EXPRESSION OF PROINFLAMMATORY CYTOKINES IN FOUR REGIONS OF THE BRAIN IN MACAQUE MULATTA (Rhesus) MONKEYS INFECTED WITH PLASMODIUM COATNEYI

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Abstract. We have characterized brain cytokine expression profiles in the Plasmodium coatneyi/rhesus (Macaca mulatta) malaria model. Eight rhesus monkeys were included in the study; four were infected with P. coatneyi, and four were used as uninfected controls. All inoculated animals became infected. Eleven days after parasite inoculation, the rhesus monkeys were killed and tissue samples from 4 regions of the brain (cortex and white matter of the cerebrum, cerebellum, and midbrain) were collected for quantitation of mRNA expression of cytokines, adhesion molecules, and inducible nitric oxide synthetase (iNOS) by reverse transcriptase-polymerase chain reaction (RT-PCR). The expression levels of tumor necrosis factor-alpha (TNF-α), gamma interferon (IFN-γ), interleukin-1-beta (IL-1β), intercellular adhesion molecule-1 (ICAM-1) and inducible nitric oxide synthetase (iNOS) were highest in the cerebellum of infected animals, correlating well with pathologic observations of sequestration of parasitized erythrocytes in this region of the brain. Infected animals also had higher TNF-α expression levels in the cortex and IL-1β expression levels in the cortex, white matter, and midbrain. Thus, the expression of pro-inflammatory and T helper-1 (TH-1) cytokines, adhesion molecules, and iNOS appears to predominate in the cerebellum of infected rhesus monkeys.

INTRODUCTION

Malaria parasites infect 300–500 million people annually, of which 2.7 million people (especially among children in sub-Saharan Africa) die from complications of primarily cerebral malaria and severe anemia. Despite the high mortality rates, the pathogenic mechanisms of cerebral malaria have not been well elucidated. The unavailability of infected human specimens and suitable animal models has hindered thorough understanding of the pathogenesis.

Recently, an alternative nonhuman primate model has been used in studies of cerebral malaria. The model employs Plasmodium coatneyi infection in rhesus monkeys (Macaca mulatta). Previous studies have shown that the clinical and pathologic characteristics of infected animals appear similar to those of infected humans. Immunologically, the model resembles human malaria in terms of cytokine and prostaglandin production. In this study, we have further examined the P. coatneyi/rhesus model by characterizing mRNA expression of pro-inflammatory and TH-1 cytokine, intercellular adhesion molecule-1 (ICAM-1), and inducible nitric oxide synthetase (iNOS) in 4 regions of the brain (cerebellum, cortex and white matter of the cerebrum, and midbrain). Results of this study further support the immunologic similarities between the P. coatneyi/rhesus model and human cerebral malaria.

MATERIALS AND METHODS

Samples. Eight non-splenectomized adult rhesus monkeys were used in the study. Four were inoculated intravenously with 1x10⁶ P. coatneyi-infected rhesus monkey erythrocytes. The other four remained uninfected as controls. All monkeys were monitored daily for clinical manifestations and for parasitemia on Days 3, 4, 5, 6, 7, 10, and 11. On Day 11, after the onset of several clinical symptoms in infected animals, all monkeys were killed. Tissue samples from 4 regions of the brain (cortex and white matter of the cerebrum, cerebel- lum, midbrain) were collected and immediately frozen on dry ice and kept at −70°C. Brain tissues were not obtained from one infected animal that died of cerebral malaria overnight on Day 10. Laboratory animal use protocols were reviewed and approved by the Centers for Disease Control and Prevention Institutional Animal Care and Use Committee. The experimental design and infection procedures were described previously.

Sequestration. To document parasitized erythrocyte (PRBC) sequestration, multiple brain tissue samples from the 4 different brain regions were fixed in formaldehyde, sectioned, and stained with hematoxylin and eosin. Microvessels in 4 regions of the brain were examined for parasite sequestration in the 3 killed animals (animal # I89–05, # I89–27, and # I89–28). The percentage of microvessel sequestration was calculated by determining the presence or absence of parasitized red blood cells in 100–200 microvessels for each region of the brain.

Primers and probes. Oligonucleotides were synthesized at the Biotechnology Core Facility, Centers for Disease Control and Prevention (Atlanta, GA). All 5′ primers were conjugated with biotin, whereas all probes were labeled with digoxigenin, using 5′ labeling techniques standardized in the laboratory. The primers and probes were designed based on GenBank sequences from rhesus monkeys or regions of sequences conserved between human and mice (Table 1). Tumor necrosis factor alpha (TNF-α), interferon-gamma (IFN-γ), and interleukin-1-beta (IL-1β) primers and probes were standardized by using control and phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) of rhesus monkeys.

RNA extraction. The total RNA was extracted from homogenized tissue in RPI (GIBCO, Grand Island, NY) by UltraSpec (Biotex Laboratories, Houston, TX). The extracted RNA was further purified by using RNeasy Mini kit (Qiagen, Valencia, CA). Reverse transcriptase-polymerase chain reaction. Quantitative reverse transcriptase-polymerase chain reaction
Table 1

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Sequence</th>
<th>PCR product size</th>
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<tbody>
<tr>
<td>TNF-α</td>
<td>Forward: TCTCGAACCCCCGAGTGGACAA</td>
<td>124 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: TATCTCTAGTCCTACACACACA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe: TGAAGGTCTGCTCAACTAGGCGCTA</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Forward: AGTTATATGTCTGTTTCTCA</td>
<td>316 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACCAAATTAGTGATAGGCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe: ATTGGGCTGTCATATTATTTTCTG</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward: GACACATGGGATAGCAAGGCC</td>
<td>563 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACCAGGAGACCTAGCAGGTT</td>
<td></td>
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<tr>
<td></td>
<td>Probe: AGCTTTTTTCGCTGAGTCGTCGA</td>
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<td>IL-12 p40</td>
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<td>431 bp</td>
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<tr>
<td></td>
<td>Reverse: GGCCCGCACGCTATTGCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe: TGGCTCTGCTGCTGACC</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Forward: GTGCCTGCTGCTGCTG</td>
<td>463 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTGCGCAGAGAAATGCTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe: TGGGCCTACACTCTGCTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe: ATTTGGCTCTGCATTGTCT</td>
<td></td>
</tr>
<tr>
<td>INOS</td>
<td>Forward: GTCCGGGAGAGGCTGAGTAG</td>
<td>296 bp</td>
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<tr>
<td></td>
<td>Reverse: TTGCCTGCTGCTGCTG</td>
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</tr>
<tr>
<td></td>
<td>Probe: TGGGACATGAGACCTGAGGCTC</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward: TGGAAATCTGGCCATGCTGAA</td>
<td>480 bp</td>
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<tr>
<td></td>
<td>Reverse: TAAACCGAGTCTAGTACAGTCCG</td>
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</tr>
<tr>
<td></td>
<td>Probe: TGGTACCCCGACACACTGTCTGCG</td>
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(RT-PCR) was performed as described by Xiao and others. cDNA was synthesized from 10 μg of total RNA by using 10 μM of Oligo-dT primer and the SuperScript® Preamplification System for First Strand cDNA Synthesis System (GIBCO, Grand Island, NY). PCR assay was then performed using 100 nM specific primers (forward primer biotin labeled), 200 μM of each deoxynucleotide triphosphate (dNTP), 1X PCR buffer (Perkin-Elmer Cetus, Norwalk, CT) with denaturation at 94°C for 5 min, 30 cycles of PCR were performed in the GeneAmp 9700 (Perkin-Elmer Cetus, Norwalk, CT) with denaturation at 94°C for 45 sec, annealing at 53°C for 45 sec, and extension at 72°C for 60 sec. A final extension was done at 72°C for 5 min. Positive and negative controls were run in parallel in the PCR and hybridization assay.

Quantitation of RT-PCR products. Five microliters of amplified PCR product was added to 220 μl of hybridization buffer (4X SSC [1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 20 mM HEPES, 2 mM EDTA, and 0.15% Tween 20) containing 100 nM of digoxigenin-conjugated probes. After denaturation at 94°C for 10 min and annealing at 37°C for 30 min, 100 μl were transferred in duplicate to wells of streptavidin-coated microtiter plates and incubated at 37°C for 1 hour. After the plates were washed with phosphate buffered saline (PBS)/0.05% Tween 20/2 mM EDTA, 5 ng of AquaLite® Aequorin-labeled anti-digoxigenin antibodies (SeaLite®, Norcross, GA) in assay buffer (0.5% gelatin and 0.15% Tween 20 in PBS) were added and incubated at 37°C for 1 hour. After 4 final washes with PBS/0.05% Tween 20/2 mM EDTA, the plates were read in a Dynatech MLX® Luminometer (Dynatech, Chantilly, VA) set on the integrate-flash mode with automatic gain. Tris buffer (50 mM, pH 7.5) containing 100 mM CaCl₂, was added to trigger the aequorin-coelenterate luciferin reaction. The amount of PCR product in the sample was expressed in relative light units (RLU).

FIGURE 1. Parasitemia of rhesus monkeys infected with Plasmodium coatneyi. Four rhesus monkeys were infected on Day 0 and parasitemia was followed for 11 days at which time all of the monkeys were killed. Control monkeys were aparasitemic throughout the experiment. Monkey i89–25 died on Day 10.

RESULTS

Parasitemia. Four animals inoculated with P. coatneyi blood-stage parasites became parasitemic 4 days after inoculation (Figure 1). Parasitemia increased gradually with synchronized appearance of ring-stage parasites in the periphery and sequestration of late-stage parasites in the microvasculature. Parasitemia reached high levels (712,000 to 880,000 PRBC/mm³) 10 days after parasite inoculation. The infection was so severe that one animal became comatose on Day 10 and died. The remaining rhesus monkeys were killed on Day 11.

Sequestration. Microvessels in 4 regions of the brain were examined for parasite sequestration in the 3 killed animals. Sequestration of infected erythrocytes and mononuclear cells was the highest in the cerebellum, with 63.3% of...
microvessels having sequestration (Table 2). Moderate sequestration was also seen in the cortex and white matter of the cerebrum, with 41.8% and 32.0% of sequestration, respectively. Lower sequestration (30.0%) was observed in the midbrain.

**Tumor necrosis factor alpha.** Localized mRNA expression of TNF-α was high in the cerebellum of the infected animals (486.4 ± 78.2 RLU) compared to that of the uninfected controls (258.1 ± 12.1 RLU). TNF-α mRNA expression levels were also higher in the cortex of infected animals (207.7 ± 11.4 RLU) than in that of the uninfected controls (112.6 ± 39.2 RLU). TNF-α mRNA expression levels in the white matter and midbrain of the infected animals were similar to those of the uninfected controls (Figure 2).

**Gamma interferon.** As with TNF-α, the highest level of IFN-γ mRNA expression was observed in the cerebellum of the infected group (301.1 ± 33.2 RLU in infected animals versus 34.6 ± 11.2 RLU in uninfected animals). IFN-γ mRNA expression was also high in the cortex of one infected animal. IFN-γ mRNA expression levels of the infected animals were similar to those of the uninfected animals (Figure 3).

**Interleukin 1-beta.** Infected and control animals differed in IL-1β expression in all regions of the brain. IL-1β mRNA expression levels were highest in the cerebellum of the infected animals (609.55 ± 128.85 RLU in infected animals versus 63.1 ± 20.6 RLU in uninfected animals). Infected animals also had higher IL-1β mRNA expression levels than did uninfected animals in the cortex (423.0 ± 353.8 RLU versus 50.0 ± 31.1 RLU), white matter (61.4 ± 24.0 RLU versus 11.2 ± 9.6 RLU), and midbrain (335.3 ± 158.1 RLU versus 70.1 ± 5.7 RLU) (Figure 4).

**Intercellular adhesion molecule-1.** Intercellular adhesion molecule-1 (ICAM-1) mRNA expression levels were higher in the cerebellum of the infected animals (644.9 ± 192.7 RLU) than in that of the uninfected animals (381.7 ± 20.6 RLU). ICAM-1 mRNA expression levels in the cortex, white matter, and midbrain of the infected animals were similar to those of the uninfected (Figure 5).

**Inducible Nitric Oxide Synthetase.** Levels of iNOS mRNA expression were remarkably higher in the cerebellum of infected animals (23.3 ± 12.1 RLU) than in that of the uninfected (4.7 ± 1.4 RLU). Levels of iNOS mRNA expression in the cortex, white matter, and midbrain of the infected animals were similar to those of the uninfected controls (Figure 6).

**Interleukin 12.** Interleukin-12 mRNA expression levels

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**Table 2**

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Cerebellum</th>
<th>White brain</th>
<th>Cortex</th>
<th>Midbrain</th>
</tr>
</thead>
<tbody>
<tr>
<td>I89-05</td>
<td>NA</td>
<td>26.1</td>
<td>38.2</td>
<td>21.5</td>
</tr>
<tr>
<td>I89-27</td>
<td>47.4</td>
<td>38.7</td>
<td>43.9</td>
<td>22.1</td>
</tr>
<tr>
<td>I89-28</td>
<td>79.1</td>
<td>31.3</td>
<td>43.2</td>
<td>46.3</td>
</tr>
</tbody>
</table>

Mean ± SD 63.3 ± 22.4 32.0 ± 6.3 41.8 ± 3.1 30.0 ± 14.1

NA = tissue section insufficient for quantitation of sequestration; SD = standard deviation.

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**Figure 2.** Expression of tumor necrosis factor-α (TNF-α) mRNA as quantitated by reverse transcriptase-polymerase chain reaction (RT-PCR) in 4 regions of the brain in *Plasmodium coatneyi*-infected and uninfected rhesus monkeys are presented. White bars = cortex. Black bars = white matter. Brick bars = cerebellum. Diagonal bars = midbrain. Error bars = the standard deviation of duplicate TNF-α quantitation in the same brain tissue sample.

**Figure 3.** Expression of gamma interferon (IFN-γ) mRNA as quantitated by reverse transcriptase-polymerase chain reaction (RT-PCR). White bars = cortex. Black bars = white matter. Brick bars = cerebellum. Diagonal bars = midbrain. Error bars = the standard deviation of duplicate IFN-γ quantitation in the same brain tissue sample.

**Figure 4.** Expression of interleukin-1β (IL-1β) mRNA as quantitated by reverse transcriptase-polymerase chain reaction (RT-PCR). White bars = cortex. Black bars = white matter. Brick bars = cerebellum. Diagonal bars = midbrain. Error bars = the standard deviation of duplicate IL-1β quantitation in the same brain tissue sample.
in the 4 brain regions of the infected animals were similar to those of the uninfected animals (data not shown).

Expression of actin. No difference was observed in the expression levels of the housekeeping gene actin between the two animal groups among the 4 regions of the brain, indicating that similar amounts of total mRNA were present in the total RNA used in the quantitation of mRNA expression (data not shown).

DISCUSSION

Several hypotheses have been developed to explain the pathogenesis of cerebral malaria. Sequestration of PRBC, the production of proinflammatory cytokines, and their downstream reactions such as mechanical blockage, aschemia, acidosis, hemorrhages, and nitric oxide production have been implicated in the pathogenesis. Several studies using the Plasmodium berghei model support the role of TNF-α, ICAM-1, and nitric oxide in the pathogenesis. This model also reveals the importance of mechanical blockage in microvasculature damage. However, difficulty arises in extrapolating the underlying pathogenic mechanisms of human cerebral malaria from the murine findings, because the sequestration in humans involves PRBC rather than leukocytes and because the regulation of the cytokine network and nitric oxide production are fundamentally different between humans and mice.

The P. coatneyi/rhesus model avoids some of the problems associated with the murine model in terms of parasite biology, cellular sequestration, and the phylogenetic similarities of the host to humans. The PRBC sequestration patterns in the P. coatneyi model have been shown to mimic those of the P. falciparum infection in humans including synchronized appearance of ring-stage parasites, the absence of late trophozoites and schizonts in the periphery, the temporal reduction in parasitemia following the appearance of ring-stage parasites, and the preferential binding of PRBC to endothelium in the cerebellum. The results of another recent study we conducted indicate that cytokine profiles in the P. coatneyi/rhesus model are similar to those observed in humans. The peripheral production of pro-inflammatory cytokines (IL-1β and TNF-α) and TH-1 (IFN-γ) cytokines increased in the rhesus monkeys after P. coatneyi infection. These observations agree with previous conclusions based on pathologic studies of humans and murine models that implicate an overproduction of pro-inflammatory and TH-1 cytokines as an important factor in the immunopathogenesis of cerebral malaria.

Results of this study indicate differences in local cytokine response in various regions of the brain after plasmodial infection. The pro-inflammatory cytokines (IL-1β and TNF-α) and TH-1 cytokine (IFN-γ) had the highest levels of mRNA expression in the cerebellum during late P. coatneyi infection in rhesus monkeys. These observations agree with histopathologic observations on the preferential sequestration of PRBC in the cerebellum. Thus the increased cytokine expression in the cerebellum of infected animals was probably the direct result of increased local antigen stimulation. As a result of increased expression of proinflammatory cytokines, ICAM-1 and iNOS mRNA expression levels were upregulated in the cerebellum of infected monkeys. These results suggest that both PRBC sequestration and cytokine production probably play important roles that culminate in cerebral pathology. Therefore, vascular damage associated with mechanical blockage and immunological disorders associated with cytokine production may work synergistically in the induction of cerebral malaria. The P. coatneyi/rhesus model provides an alternative model for examining the role of these and other factors in the pathogenesis of human cerebral malaria.

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