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 to cause 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.

**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 kindly donated by P. A. Cazenave (Institut Pasteur). Body temperature. Body temperature was measured with a digital thermometer (Quick-check; Polylabo, 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).

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

<table>
<thead>
<tr>
<th>Exp</th>
<th>Cumulative incidence of ECM (%)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>66.6</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>58.3</td>
</tr>
</tbody>
</table>

* PE of the *P. berghei* ANKA polyclonal line were obtained from infected C57BL/6 mice, and 10° PE from various batches were injected i.p. into mice (7 to 12 mice per group).

 arbitrary treatments were performed on each day after the start of infections.
A 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 damage). 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).
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 various 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 $2 \times 10^6$ 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 stabiles 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.
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 because 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).
Infected blood was obtained from these different mouse strains, the PE had an effect on the development of ECM in recipient mice (five mice per group). Quantities of PE from the same batch of each cloned line were injected i.p. into recipient mice, which varied from a low (BALB/c) to a high (C57BL/6) degree. Infection with cloned line 1.49L was always lethal in all strains. Some strains were very susceptible to ECM, some were partially susceptible, and others were not susceptible at all. PE of various quantities from the same batch of each cloned line were injected i.p. into recipient mice (five mice per group).

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

<table>
<thead>
<tr>
<th>Cloned line</th>
<th>Dose of PE</th>
<th>Cumulative incidence of ECM (%)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.49L</td>
<td>1 × 10⁴</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>1 × 10⁵</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>2 × 10⁶</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>1 × 10⁷</td>
<td>20</td>
</tr>
<tr>
<td>1.97L</td>
<td>1 × 10⁴</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>1 × 10⁵</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1 × 10⁶</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>2 × 10⁶</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1 × 10⁷</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1 × 10⁴</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>1 × 10⁵</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1 × 10⁶</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>2 × 10⁶</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>1 × 10⁷</td>
<td>40</td>
</tr>
</tbody>
</table>

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).

Effect of the mouse strain from which PE were taken on ECM induction by P. berghei ANKA cloned line 1.49L. 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).

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

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Haplotype</th>
<th>No. of experiments</th>
<th>Total no. of mice</th>
<th>Cumulative incidence of ECM (%)a</th>
<th>Parasitemia (%)b</th>
<th>Temperature (°C)c</th>
<th>Time taken to neurological signs (days)d</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBA/2</td>
<td>d</td>
<td>4</td>
<td>25</td>
<td>0</td>
<td>24.73 ± 5.4</td>
<td>35.7 ± 0.56</td>
<td>7–11</td>
</tr>
<tr>
<td>BALB/c</td>
<td>d</td>
<td>5</td>
<td>35</td>
<td>35</td>
<td>28.37 ± 2.67</td>
<td>29.52 ± 2.67</td>
<td>8–11</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>k</td>
<td>2</td>
<td>10</td>
<td>50</td>
<td>19.13 ± 2.25</td>
<td>28.55 ± 0.64</td>
<td>7–10</td>
</tr>
<tr>
<td>129Sv/eV</td>
<td>k</td>
<td>2</td>
<td>12</td>
<td>66</td>
<td>16.9 ± 0.52</td>
<td>33.12 ± 0.75</td>
<td>7–10</td>
</tr>
<tr>
<td>CBA/Ca</td>
<td>k</td>
<td>2</td>
<td>21</td>
<td>57.1</td>
<td>16.11 ± 0.52</td>
<td>21.27 ± 2.44</td>
<td>7–12</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>b</td>
<td>3</td>
<td>19</td>
<td>68.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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).

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-
This phenomenon into account in studies of pathophysiology, of ECM. Our results also emphasize the importance of taking Santes to Laurent Renia.

A total of 106 PE were injected i.p. into C57BL/6 mice. There were six to 12 between days 6 and 12.

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 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 an increased tumor necrosis factor production and macrophage accumulation. J. Exp. Med. 168:1499–1504.

This phenomenon into account in studies of pathophysiology, virulence, or protection in rodent or human malaria.

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REFERENCES


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