a neurovascular disease, experimental cerebral malaria (ECM), resulting in death 6 to 14 days after inoculation with the parasite. These two parasite strains have been extensively used in experimental models for cerebral malaria (13, 20, 38). However, in recent years the relevance of this model system has often been called into question. It has been suggested that the major difference between human and murine cerebral malaria is that there is no major cerebral sequestration of parasitized erythrocytes (PE) in the vascular endothelium of the mouse brain (4, 45). Lymphocytes and monocytes are the major cell populations sequestered in the venules of rodents, but recent studies have suggested that PE can also be sequestered (8, 15, 29, 36). Margination and sequestration of mononuclear cells have also been reported in the brains of patients who died of cerebral malaria (35, 37).

The idea that erythrocyte sequestration is the key factor in the pathogenesis of the human syndrome has been challenged (4, 6, 23, 33), and it has been suggested that this process is actually one of the terminal events of cerebral malaria (33). Cytokine induction and nitric oxide generation have also been implicated in the pathogenesis of cerebral malaria in both humans and rodents (5, 6). Recent studies have shown an association between tumor necrosis factor alpha (TNF- α) concentrations, interleukin 1 (IL-1) concentrations, and disease severity (19, 30, 31). Grau et al. used a polyclonal line of *P. berghei* ANKA in CBA/Ca mice and monoclonal or polyclonal antibodies to cytokines to demonstrate the involvement of gamma interferon (IFN- γ), TNF- α , IL-3, and granulocyte-macrophage colony-stimulating factor (16, 17, 20, 21). Different results were obtained when the K173 strain of *P. berghei* was used in C57BL/6 mice; antibodies to IFN- γ and TNF- α

were unable to prevent ECM (10, 13). In a recent study, Hermesen et al. reported that circulating TNF- α was not involved in the development of ECM in C57BL/6 mice infected with *P. berghei* K173 (27). This finding demonstrated the importance of defining precisely the host-parasite combination.

The genetic backgrounds in mice (12, 21, 32, 38) and in humans (28) affect the development of cerebral malaria, whereas there is no firm evidence as yet that clonal variation of the parasite has any effect. In this study, we investigated the capacity of various cloned lines of *P. berghei* ANKA to cause ECM.

MATERIALS AND METHODS

Mice. Six- to 8-week-old pathogen-free female C57BL/6, BALB/c, C3H/HeN, DBA/2, and Swiss mice were purchased from Charles River Breeding Laboratories (Saint Aubin Les Elbeuf, France). CBA/Ca (6- to 8-week-old) mice were obtained from Harlan Olac (Leicester, United Kingdom). C3H/HeJ and 129Sv/eV (6- to 8-week-old) mice were kindly donated by P. A. Cazenave (Institut Pasteur).

Body temperature. Body temperature was measured with a digital thermometer (Quick-check; Polyabo, Strasbourg, France) inserted into the rectum and read when the readout stabilized (20 s).

Parasites. Lines 1.49L, 1.97L, 4, and 5 of *P. berghei* ANKA were cloned by one of us (D.W.) from an initially polyclonal line of *P. berghei* ANKA obtained from M. Wéry (Institute of Tropical Medicine, Antwerp, Belgium) as previously described (44). Cloned lines 1.49L and 1.97L were derived from cloned line 1 and differ in the numbers of passages in mice. The polyclonal line used in this study was obtained from G. Grau (University of Geneva, Geneva, Switzerland). This

TABLE 1. Cumulative incidence of ECM in C57BL/6 mice infected with the *P. berghei* ANKA polyclonal line^a

Expt	Cumulative incidence of ECM (%) ^b
1.....	66.6
2.....	100
3.....	90
4.....	58.3

^a PE of the *P. berghei* ANKA polyclonal line were obtained from infected C57BL/6 mice, and 10⁶ PE from various batches were injected i.p. into mice (7 to 12 mice per group).

^b Between days 6 and 12 after infection with the *P. berghei* ANKA polyclonal line.

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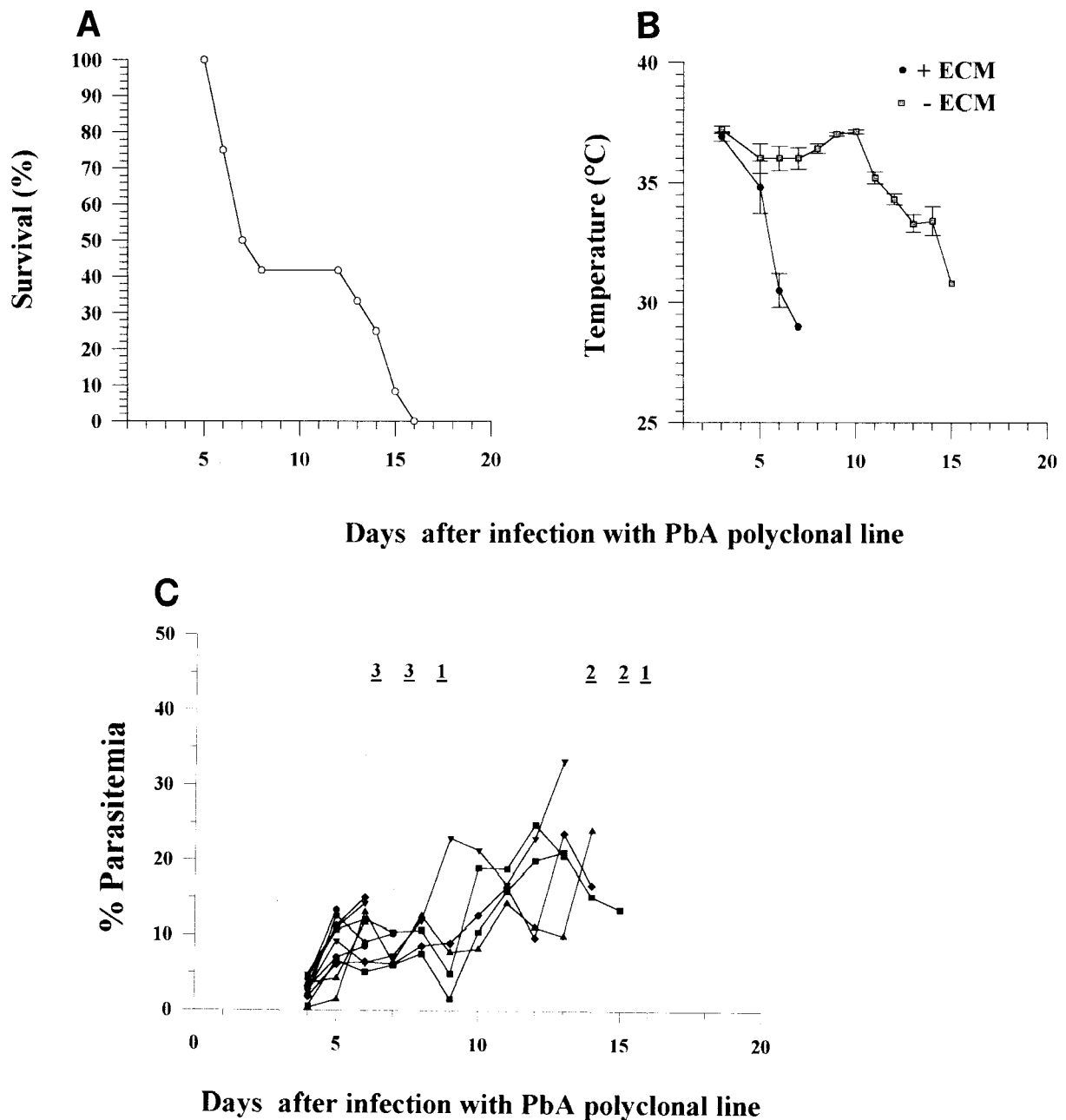


FIG. 1. Survival (A), body temperature (B), and parasitemia (C) after infection of 12 C57BL/6 mice with 10^6 PE of the *P. berghei* ANKA polyclonal line. This experiment is experiment 4 reported in Table 1. + ECM, mice with neurological signs of ECM; - ECM, mice with no neurological signs of ECM. Mortality is indicated at the top of panel C as the number of mice that died on that day, and each curve represents the progression of a single mouse.

line was derived from the same polyclonal line of *P. berghei* ANKA as that used to derive the various cloned lines and was maintained as a stabilate in liquid nitrogen. It was passaged sequentially in OF1, BALB/c, and CBA/Ca mice in Geneva (7, 15) and then in C57BL/6 mice in our laboratory. Blood stages of the cloned lines were stored as stabilates (10^7 or 10^8 PE/ml in Alsever's solution containing 10% glycerol) in liquid nitrogen. Mice were infected intraperitoneally (i.p.) with various doses of PE. Parasitemia was monitored daily by counting the number of infected cells per 2,000 erythrocytes in Giemsa-stained slides.

Disease assessment. Mice were considered to have ECM if they had neurological signs (ataxia, paralysis, deviation of the head, and convulsions). Some mice were killed when they displayed clinical signs. Brains were removed and fixed in 10% paraformaldehyde for histological examination by standard techniques to detect neurological lesions (hemorrhage, edema, and endothelial dam-

age). The cumulative incidence of ECM was defined as the appearance of neurological signs followed by death after *P. berghei* ANKA infection.

RESULTS

Induction of cerebral malaria by a polyclonal line of *P. berghei* ANKA. We found that different aliquots of 10^6 PE obtained from frozen stabilates of blood from a C57BL/6 mouse infected with a polyclonal line of *P. berghei* ANKA induced ECM in replicated experiments with an efficiency of 50 to 100% (Table 1). In each experiment, the mice that developed ECM showed neurological signs and early hypothermia (Fig. 1B)

TABLE 2. Cumulative incidence of ECM in mouse strains infected with the *P. berghei* ANKA polyclonal line passaged in various mouse strains

Expt	Mouse strain ^a		Cumulative incidence of ECM (%) ^b
	PE donor	Recipient	
1	Swiss	C57BL/6	33
		CBA/Ca	20
2	C57BL/6	C57BL/6	62.5
		BALB/c	20
		CBA/Ca	70
3	BALB/c	C57BL/6	0
		BALB/c	0
4	CBA/Ca	C57BL/6	70
		CBA/Ca	80

^a PE of the *P. berghei* ANKA polyclonal line were obtained from an infected C57BL/6 mouse, and 10^6 PE were injected i.p. into recipient mice (five to eight mice per group).

^b Between days 6 and 12 after infection with the *P. berghei* ANKA polyclonal line.

between days 6 and 12 and had about 5 to 15% parasitemia (Fig. 1C). Histological studies showed that mice with ECM had widespread damage to the microvasculature in the brain, with edema and hemorrhage (data not shown).

We also found that the mouse strains from which the PE originated affected the incidence of ECM. PE taken from C57BL/6 or CBA/Ca mice consistently induced ECM in a high proportion of C57BL/6 or CBA/Ca mice but not in BALB/c mice (Table 2). PE taken from Swiss mice induced ECM in few C57BL/6 or CBA/Ca mice. PE taken from BALB/c mice did not induce ECM in C57BL/6 or BALB/c mice.

Induction of cerebral malaria by various cloned lines of *P. berghei* ANKA. The results described above suggested that the line of *P. berghei* ANKA used in these experiments contained a mixture of clones with various degrees of virulence. They also suggested that the proportion of these clones could be altered by the genetic background of the mouse or could differ between mice depending on the proportions of the var-

TABLE 3. Cumulative incidence of ECM for various cloned lines of *P. berghei* ANKA in C57BL/6 mice^a

Cloned line	Cumulative incidence of ECM (%)	Time taken to neurological signs (days) ^b
1.49L	70	6-12
1.97L	100	6-7
4	86	6-7
5	80	6-14

^a PE of different *P. berghei* ANKA cloned lines were obtained from C57BL/6 mice previously infected with the respective cloned lines. An aliquot of 2×10^6 PE of each cloned line was injected i.p. into recipient mice (5 to 12 mice per group).

^b Time between inoculation with PE and appearance of the neurological signs.

ious clones in the infectious inoculum or the growth capacity of each clone.

We used cloned lines derived from a polyclonal line of *P. berghei* ANKA (44) from which our own polyclonal line was derived (7, 15). PE of four cloned lines were obtained from C57BL/6 mice, and their capacity to cause ECM was compared with that of the polyclonal line of *P. berghei* ANKA. We observed that all of the cloned lines induced ECM in C57BL/6 mice infected with 2×10^6 PE. However, there were differences between the cloned lines in survival time and the time taken to develop hypothermia and neurological manifestations. In a typical experiment (Table 3 and Fig. 2), cloned lines 1.97L and 4 induced ECM in 6 to 7 days, whereas cloned lines 1.49L and 5 induced neurological signs over a longer period (6 to 14 days) (Table 3). Mice died 2 days after the onset of neurological signs; therefore, extension of the time taken to develop ECM manifestations led to a delay in mortality (Fig. 2A) and in the development of hypothermia (Fig. 2B). The time taken to develop ECM in a single mouse strain could not be used as a feature to characterize a parasite cloned line, since the values obtained were highly variable in different experimental inoculations with parasite stabilates of the same or different batches (data not shown). Nonetheless, the proportion of mice which developed ECM was constant for the different clones.

All of the cloned lines produced similar parasitemia curves

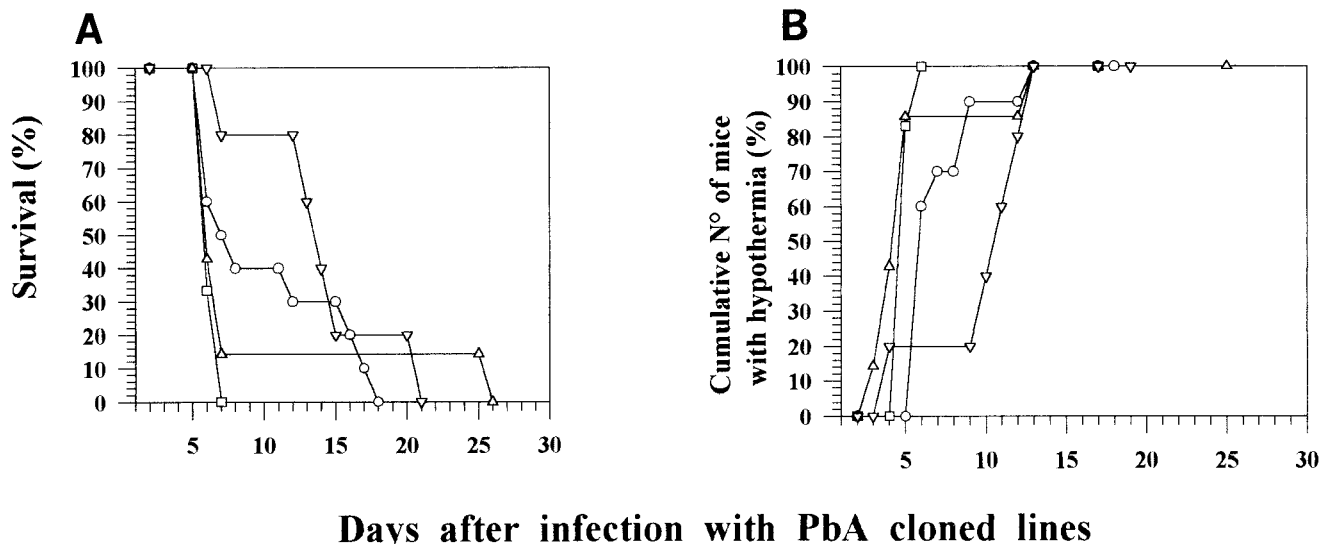


FIG. 2. Survival (A) and cumulative percentage of C57BL/6 mice displaying a body temperature below 35°C (B) after infection with 2×10^6 PE of four *P. berghei* ANKA cloned lines. Symbols: ○, line 1.49L; □, line 1.97L; △, line 4; ▽, line 5.

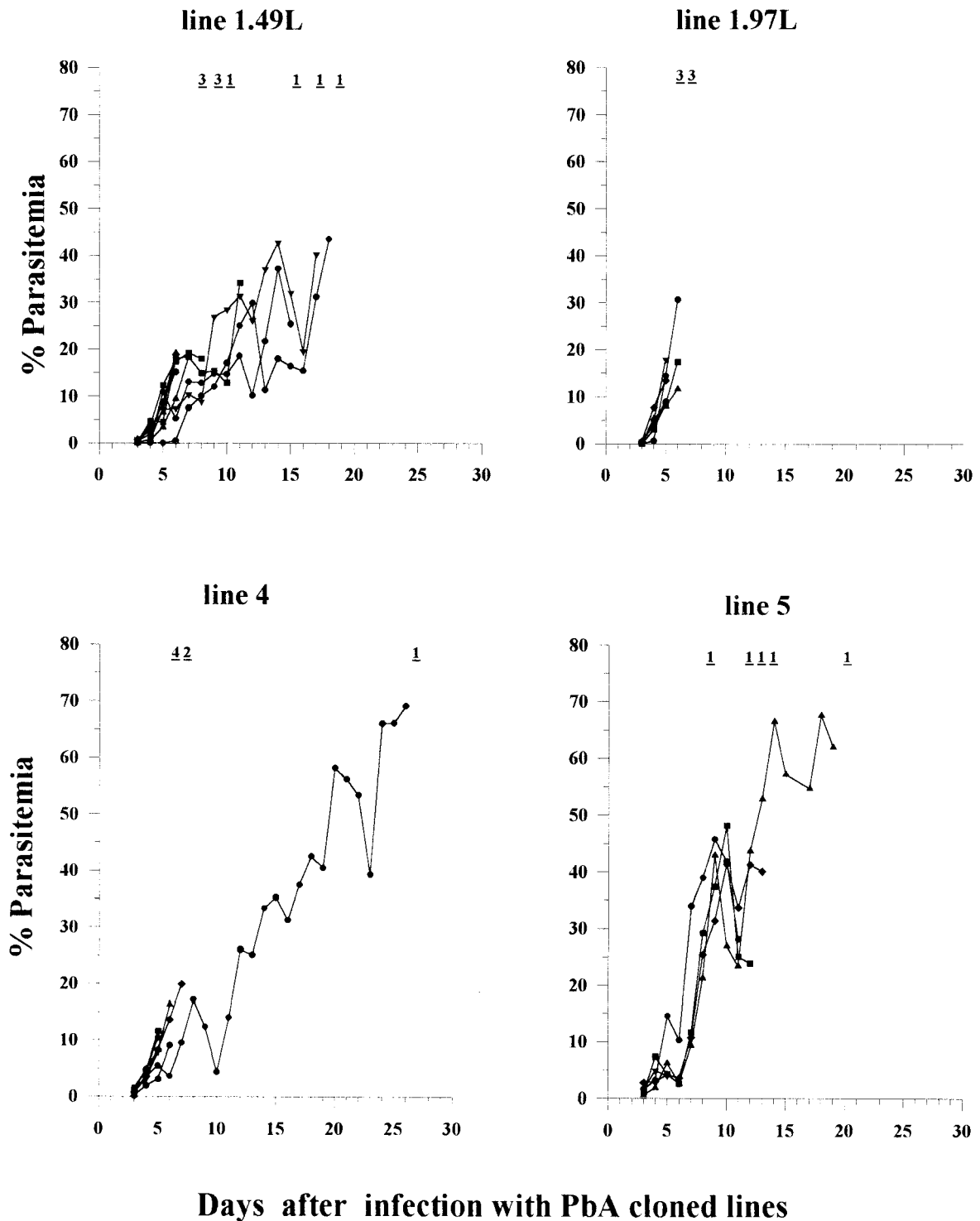


FIG. 3. Parasitemia of C57BL/6 mice infected with four *P. berghei* ANKA cloned lines. Mortality is indicated at the top of each panel as the number of mice that died on that day. Each curve represents the progression of a single mouse.

(Fig. 3). However, cloned lines 1.49L, 1.97L, and 4 resulted in lower parasitemia levels (10 to 20%) when they induced ECM than did cloned line 5 (20 to 40%).

Effects of the amount of infectious inoculum of three cloned lines on the incidence of ECM. We investigated the effects of the amount of infectious inoculum of three cloned lines be-

cause it was previously shown that this factor could affect the incidence of ECM (9). For cloned lines 1.49L and 5, the injection of a large or small quantity of PE resulted in a smaller proportion of mice having ECM than for the other cloned line. For cloned line 1.97L, only a very large amount of inoculum (10^7 PE) affected the incidence of ECM (Table 4).

TABLE 4. Effect of the amount of inoculum of the various cloned lines of *P. berghei* ANKA on the cumulative incidence of ECM in C57BL/6 mice^a

Cloned line	Dose of PE	Cumulative incidence of ECM (%) ^b
1.49L	1 × 10 ⁵	40
	1 × 10 ⁶	60
	2 × 10 ⁶	80
	1 × 10 ⁷	20
1.97L	1 × 10 ⁴	70
	1 × 10 ⁵	100
	1 × 10 ⁶	75
	2 × 10 ⁶	100
	1 × 10 ⁷	0
5	1 × 10 ⁴	40
	1 × 10 ⁵	10
	1 × 10 ⁶	20
	2 × 10 ⁶	80
	1 × 10 ⁷	40

^a PE of various *P. berghei* ANKA cloned lines were obtained from C57BL/6 mice previously infected with the respective cloned lines. Aliquots of various quantities of PE from the same batch of each cloned line were injected i.p. into recipient mice (five mice per group).

^b Between days 6 and 12 (cloned lines 1.49L and 1.97L) and between days 6 and 14 (cloned line 5).

Infection of several mouse strains with cloned line 1.49L. We (Table 2) and others (7, 12, 18, 21, 24–26, 32, 34, 38, 41) have shown that the mouse genetic background affects the incidence of ECM caused by polyclonal *P. berghei*, so cloned line 1.49L (10⁶ PE from C57BL/6 mice) was injected into various mouse strains. Some strains were very susceptible to ECM, some were partially susceptible, and others were not susceptible at all (Table 5). Mice developed ECM between days 7 and 12, had moderate parasitemia at that time, and suffered early hypothermia, which varied from a low (BALB/c) to a high (C57BL/6) degree. Infection with cloned line 1.49L was always lethal in all of the mouse strains and resulted in similar parasitemia curves in all of the strains (data not shown). Mice which did not die of ECM died of anemia and hyperparasitemia.

Effect of the mouse strain from which PE were taken on ECM induced by *P. berghei* ANKA cloned line 1.49L. The origin of the PE had an effect on the development of ECM in recipient mice (Table 2). Three strains of mice were infected with PE taken from a C57BL/6 mouse infected with cloned line 1.49L. Infected blood was obtained from these different mouse strains, and 2 × 10⁶ PE were injected into C57BL/6 mice. Compared with PE taken from C57BL/6 mice, PE taken from BALB/c

mice caused less ECM and PE taken from C3H/HeJ mice caused more ECM (Table 6).

DISCUSSION

In this study, we have shown that cloned lines of *P. berghei* ANKA can differ in their ability to induce ECM in the same host. Unlike the polyclonal line, aliquots from the same stock of cloned lines induced consistently similar levels of ECM in C57BL/6 mice. All of the cloned lines caused ECM, but the degree of ECM induction was dependent on the amount of inoculum and thus differed for the cloned lines (Table 4). This finding is consistent with a previous report showing that the amount of infectious *P. berghei* K173 inoculum used affects the development of ECM in C57BL/6 mice (9). ECM is thought to develop via a complex pathway. Grau et al. have suggested that *P. berghei* ANKA antigens induce the release of IL-3, granulocyte-macrophage colony-stimulating factor, and IFN-γ by CD4 Th1 cells (20). These lymphokines activate macrophages, causing them to release TNF-α. TNF-α then induces adhesion molecules (e.g., intracellular adhesion molecule 1) in the brain endothelium, leading to leukocyte adhesion (14, 22, 40). Changes in the amount of the inoculum could lead to the release of a different array of lymphokines (e.g., IL-4 and IL-10) by activation of CD4 Th2 cells. Consistent with this notion, we previously reported that IL-10 prevents the induction of ECM (11).

We used four cloned lines in this study. Two of these lines (1.49L and 1.97L) were derived from the same clone and differed only in the number of passages in mice. If differences between clones can be accounted for by antigenic differences, the differences between lines 1.49L and 1.97L showed that a clonal phenotype may not be stable. A clone may also generate new clones after propagation. Such clonal variation has been demonstrated for *P. falciparum*, in which it occurred at a rate of 2% per generation in vitro (39). Clonal variation is linked to the adhesion capacity of clones and has been shown to be due to antigenic variation of a parasite protein, PfEMP-1 (3, 42, 43). No homologous molecules have been described for *P. berghei*, but there is evidence that antigenic clonal variation exists in this parasite species. Antigenic variation has been demonstrated by homologous and heterologous challenges of white mice with various clones of *P. berghei* ANKA (1, 2, 44).

It has been shown that the genetic background of a mouse affects the course of parasitemia and disease (7, 12, 18, 21, 24–26, 32, 34, 38, 41). We assessed the development of ECM in six inbred mouse strains infected with 10⁶ PE of *P. berghei* ANKA cloned line 1.49L. There were marked differences in susceptibilities to ECM between the strains, but the levels of parasitemia were similar in all of the strains (Table 5). Mice were either susceptible or resistant to the neurological com-

TABLE 5. Incidence of ECM in various mouse strains infected with *P. berghei* ANKA cloned line 1.49L^a

Mouse strain	Haplotype	No. of experiments	Total no. of mice	Cumulative incidence of ECM (%) ^b	Parasitemia (%) ^c	Temperature (°C) ^c	Time taken to neurological signs (days) ^d
DBA/2	<i>d</i>	4	25	0			
BALB/c	<i>d</i>	5	35	35	24.73 ± 5.4	35.7 ± 0.56	7–11
C3H/HeN	<i>k</i>	2	10	30	28.37 ± 2.67	29.52 ± 2.67	8–11
129Sv/eV	<i>k</i>	2	12	66	19.13 ± 2.25	28.55 ± 0.64	7–10
CBA/Ca	<i>k</i>	2	21	57.1	16.9 ± 0.52	33.12 ± 0.75	7–10
C57BL/6	<i>b</i>	3	19	68.4	16.11 ± 0.52	21.27 ± 2.44	7–12

^a PE of *P. berghei* ANKA cloned line 1.49L were obtained from an infected C57BL/6 mouse, and 10⁶ PE were injected i.p. into recipient mice (7 to 12 mice per group).

^b Between days 6 and 12.

^c Values are for mice with ECM on the day of death.

^d Time between inoculation with PE and appearance of the neurological signs.

TABLE 6. Cumulative incidence of ECM in C57BL/6 mice infected with *P. berghei* ANKA cloned line 1.49L passaged in various mouse strains

Mouse strain in which PE were ^a :		Cumulative incidence of ECM (%) ^b
Passaged	Injected	
C57BL/6	C57BL/6	71.4
BALB/c	C57BL/6	42.8
C3H/HeJ	C57BL/6	100

^a PE of *P. berghei* ANKA cloned line 1.49L were obtained from C57BL/6, BALB/c, and C3H/HeJ donor mice infected with PE obtained from C57BL/6 mice. A total of 10⁶ PE were injected i.p. into C57BL/6 mice. There were six to seven mice in each recipient group.

^b Between days 6 and 12.

plications. Genes in the mouse major histocompatibility complex do not seem to control ECM susceptibility, because some mouse strains with the same *H-2^d* haplotype were susceptible (BALB/c) whereas others were resistant (DBA/2). Genetic studies are required to define precisely the role of the mouse genetic background and to identify the genes involved in ECM resistance.

Studies with various host-parasite combinations revealed another level of complexity. The mouse strain from which the PE were taken affected the incidence of ECM. When the polyclonal line or cloned line 1.49L was used, the mouse strain in which the line was passaged affected the incidence of ECM (Tables 2 and 6). Similar findings have been reported with another polyclonal line of *P. berghei* ANKA (41). It is possible that passage of a *P. berghei* ANKA line in one mouse strain results in the selection of a specific group of clones which may differ from that selected in a different mouse strain. Our data may also explain the conflicting reports on the susceptibility or resistance to ECM of BALB/c mice infected with *P. berghei* ANKA polyclonal lines (15, 21, 32, 34). As these lines were passaged in different mouse strains, they may differ in parasite clonal content. There were also differences in the incidence of ECM in BALB/c mice obtained from different suppliers (unpublished results). This finding suggests that there may be differences in the genetic backgrounds of BALB/c mice from different suppliers. Thus, different clones could induce ECM in different mouse strains in specific combinations.

We used blood-stage parasites in this study. These parasite forms multiply by mitosis, with mutations occurring at a very low rate. In contrast, passage through mosquitoes may result in a large number of new clones being created by recombination, which may occur during the sexual stage when the parasite multiplies by meiosis. Consistent with this idea, we were unable to induce consistently high levels of ECM in mice through *P. berghei* ANKA-infected-mosquito bites or sporozoite injection (unpublished results).

In summary, the clonal composition of the parasite inoculum may affect the incidence of ECM, and the selection of various virulent clones is regulated by the mouse genetic background and the dynamic interplay between the clones and their hosts. Further work is needed to identify, at the molecular level, which *P. berghei* ANKA antigens are involved in the induction of ECM. Our results also emphasize the importance of taking this phenomenon into account in studies of pathophysiology, virulence, or protection in rodent or human malaria.

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