Malaria Infection Induces Rapid Elevation of the Soluble Fas Ligand Level in Serum and Subsequent T Lymphocytopenia: Possible Factors Responsible for the Differences in Susceptibility of Two Species of Macaca Monkeys to Plasmodium coatneyi Infection

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The intraerythrocytic stage of the simian malaria parasite Plasmodium coatneyi (CDC strain) was intravenously inoculated into two species of macaques with different susceptibilities to infection with this parasite, including four Japanese macaques (Macaca fuscata) and three cynomolgus macaques (M. fascicularis). The Japanese macaques infected with P. coatneyi developed severe clinical manifestations similar to those of severe human malaria and eventually became moribund, while the infected cynomolgus macaques, natural hosts of the parasite, exhibited no severe manifestation of disease except anemia and finally recovered from the infection. In the infected Japanese macaques, peripheral CD4+ and CD8+ T-cell populations were markedly decreased and fragmentation of chromosomal DNA in peripheral blood mononuclear cells was detected during the terminal period of infection, suggesting that apoptotic cell death was responsible at least in part for the T lymphocytopenia. Furthermore, soluble Fas ligand levels in sera of the infected Japanese macaques increased gradually to a markedly high level of 28.83 ± 10.56 pg/ml (n = 4) when the animals became moribund. On the other hand, none of the infected cynomolgus monkeys exhibited either T lymphocytopenia or elevated soluble Fas ligand level. These findings suggest that differences in immune response between the two species of macaque tested accounted for the contrasting outcomes after infection with the same isolate of malarial parasite, and in particular that a profound T lymphocytopenia due to Fas-derived apoptosis played a role in the fatal course of malaria in the infected Japanese macaques.

Plasmodium falciparum infection leads to severe signs and symptoms and a wide variety of clinical consequences, but not every patient becomes seriously ill or dies (28). The outcome of infection is influenced by individual susceptibility, parasite virulence, and a number of environmental factors, and recent intensive studies on the factors responsible for resistance to malarial infection have elucidated some effects of host genetic background on outcome of infection as well (8, 16).

P. coatneyi, a simian malarial parasite, causes mild infection in its natural host, the cynomolgus macaque (Macaca fascicularis) (4), but more serious infection in experimental hosts such as the Japanese macaque (M. fuscata) (11) and rhesus macaque (M. mulatta) (1). In the latter two species, infected monkeys develop a fulminating acute infection with pronounced parasitemia and became moribund with severe manifestations. Furthermore, they exhibit histopathological findings typical of cerebral malaria: sequestration of parasitized red blood cells and cytoadherence-associated knobs on parasitized red cells to endothelial cells were found in cerebral microvessels and capillaries of major organs in these monkeys. Japanese macaques and rhesus macaques infected with P. coatneyi can thus be used as powerful primate models for the pathophysiological study of severe human malaria.

The Japanese macaque belongs to the genus Macaca, the same genus as the rhesus macaque and cynomolgus macaque, and is found on three of the four major islands of Japan (15), where natural infection with simian malaria has never been reported. On the other hand, the cynomolgus macaque is widely distributed throughout Southeast Asian countries (15), where various species of simian malaria are endemic and the monkey is a natural host of malarial parasites such as P. coatneyi, P. cynomolgi, P. inui, and P. knowlesi (5). As previously proposed (29), one important factor maintaining the species integrity of cycnomolgus macaques may be ecological isolation due to genetic resistance to certain species of simian malarial infection.

In the present study, we used two species of macaques differing in susceptibility to P. coatneyi infection, Japanese macaques and cynomolgus macaques, as models nonresistant and resistant to malarial infection, respectively. The aim of this study was to clarify immunological features in infected monkeys with different susceptibilities to the parasite. The infected Japanese macaques exhibited severe peripheral T lymphocytopenia and a markedly high serum level of soluble Fas ligand (sFasL) when they became moribund, whereas the infected cynomolgus macaques exhibited none of these changes.

We report here factors possibly determining the differences in outcome between two species of macaques with different
susceptibilities to malaria infection and the applicability of the
P. coatneyi-infected Japanese macaque to the pathophysiological
study of severe human malaria.

MATERIALS AND METHODS

Experimental animals. Four Japanese macaques (J-8, J-9, J-10, and J-11) and
three cynomolgus macaques (CY-1, CY-2, and CY-3) were used in this study in
accordance with the guidelines for use of experimental animals authorized by
the Japanese Association for Laboratory Animal Science. All monkeys were bred
and grown in animal facilities in a malaria-free environment in Japan and were
2 years old when used. Each animal was kept in an individual cage in a controlled
environment at 25 to 29°C and fed commercial food pellets supplemented with
fresh fruits. At the time of infection, all animals were clinically healthy and used
without splenectomy.

Parasite and infection. P. coatneyi (CDC strain) was used in this study. This
strain was also used in previous studies and proved infective in macaques of both
species employed in this study (11, 14). Each macaque was intravenously inoc-
ulated without splenectomy. At the time of infection, all animals were clinically healthy and used
without splenectomy.

Sample collection. After inoculation, daily clinical follow-up of the monkeys was
performed; no antimalarial treatment was given during the course of infec-
tion. Hematological examination and flow cytometric analyses of the blood samples were obtained every 3 or 4 days or more frequently after infection. When
the infected macaques became moribund with high parasitemia, they were anes-
thetized with an intramuscular injection of ketamine (15 mg/kg of body weight)
and then autopsied. Thin blood films were prepared from blood obtained
to observe. Following Giemsa staining, parasitemia was counted in a total of
100 erythrocytes under an optical microscope.

Flow cytometric analyses. One or two million mononuclear cells per milli-
liter of sample blood was mixed with 20 µl of sample blood mixed with 20 µl
of MAb reagent and incubated for 15 min at room temperature. Cells were
treated with buffer (FACS lysing solution; Becton Dickinson), washed twice with
phosphate-buffered saline, resuspended in phosphate-buffered saline, and ana-
yzed using FACScalibur (Becton Dickinson). Analyses of the fluorescence inten-
sities were performed with CellQuest software (Becton Dickinson). Samples
were analyzed by setting appropriate forward and side scatter gates around the
lymphocyte populations. The number of peripheral leukocytes and their differ-
entiation was determined simultaneously. The absolute count of lymphocytes
for each subset was derived from the number of circulating lymphocytes and the
proportion of MAbs-positive cells in flow cytometric analyses. Fas-positive rates of
CD4, CD8, and CD2 lymphocytes were determined in two-color immu-
nostaining using PE-labeled anti-CD54 MAb and FITC-labeled anti-CD2, anti-
CD4, or anti-CD8 MoAb simultaneously.

DNA fragmentation assay. To detect DNA fragmentation, which is character-
istic of cells undergoing apoptosis, fresh peripheral blood mononuclear cells
(PBMCs) were obtained from the infected Japanese macaques and cynomolgus
macaques, and PBMCs from an uninfected Japanese macaque were used as a
negative control. PBMCs were separated from whole venous blood by Ficoll-
Paque PLUS (Pharmacia Biotech AB, Uppsala, Sweden) density gradient, and
106 cells were used as described previously (18) prior to isolation of chromo-
sonal DNA. The DNA was then subjected to electrophoresis in a 0.75% agarose
gel and visualized with ethidium bromide.

Fas staining in serum. Serum-FasL levels in the infected monkeys were
measured using a commercially available human FasL-messuring kit (MBL,
Nagoya, Japan), which is a sandwich enzyme-linked immunosorbent assay system
using two anti-human FasL, hamster MAb, 4H9 and 4A5 (20). The cross-
reactivity of monoclonal antibody 4A5 with rCyfasL was 32% as measured by ELISA
and was used as a standard. One hundred microliters of serum or standard solution
containing 5 to 1,000 pg of rCyfasL per ml was added to two wells of primary
antibody (4H9)-coated ELISA plate. After reaction at room temperature for 1 h,
the plate was washed five times with washing solution. Then 100 µl of diluted second
antibody (4A5) was added to each well and reacted at room temperature for additional 1 h. After another five washes, 100 µl of peroxidase substrate solution was added and allowed to incubate at 37°C for 30
min. Finally, acid solution was added to each well to terminate the enzyme
reaction. The optical density of each well was measured at 450 nm using a
dual-wavelength plate reader (THERMOMax, Molecular Devices, Sunnyvale,
Calif.). The correlation between optical densities and standard rCyfasL contents
ranging from 5 to 1,000 pg/ml was always significant (r > 0.9, p < 0.01). Serum
FasL content was determined by reference to the standard curve obtained from
the reactivity of the rCyfasL.

RESULTS

Clinical findings and parasitemia. In this study, two species of Macaca
monkeys, Japanese macaques and cynomolgus macaques, had quite different con-
sequences of infection with the same isolate of the malarial parasite P. coatneyi: the
infection was fatal to the Japanese macaques, whereas the cynomolgus
macaques persisted with the infection and eventually survived. The infected
Japanese macaques were frequently anorectic, listless, and occasionally depressed, and they finally became
lethargic and severely withdrawn just before autopsy on days
14 (J-8), 12 (J-9), 12 (J-10), and 11 (J-11) after infection. The parasite was first detected in the peripheral blood of the infected
Japanese macaques about 7 days after infection; parasite densities then increased sharply to around 30% within 2
weeks after infection (Fig. 1A). Maximum parasitemias were
30.6% (J-8), 41.0% (J-9), 29.7% (J-10), and 17.6% (J-11). On the other hand, the cynomolgus macaques exhibited no severe
manifestation except anemia (the lowest hematocrits were
18.0% [CY-1], 34.0% [CY-2], and 11.0% [CY-3]). The prepatent
period in the infected cynomolgus macaques was about 7 days; parasitemia then increased, peaked at around 5%, and subse-
quently decreased to less than 0.01% (Fig. 1B) within 35 days after infection. Maximum parasitemias were 4.8% (CY-1),
0.2% (CY-2), and 11.0% (CY-3).

Kinetics of lymphocyte subsets. Absolute counts for each lymphocyte subset at different time points in the infected
macaques are shown in Fig. 2 and 3. Of four P. coatneyi-infected
Japanese macaques, three (J-8, J-9, and J-11) had a markedly
reduced number of CD2+ lymphocytes when they were severely
ill during the rapid increase in parasitemia (Fig. 2A). None of the infected
cynomolgus macaques exhibited reduced number of CD2+ lymphocytes (Fig. 2B). The cynomolgus macaques instead exhibited a gradual increase in CD2+ lymphocyte population with one or two peaks just after infection and/or during the period in which the peak of the parasitemia was observed. In the infected Japanese macaques, differentiation
of T-cell subsets by CD4 and CD8 markers revealed that
the populations of both subsets had kinetics similar to those of
CD2+ lymphocytes (Fig. 3A) and thus that both subtypes of lymphocytes were involved in T lymphocytopenia.

Involvement of apoptotic cell death in T lymphocytopenia.
We hypothesized that T lymphocytopenia in P. coatneyi-
infected Japanese macaques was caused by apoptosis of T cells on the basis of findings of previous studies of human malarial
infection (24, 25). To confirm this hypothesis, chromosomal
DNA was prepared from fresh PBMCs of monkeys with or
without P. coatneyi infection and analyzed by agarose gel elec-
rophoresis. As shown in Fig. 4, DNA fragmentation occurred
only in PBMCs obtained from the P. coatneyi-infected Japa-
nese macaques when they became moribund, not in an unin-
fected control monkey. In contrast, no ladder pattern of DNA
fragments was observed on electrophoresis using the samples
from the infected cynomolgus macaques on days 7, 14, and 21
after infection (data not shown).

Rates of Fas expression of lymphocyte subsets. Since Fas
antigen is a cell surface protein that mediates apoptosis, the
-Fas-positive rate of each lymphocyte subset was determined before and after infection with P. coatneyi (Table 1). In both
species of macaques, Fas positivity of CD2+ cells decreased on
11 to 14 days after P. coatneyi infection, which was more pro-
nounced in the infected Japanese macaques when they became

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severely ill. In addition, the Fas expression rate of CD4$^+$ and CD8$^+$ cells also decreased in only Japanese macaques when they showed severe manifestations.

sFasL concentration in serum. We subsequently measured sFasL concentration in serum obtained from the infected monkeys. As clearly shown in Fig. 5A, the infected Japanese macaques had markedly increased levels of sFasL, and this elevation was simultaneous with the period of T lymphocytopenia. The Japanese macaques had high maximum levels of sFasL (17.38 pg/ml in J-8, 28.00 pg/ml in J-9, 42.94 pg/ml in J-10, and 27.00 pg/ml in J-11) at the time of autopsy. In the cynomolgus macaques, which were free of T lymphocytopenia, the concentration of serum sFasL remained much lower than those in the infected Japanese macaques throughout the course of infection (the highest concentrations were 3.69 pg/ml in CY-1, 7.11 pg/ml in CY-2, and 0.06 pg/ml in CY-3 [Fig. 5B]).

FIG. 1. Course of P. coatneyi parasitemia in four Japanese macaques (A) and three cynomolgus macaques (B) after infection with parasitized red blood cells.

DISCUSSION

Host genetic factors including immune response which govern susceptibility to malaria infection are well documented (8, 16). In the present study, two species of Macaca monkeys exhibited markedly different outcomes of infection with the same isolate of malaria parasite: infection was lethal to Japanese macaques but not to cynomolgus macaques. These results were consistent with those described in previous reports (4, 11).

Studies of lymphocyte kinetics revealed that profound T lymphocytopenia occurred in the P. coatneyi-infected Japanese macaques, whereas the infected cynomolgus macaques were free of T lymphocytopenia. T lymphocytopenia is also a well-known finding for human patients with acute severe malaria (3, 7, 9, 26), but little is known concerning the changes in T-cell

FIG. 2. Absolute numbers of peripheral CD2$^+$ (●) and CD20$^+$ (□) lymphocytes in four Japanese macaques (A) and three cynomolgus macaques (B) after infection with P. coatneyi.
populations following infection with falciparum malaria. Our study of lymphocyte kinetics using a primate model of severe malaria revealed that T-cell numbers decreased most markedly when the infected Japanese macaques became moribund, suggesting that T lymphocytopenia was intimately associated with severe signs of disease.

Apoptosis is considered a possible mechanism of T lymphocytopenia in human malaria, since patients with acute *P. falciparum* infection have higher in vitro percentages of lymphocyte apoptosis than do healthy individuals (24, 25). To determine whether the T lymphocytopenia observed in our study was also due to apoptosis, we examined fragmentation of chromosomal DNA, one of the characteristics of cells undergoing apoptotic death. We found DNA fragmentation in PBMCs of the infected Japanese macaques suffering from severe disease but not in those of the infected cynomolgus macaques. These findings support our hypothesis that apoptotic T-cell death was, at least in part, responsible for peripheral T lymphocytopenia in the infected Japanese macaques.

Fas is a membrane protein which mediates apoptosis when it is cross-linked with its ligand. Fas-derived apoptosis plays roles in physiological immune regulatory mechanisms (2, 6, 10), but it can also be harmful (12) and even lethal (21). To examine whether the apoptosis in the infected Japanese macaques was mediated by Fas and its ligand, we measured both Fas expression rates on the surface of lymphocytes and serum sFasL concentration. We found marked elevation of serum sFasL in nonresistant Japanese macaques, which was simultaneous with the decrease in the T-cell population. Interestingly, the resistant cynomolgus macaques, in which T lymphocytopenia was absent, had outcomes very different from those of the nonresistant Japanese macaques: in the former, the serum level of sFasL remained very low throughout the course of infection. In addition, we found that the Fas-positive CD2⁺, CD4⁺, and CD8⁺ cell counts markedly decreased just after the elevation in physiological immune regulatory mechanisms (2, 6, 10), but it can also be harmful (12) and even lethal (21). To examine whether the apoptosis in the infected Japanese macaques was mediated by Fas and its ligand, we measured both Fas expression rates on the surface of lymphocytes and serum sFasL concentration. We found marked elevation of serum sFasL in nonresistant Japanese macaques, which was simultaneous with the decrease in the T-cell population. Interestingly, the resistant cynomolgus macaques, in which T lymphocytopenia was absent, had outcomes very different from those of the nonresistant Japanese macaques: in the former, the serum level of sFasL remained very low throughout the course of infection. In addition, we found that the Fas-positive CD2⁺, CD4⁺, and CD8⁺ cell counts markedly decreased just after the elevation

<table>
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<tr>
<th>Animal</th>
<th>Day after infection</th>
<th>Rate of Fas positivity (%)</th>
<th>CD2⁺</th>
<th>CD4⁺</th>
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<td>51.1</td>
<td>86.4</td>
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</tr>
<tr>
<td>J-11</td>
<td>11 (severe illness)</td>
<td>58.7</td>
<td>45.3</td>
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of serum sFasL concentration only in the infected Japanese macaques. These results strongly suggest that Fas/FasL-mediated apoptosis involves the elimination of peripheral T cells, resulting in severe lymphocytopenia and manifestations of disease in the infected Japanese macaques. The difference in immune responses including the function of FasL-producing cells during the course of malaria infection might explain the difference in susceptibility between Japanese macaques and cynomolgus macaques.

As described for other infectious diseases (13, 19, 23, 27), falciparum malaria infection also induces host immune dysfunction including a decreased number of circulating T cells (3, 7, 9, 26) and in vitro depression of proliferative response of PBMCs to parasite antigens (17, 22), which may be responsible for the severe disease of the patients with falciparum malaria. In fact, T lymphocytopenia is also considered one of the typical features of human acute falciparum malaria, and the degree of T lymphocytopenia is correlated with disease severity: it is higher in patients with severe malaria and those with cerebral malaria than in patients with uncomplicated malaria (9). Although the pathogenic process of severe malaria is considered multifactorial, our findings suggest the additional possibility that malarial infection induces a high level of sFasL in the periphery of nonresistant hosts, which causes T lymphocytopenia and subsequent immune dysfunction and severe disease. These immunopathological changes in severe malaria should be taken into account in the design and analysis of cellular investigations and the clinical treatment of patients with severe manifestations of falciparum malaria.

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FIG. 5. Time course of serum sFasL levels in four Japanese macaques (A) and three cynomolgus macaques (B) after infection with P. coatneyi.