Animal Model

Immunopathology and Ehrlichial Propagation Are Regulated by Interferon-γ and Interleukin-10 in a Murine Model of Human Granulocytic Ehrlichiosis

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Previous studies of human granulocytic ehrlichiosis (HGE) suggest a role for host immune response in resolving infection and in causing histopathological lesions. We hypothesize that interferon (IFN)-γ allows tissue injury that is suppressed by interleukin (IL)-10 after initiation by ehrlichia infection. Thus, parental C57BL/6, IL-10−/−, and IFN-γ−/− strains of mice were infected and then assayed for hepatic histopathological lesions, ehrlichial burden, and cytokine responses to ehrlichial antigen in primary splenic cultures during the first 21 days after infection. Histopathological severity in C57BL/6 of mice rose in parallel through day 7, but then diverged as pathology in IL-10−/− mice continued to increase and remained high throughout the course of the study. The histopathological rank of C57BL/6 of mice decreased at day 10 and returned to baseline levels at days 14 and 21. In contrast, the IFN-γ−/− strain had baseline pathology scores throughout the course of the infection, yet had significantly higher ehrlichial burden both in the blood and tissues than C57BL/6 or IL-10−/− mice. This suggests that histopathological lesions in the HGE murine model do not result from direct ehrlichia-mediated injury but from immunopathological mechanisms initiated by ehrlichial infection. The similarities with lesions in humans suggest an immunopathological basis for HGE. (Am J Pathol 2001, 158:1881–1888)

Materials and Methods

Mice
Pathogen-free male mice (3 to 6 weeks of age) were obtained from Jackson Labs (Bar Harbor, ME) and main-

Murine models have proven useful for studying various aspects of the pathogenesis of human granulocytic ehrlichiosis (HGE), an acute, febrile illness caused by Ehrlichia phagocytophila. Although mice do not develop clinical signs, they do develop pathological lesions closely resembling those seen in humans and other species with granulocytic ehrlichiosis.1–4 Sero-epidemiological studies in humans and horses suggest that the disease is often mild or inapparent.5,6 However, severe infection in humans may be complicated by acute respiratory distress syndrome and opportunistic infections.7–9 The precise mechanism by which pathological injury occurs in HGE is not known. The temporal, microanatomical, and quantitative disparity between ehrlichiae detected in tissue and the degree of histopathological injury does not support a role for a direct bacterial cytolytic mechanism. Some aspects of the clinical disease and histopathological lesions of HGE suggest the potential for host-mediated immunological injury.1 Therefore, previous studies in our laboratory have examined host immune response in a murine model, focusing on the role of cytokines in relation to developing pathology and ehrlichial quantity. In C3H/HeJ mice, a strain known to be susceptible to rickettsial infections, levels of plasma interferon (IFN)-γ peak before maximal pathological injury when ehrlichiae are absent in tissues, supporting a role for host immunity in the pathogenesis of HGE.3 The purpose of this study was to examine the roles of proinflammatory and anti-inflammatory cytokines and ehrlichial quantity on host pathology in a murine model using IFN-γ- and interleukin (IL)-10-deficient mice.

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tained in microisolator cages in accordance with institutional guidelines. 48 mice of each of the following strains were obtained: C57BL/6-Ifngtm1Ts (IFN-γ−/−), C57BL/6-IL10tm1Cgn (IL-10−/−), and parental C57BL/6 (hereafter referred to as C57BL/6). They were allowed to acclimate for 5 days before manipulation.

Inoculum

Webster strain HGE agent *E. phagocytophila* was grown in HL-60 cells, a human promyelocytic cell line, until 100% of the cells contained morulae. On the day of inoculation, infected and uninfected cells were centrifuged, then resuspended in serum-free RPMI 1640 medium to a concentration of 2 × 10⁶ cells/ml. Twenty-four mice of each strain were inoculated intraperitoneally with 0.5 ml (1 × 10⁶) uninfected HL-60 cells (sham-inoculum), or HL-60 cells infected with *E. phagocytophila* (passage 4). Mice were observed daily for evidence of clinical illness, such as fur ruffling, hunched posture, depression, anorexia, or tachypnea.

Necropsy

Eight mice of each strain (four inoculated with *E. phagocytophila*-infected cells and four with uninfected HL-60 cells) were necropsied at six time points: days 0 (4 hours after inoculation), 4, 7, 10, 14, and 21. The mice were sedated with methoxyflurane, then exsanguinated by cardiac puncture, followed by cervical dislocation. The spleen was sterilely harvested, followed by collection of lung, liver, sublumbar lymph nodes, and brain. Bone marrow was obtained by flushing the femur with 1 ml of sterile phosphate-buffered saline (PBS). Half of the spleen was placed in sterile culture medium (RPMI 1640 and 2% fetal bovine serum), a quarter fixed in 10% formalin, and a quarter was frozen at −80°C. The remainder of the tissues were formalin-fixed and paraffin-embedded for hematoxylin and eosin (H&E) staining and immunohistological examination. Tissues were examined for lymphohistiocytic cell aggregates, necrosis, and apoptosis. The degree of liver pathological changes observed on histological examination. Tissues were examined for lympohiphistic cell aggregates, necrosis, and apoptosis.

Cytokine Assays

Levels of IFN-γ and IL-10 were assayed in the supernatant of the splenic cultures by sandwich enzyme-linked immunosorbent assay. Capture and detection monoclonal antibodies and recombinant antigen for IFN-γ and IL-10 were obtained from Pharmingen (San Diego, CA). Briefly, 96-well plates were coated with capture antibody and incubated overnight at 4°C. The wells were blocked with PBS with 3% bovine serum albumin for 2 hours, and then washed with PBS/Tween. Doubling dilutions of recombinant antigen (diluted in PBS with 3% bovine serum albumin) were used for a standard curve. Then recombinant antigen standards or supernatants were added to duplicate wells and incubated overnight at 4°C. Plates were washed and reacted with biotinylated secondary antibody, and developed at room temperature for 45 minutes. After washing, plates were reacted with streptavidin alkaline phosphatase (DAKO, Carpinteria, CA) at room temperature for 30 minutes, and washed again with PBS/Tween. Color was developed with the TMB peroxidase system (KPL, Gaithersburg, MD) and the optical density measured at 630 nm on an enzyme-linked immunosorbent assay plate-reader.

Serology

Plasma was assayed for *E. phagocytophila* group antibodies by indirect immunofluorescence assay, as described elsewhere.²,³ using *E. phagocytophila*-infected HL-60 cells (Webster strain) as antigen. Plasma samples reactive at a 1:80 dilution were titrated to endpoint.

Measurement of Ehrlichial DNA Quantity

Plasma was separated from packed blood cells, and nucleic acids were prepared from the cellular fraction.
(~200 to 400 μl) using the Gentra PureGene kit (Gentra Systems, Minneapolis, MN). The samples obtained from mice inoculated with uninfected HL-60 cells were tested for E. phagocytophila DNA by polymerase chain reaction (PCR) using the primers ge9f and ge10r. DNA extracted from the blood of mice inoculated with infected cells was tested with a quantitative PCR method using an ABI TaqMan 7700 instrument (PE Biosystems, Foster City, CA). The TaqMan reporter probe and primer sequences were modified from Pusterla and colleagues.10 The fluorescent reporter dye at the 5’ end of the TaqMan probe (ehr 80p, CCTATGCATTACTCACCCTGCTGCCACT) was 6-carboxy-fluorescein (FAM); the quencher at the 3’ end was 6-carboxy-tetramethyl-rhodamine (TAMRA). Primers were amplified from a 106-bp fragment of the 16S rRNA gene (ehr 50r, 5’-TTCGACCGATTATCTTTTAGCTTG-3’, and ehr 145f, 5’-CCATTTCTAGTGCTATCCCATAC-TAC-3’). The standard curve was developed using DNA prepared from a known quantity of E. phagocytophila-infected HL-60 cells added to ethylenediaminetetraacetic acid-anti-coagulated mouse blood from which 10-fold dilutions were made in sterile water. The 50-μl PCR mixture contained 900 nmol/L each of forward and reverse primers, 150 nmol/L probe, 25 μl TaqMan Universal PCR Master Mix (Roche, Branchburg, NJ), and ~50 ng (range, 25 to 65 ng) of DNA. After target denaturation for 3 minutes at 95°C, amplification conditions were five cycles of 30 seconds at 95°C and 20 seconds at 62°C, followed by 45 cycles of 40 seconds at 85°C and 60 seconds at 62°C. Results were expressed as quantity of infected cells per μl host DNA template where 1 μl was determined to represent ~106 host cells.

Immunohistochemistry

Five-μm thick paraffin-embedded tissue sections were prepared and examined by immunohistological techniques with rabbit anti-E. phagocytophila Webster strain as the primary antibody, as described elsewhere.2 Slides were examined by light microscopy for the presence of intracytoplasmic morulae stained red in color. The total number of infected cells per volume of tissue was calculated.

Results

Animals and Pathology

Consistent with previous studies, none of the mice exhibited any clinical signs of illness. C57BL/6 mice had mild to moderate histopathological lesions (lymphohistiocytic aggregates, apoptotic cells, mild hepatitis) evident at 7 to 14 days, which were resolving by day 21. In the IL-10−/− mice, hepatic histopathological lesions were much more severe at each time point and included marked lymphohistiocytic accumulations, severe hepatitis with numerous apoptoses, and necrosis. The IFN-γ−/− mice had minimal hepatic pathology throughout the course of the experiment (Figure 1). The quantitative and kinetic results of histopathological examinations are summarized in Figure 2. Mice of the C57BL/6 and IL-10−/− strains had mildly elevated histopathology scores as compared to the IFN-γ−/− strain at day 0 (P = 0.011, P = 0.21, respectively, t-test). Histopathological severity in C57BL/6 and IL-10−/− mice rose in parallel through day 7, but then diverged as histopathological lesions in IL-10−/− mice continued to increase and remained high throughout the course of the study (P = 0.023, t-test). The histopathology rank of C57BL/6 strain of mice decreased at day 10 and returned to baseline levels at days 14 and 21. In contrast, the IFN-γ−/− strain had baseline pathology scores throughout the course of the infection.

Serology

All sham-inoculated mice remained seronegative throughout the course of the study, whereas all mice inoculated with E. phagocytophila-infected HL-60 cells seroconverted. Serum titers of the E. phagocytophila-infected mice are shown in Figure 3, and confirm results obtained in pilot studies. At day 7, the serological titer of the IFN-γ−/− mice was significantly, or near significantly, higher than the C57BL/6 (P = 0.063) or IL-10−/− (P = 0.024) mice. At day 10, the serum titer of the IL-10−/− mice continued to increase, so that by days 14 and 21 it was not significantly different from the IFN-γ−/− mice. In contrast, the C57BL/6 mice had a significantly lower serum titer than either of the other two strains at days 10 and 14 (P = 0.002, P = 0.024). By day 21, the C57BL/6 mice had a marked increase in serological titer, approaching that of the other two strains (P = 0.060).

Cytokine Measurements

To control for endogenous cytokine production by the cells in the splenic cultures, the cytokine measurements from the sham-inoculated mice were subtracted from that of the E. phagocytophila-inoculated mice. This adjusted value was plotted by averaging the duplicate cytokine measurements of each mouse strain per day in duplicate cultures (Figure 4). Splenic cultures stimulated with 50 μg/ml and 5 μg/ml of E. phagocytophila antigen produced nearly identical quantities and kinetics of IFN-γ and IL-10 whereas those stimulated with 0.5 μg/ml had little cytokine production greater than baseline levels. Day 7 results were eliminated from the calculations as the splenic cultures from that day did not respond to either ConA or lipopolysaccharide (control) stimulation. The reason for the lack of splenic culture stimulation on that day is unclear; however, the results suggest a technical problem with culture on that day alone. For the IFN-γ−/− mice, no IFN-γ was produced during the 21-day trial. The IL-10−/− and C57BL/6 strains both had IFN-γ production by days 4 to 10, which later became undetectable. IL-10 was undetectable in IL-10−/− mice throughout the study. In both IFN-γ−/− and C57BL/6 mice, IL-10 was detected early in the infection (day 0 to 4) and in the IFN-γ−/− mice again at day 21.
PCR

All sham-inoculated mice were negative by qualitative PCR. The *E. phagocytophila*-inoculated mice were tested in triplicate by quantitative PCR (Figure 5). Both C57BL/6 and IL-10−/− strains had very low quantities of *E. phagocytophila* DNA detected. The IFN-γ−/− mice on average had ehrlichial DNA detected at every time point except days 10 and 21, but not all inoculated mice had *E. phagocytophila* DNA detected at each time point. When IFN-γ−/− strain values were compared to the combined grouping of C57BL/6 and IL-10−/− strains, there were significant, or near significant, differences at days 0, 4, and 7 (P < 0.025, <0.100, and 0.010, respectively; Wilcoxon rank sum).

Immunohistochemistry

Ehrlichial tissue infection detected by immunohistochemistry is summarized in Table 1. For both the C57BL/6 and IL-10−/− mice, small numbers of infected cells were detected only in the lung, and at only one time point. The IFN-γ−/− mice had ehrlichia-infected cells detected in the lungs at days 4 and 7 and in the spleen at days 7 and

Figure 1. Hepatic pathology with HGE agent infection in IL-10−/− mice at day 14 after inoculation (A and B), C57BL/6 mice at day 21 after inoculation (C and D), and IFN-γ−/− mice at day 14 after inoculation (E and F). Note the large number of lesions and extensive necrosis/apoptosis in the IL-10−/− mice that is ameliorated in the control C57BL/6 mice, and absent in the IFN-γ−/− mice. H&E, original magnifications: ×40 (A, C, and E) and ×160 (B, D, and F).
No infected cells were detected in the liver in any strain, at any time point.

**Discussion**

Our results indicate that IFN-γ plays a crucial role in the clearance of the organism, and is a major determinant of histopathology associated with HGE. Mice that were deficient in IFN-γ developed minimal hepatic pathology, but demonstrated a significantly higher quantity of ehrlichiae in the blood and tissues than either of the other two strains examined. In contrast, IL-10/H11002 and C57BL/6 mice had a significantly greater degree of hepatic pathology, despite often undetectable levels of ehrlichiae in the blood at the same time point.

The mechanisms of tissue injury with *E. phagocytophila* infection are poorly understood. Unlike the situation with vasculotropic rickettsioses,11,12 vasculitis because of rickettsia-mediated endothelial injury does not occur with the ehrlichioses. Although *in vitro* propagation of *E. phagocytophila* strains clearly leads to significant host cell cytolysis, necrosis, and changes in apoptotic activity,13–15 it is unclear whether these bacteria-driven mechanisms have a role *in vivo*. Previous studies in humans and other animals with granulocytic ehrlichiosis have shown a stark disparity in tissue localization and quantities of ehrlichiae as opposed to the degree of pathological injury.1 Moreover, certain aspects of the clinical course including toxic shock-like manifestations and acute respiratory distress syndrome9,16–18 and pathological findings such as hepatic apoptoses and erythro-
Comparison of the quantity of *E. phagocytophila* DNA in the blood of infected C57BL/6, IFN-γ−/−, and IL-10−/− mice. C57BL/6 and IL-10−/− mice had very low quantities of *E. phagocytophila* DNA detected. The IFN-γ−/− mice had significantly increased ehrlichial DNA quantities as compared with the combined group of C57BL/6 and IL-10−/− mice at days 0 and 7 (P < 0.025 and P = 0.010, respectively, Wilcoxon rank sum) and approached significance (P = 0.100) at day 4.

Figure 5. Comparison of the quantity of *E. phagocytophila* DNA in the blood of infected C57BL/6, IFN-γ−/−, and IL-10−/− mice. C57BL/6 and IL-10−/− mice had very low quantities of *E. phagocytophila* DNA detected. The IFN-γ−/− mice had significantly increased ehrlichial DNA quantities as compared with the combined group of C57BL/6 and IL-10−/− mice at days 0 and 7 (P < 0.025 and P = 0.010, respectively, Wilcoxon rank sum) and approached significance (P = 0.100) at day 4.

Table 1. Quantity of *Ehrlichia phagocytophila* Morulae Detected by Immunohistochemistry in C57BL/6, IFN-γ−/−, and IL-10−/− Mice

<table>
<thead>
<tr>
<th>Mouse strain (tissue)</th>
<th>Day after inoculation</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
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<tr>
<td>C57BL/6 (lung)</td>
<td>0</td>
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<tr>
<td>IL-10−/− (lung)</td>
<td>0</td>
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<tr>
<td>IFN-γ−/− (lung)</td>
<td>0</td>
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<tr>
<td>IFN-γ−/− (spleen)</td>
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Results are expressed as number infected cells (No. infected cells/μl tissue).
cific mechanisms responding to ehrlichial components early in infection. To what degree these responses result from biological variations and sampling intervals is uncertain. Further investigation is needed to elucidate which other components of early intrinsic Th1 immunity are important in restricting ehrlichial growth.

It is interesting to note that no ehrlichial DNA or morulae were detected at day 10 in the IFN-γ−/− mice by quantitative PCR or immunohistochemistry, yet at day 14 low levels of ehrlichiae were detected by both techniques. Whether this is within biological variation, or is the result of biologically meaningful change is not known. E. phagocytophila strains are known to be antigenically diverse and it is possible that the second peak in ehrlichial quantity on day 14 represents the emergence of E. phagocytophila antigenic variants. In other rickettsial species, such as Anaplasma marginale, Ehrlichia risticii, Ehrlichia canis, and Ehrlichia chaffeensis, antigenic variation results from the differential expression of one or more major surface proteins encoded by a family of paralogous genes. It is speculated that the ability of ehrlichiae to modify major surface protein profiles may allow immune evasion and persistence of infection. This observation needs further investigation, but may explain some disparity in ehrlichial quantity at different time points.

In summary, IFN-γ-related mechanisms are important in clearance of the organism in the early phases of HGE. However, IFN-γ also plays an essential role in pathology associated with the infection. IL-10 moderates the pathology, perhaps through down-regulatory effects on IFN-γ or through other anti-inflammatory mechanisms. Further studies are needed to determine what components of the ehrlichial organism stimulate the inflammatory response and drive the IFN-γ production, as well as the mechanisms of disease resolution and control of immunopathology. Whether results derived from murine models have relevance to human and veterinary granulocytic ehrlichiosis has yet to be proven. However, the mouse model that implicates immunopathological responses in the pathogenesis of HGE does provide a good pathological and experimental mimic of infection and should provide insights that can be extrapolated to humans and tested in other infection models.

References


