Pyrimethamine Impairs Host Resistance to Infection with *Listeria monocytogenes* in BALB/c Mice


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Increased mortality has been observed when HIV-infected patients were treated with pyrimethamine (Pyr) as prophylaxis for toxoplasmonic encephalitis, suggesting that Pyr might possess immunosuppressive activity. To analyze this in an animal model, immune function was assessed in BALB/c mice using a battery of in vivo and ex vivo assays and an in vivo model of host resistance to *Listeria monocytogenes* infection. Treatment for 30 days with 60 mg/kg Pyr decreased circulating white blood cell and lymphocyte counts but not neutrophil, red blood cell, or platelet counts or hemoglobin levels. Splenic B cell percentages and lipo polysaccharide-induced B cell proliferation decreased significantly after treatment with 60 mg/kg Pyr, as did levels of anti-keyhole limpet hemocyanin (KLH) IgM in serum 7 days after immunization with KLH. Anti-KLH IgG levels 14 days after immunization were not affected. Percentages of splenic T cells and macrophages and T cell proliferation in the presence of concanavalin A or allogeneic cells were not decreased by Pyr treatment. An ex vivo assay of T-cell-mediated cytotoxicity was also unaffected. When host resistance to *L. monocytogenes* infection was assessed, dramatic increases in mortality were observed in Pyr-treated compared to control mice. Increased numbers of *L. monocytogenes* organisms were observed in liver and spleen of Pyr-treated mice, compared to controls. The reduction in *Listeria* resistance, which is T cell mediated, contrasts with the fact that no significant changes in T-cell-mediated immunity were observed. It is possible that Pyr affects parameters of innate immunity, which were not monitored in this study. © 1998 Society of Toxicology.

Pyrimethamine (Pyr; 2,4-diamino-5-(p-chlorophenyl)-6-ethylpyrimidine) is a dihydrofolate reductase (DHFR) inhibitor that blocks the conversion of dihydrofolate to tetrahydrofolate ("Physicians Desk Reference," 1994). Pyr has been used extensively to treat opportunistic infections such as malaria and is one of the primary therapeutics against toxoplasmonic encephalitis and toxoplasmonic encephalitis (Israelski et al., 1990).

In a study performed by the Community Program for Clinical Research on AIDS (CPCRA), HIV-infected patients treated with Pyr as a primary prophylaxis against toxoplasmonic encephalitis (TE) showed a higher mortality rate than patients treated with a placebo (Jacobson et al., 1994). Since the occurrence of TE was low in both treatment groups, it was postulated that Pyr might have inhibited hematopoietic stem cell or T lymphocyte proliferation. To determine whether treatment with Pyr suppressed the immune response in a murine model, we performed an immunotoxicology screening study using BALB/c mice. This included a battery of in vivo and ex vivo tests of immune function and a study of the effect of Pyr on host resistance to *Listeria monocytogenes*

The battery of assays was modeled after the guidelines of the National Toxicology Program (NTP) (Luster et al., 1988) and included measurement of splenic lymphocyte phenotypes, T and B cell proliferation in response to mitogens, T cell proliferation in response to allogeneic cells, and in vitro T-cell-mediated cytotoxicity. The ability of mice to mount an antigen-specific antibody response to keyhole limpet hemocyanin (KLH), a T-cell-dependent antigen was also determined by measuring serum IgM and IgG levels using an enzyme-linked immunosorbent assay (ELISA).

Host resistance to *L. monocytogenes* was selected as the model in which to study the effect of Pyr, because resistance to this organism (like resistance to TE) is mediated primarily through T cells rather than antibodies (Bradley, 1995; North, 1973; Gazzinelli et al., 1992) as well as through the innate immune response (Rogers et al., 1995). An unexpected finding in this study was that Pyr caused significant increases in mortality after infection with *L. monocytogenes* although it did not have significant effects on the functional assays of T-cell-mediated immunity.

**MATERIALS AND METHODS**

**Chemicals.** Pyrimethamine, methylcellulose (MC), cyclophosphamide (CY), concanavalin A (Con A), KLH, Tween 20 (polyoxyethylene sorbitan monolaurate), and o-phenylenediamine dihydrochloride (OPD) were obtained...
from Sigma (St. Louis, MO). Lipopolysaccharide (LPS) was purchased from List Biologicals (Sunnyvale, CA). Brain–heart infusion broth was obtained from Difco (Detroit, MD) and tryptic soy agar with 5% defibrinated sheep blood (TSA-SB) was from BBL (Cockeysville, MD). RPMI 1640 with 25 mM Hepes was obtained from BioWhittaker (Walkersville, MD). Iscove's modified Dulbecco's medium (IMDM), fetal calf serum (FCS), t-glutamine, penicillin, streptomycin, 2-mercaptoethanol (2-ME), streptavidin red 670, and monoclonal antibodies to CD3, CD4, and CD8 were obtained from Gibco BRL (Grand Island, NY). Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies to Mac-1 and CD45R were obtained from Boehringer Mannheim (Indianapolis, IN). [methyl-3H]Thymidine (6.7 Ci/mmol) was purchased from NEN Life Sciences Products (Boston, MA). Peroxidase-conjugated goat antirabbit IgG and goat anti-mouse IgM were obtained from Jackson ImmunoResearch (Bar Harbor, ME). Alum was purchased from E. M. S尔斯ant (Clifton, NJ) and methoxyflurane was from Pitman-Moore (Mundeilen, IL).

**Animals/housing.** Four- to five-week-old female BALB/c mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and quarantined before initiation of the study. Three animals were selected randomly for a quarantine necropsy, which included examination of all tissues and determination of tissue weights of the brain, kidneys, liver, and spleen. Females were examined for endo- and ectoparasites. Mice were approximately 5 weeks of age at study initiation and were housed five per cage in polycarbonate cages. Animal rooms were environmentally controlled with a 12-h light/dark photoperiod. All animals received food and water ad libitum. All mice were monitored daily for general appearance, behavior, appetite, elimination, mortality, and clinical signs of ill health. Individual body weights were determined on Day 0, once each week thereafter, and at study termination.

**Administration of test articles.** Test articles were administered in two distinct protocols for measuring general effects on immunological parameters and host resistance:

1. To measure the effect of treatment with Pyr on specific immune functions, six mice per treatment group were treated with Pyr (30 or 60 mg/kg) or vehicle (0.5% MC) by oral gavage for 30 days. Pyr was administered in 0.5% MC in a volume of 5 ml/kg. CY, a known immunosuppressive agent, was used as a positive control and administered by intraperitoneal (ip) injection of 25 mg/kg.

2. To evaluate effects on host resistance, mice were treated with Pyr or vehicle at the doses described above daily by oral gavage for 21 days before injection with L. monocytogenes and for 14 days following infection. As a positive control, mice were treated with CY as a single 200 mg/kg ip dose 1 day before infection (Morhan et al., 1984). Intraperitoneal saline injection was used as a control for CY.

**Analysis of dosage preparations.** Suspensions of Pyr were prepared weekly in 0.5% MC. In the suspensions was quantified by high-performance liquid chromatography (HPLC) using a Spectra-Physics Model 8700 liquid chromatograph. Aliquots of dose suspensions were diluted in acetonitrile and 0.025 M HCl and chromatographed on a reverse-phase ODS column (ODS II, HiChrom, 4.6 × 250 mm, Regis Technologies Inc., Morton Grove, IL). The mobile phase was 0.1 M KH₂PO₄ (pH 3.5)acetonitrile (60/40, v/v) at a flow rate of 0.8 ml/min. Pyr was detected at 229 nm and under these conditions eluted at 6.1 min. Its estimated purity was 99.8%. Dose suspensions were shown to be stable for longer than 1 week.

**Hemological analysis.** Blood samples for clinical laboratory studies were obtained from the retroorbital sinus under methoxyflurane anesthesia after mice had been treated for 30 days with Pyr. All hematology procedures were performed on whole blood samples with 1 mg/ml EDTA as an anticoagulant. The following parameters were evaluated: red blood cell (RBC) count, white blood cell (WBC) count, WBC differential count, platelet count, reticulocyte count, and hemoglobin level. Blood samples were analyzed by Consolidated Veterinary Diagnostics, Inc. (CVD, West Sacramento, CA).

**Cell lines and reagents.** The murine EL-4 cell line, a dimethyl-1,2-3

**Spleen cell isolation.** Spleens were weighed and cell suspensions were prepared using a Dounce homogenizer in medium containing RPMI 1640 with 25 mM Hepes, 2 mM t-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Red blood cells were lysed using a solution containing 0.16 M ammonium chloride, 0.01 M potassium bicarbonate, and 0.096 M EDTA.

**Lymphocyte phenotyping.** Spleen cell phenotypes were analyzed by staining spleen cell suspensions with monoclonal antibodies to T cell, B cell, and macrophage/monomocyte surface antigens conjugated with FITC, phycoerythrin (PE), or biotin. Analysis was performed on a Coulter Epics Elite flow cytometer (Hialeah, FL). T cells were labeled with FITC-conjugated antibodies to CD3, biotin-conjugated antibodies to CD4, and PE-conjugated antibodies to CD8 and counted using three-color analysis. Staining of biotin-conjugated CD4⁺ cells was developed using streptavidin red 670. Macrophages/monocytes stained with FITC-conjugated monoclonal antibodies to Mac-1, and B cells stained with PE-conjugated antibodies to the CD45R molecule, were counted in a two-color analysis. Granulocytes and natural killer (NK) cells are also detected with antibody to Mac-1.

Spleen cells (10⁶ cells) from each mouse were pelleted and Fe receptors were blocked by resuspending cells on ice in the appropriate isotype-matched antibodies for 30 min. Cells were again pelleted and resuspended in the appropriate antibodies at 1 μg/10⁵ cells in 100 μl volume and then incubated on ice, protected from light, for 30 min. Cells were washed twice with phosphate-buffered saline (PBS) containing 1% bovine serum albumin and 0.01% sodium azide and stained with propidium iodide to distinguish live from dead cells. Ten thousand cells from each sample were analyzed using the Immuno-4 data analysis package (Coulter).

**Assay of mitogen-induced T and B cell proliferation.** Spleen cells were plated in 96-well, round-bottomed microtiter plates at 1 × 10⁵ cells per well in 100 μl of proliferation medium containing IMDM, 5% FCS, 2 mM t-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 × 10⁻⁴ M 2-ME. Con A or LPS was diluted in the above medium and added to four replicate wells so that the final concentrations per well were 2.5 μg/ml and 10 μg/ml, respectively, in a total volume of 200 μl. Optimum concentrations of mitogens were determined in preliminary experiments. Cells were incubated in the absence or presence of mitogens for 72 h at 37°C in a humidified 10% CO₂ incubator. The effect on DNA synthesis was measured by [³H]thymidine incorporation (μCi per well) during the last 6 h of incubation. Cells were harvested using a Tomtec Harvester 96 (Tomtec, Orange, CT), and incorporation of radioactive thymidine was measured on a Betaplate liquid scintillation counter (Wallac Instruments, Gaithersburg, MD).

**Mixed lymphocyte reactivity (MLR).** The effect of Pyr on the ability of T cells to proliferate in response to stimulation with allogeneic cells was measured by incubating 1 × 10⁵ spleen cells per well with 1 × 10⁵ ¹²⁵I-irradiated allogeneic EL-4 cells per well of a 96-well, round-bottomed plate. Cells were incubated in a total volume of 200 μl of proliferation medium for 4 days at 37°C in a humidified 10% CO₂ incubator. DNA synthesis in cells from control and drug-treated mice was measured by uptake of [³H]thymidine as described above, during the last 16 to 18 h of incubation. Cells were harvested and counted as above.

**T-cell-mediated cytotoxicity.** Spleen cells (1 × 10⁶/ml) from Pyr-treated and control BALB/c mice were incubated with irradiated (1500 rad) allogeneic EL-4 cells (2 × 10⁵/ml) for 5 days in a mixed lymphocyte culture at 37°C in 10% CO₂ with humidity. Viable effector cells were isolated by Ficoll–Paque gradient centrifugation (Pharmacia, Gaithersburg, MD) and tested for their ability to lyse ¹²⁵I-labeled EL-4 cells in a 4-h chromium release assay according to the methods of Brunner et al. (1968). The decrease in ¹²⁵I release in test samples compared to ¹²⁵I release in controls was a measure of the toxic effect of the test agent on cell-mediated lysis.

**IgM and IgG production after in vivo immunization with KLH.** Separate sets of six mice per treatment group were treated with Pyr for 30 days. On Day
27 after the start of the treatment, mice were immunized ip with 0.2 ml of 50 
μg/ml KLH and 1.0 mg/ml alum in PBS. Peripheral blood was collected from the 
retroorbital sinus on Days 7 and 14 after immunization. IgM levels on Day 7 and IgG levels on Day 14 were measured by ELISA as follows: Wells of 
96-well high-protein-binding ELISA plates (Corning Glassworks, Corning, 
NY) were coated overnight with 25 μg/ml KLH in PBS. After the wells were 
washed with 0.05% Tween 20 in PBS, blocked with 5% skim milk, and washed 
again, diluted mouse serum was added to each well (three replicates per 
dilution). Wells were incubated at 37°C for 1 h and washed as above, and 
peroxidase-conjugated goat anti-murine IgG or goat anti-murine IgM was 
added. After a 1-h incubation at 37°C, the chromagen, OPD, was added with 
0.03% hydrogen peroxide in 0.01 M citrate buffer. After 30 min, 2 N sulfuric 
acid was used to stop the reaction. Color generated by enzymatic cleavage of 
the chromagen was monitored using a Vmax kinetic microplate reader (Mo-
olecular Devices, Menlo Park, CA). OD980 values at each dilution were com-
pared to determine the effect of treatment.

L. monocytogenes. L. monocytogenes, strain 19303, was obtained from 
Dr. Kimber White (Medical College of Virginia, Virginia Commonwealth 
University, Richmond, VA). Colonies were isolated and used to seed a large 
stock cultured in brain–heart infusion broth (BHI). Aliquots were frozen from 
this stock. For each experiment, a frozen aliquot was inoculated into a 50-ml 
flask containing BHI and grown overnight at 37°C. The turbidity of the culture 
was monitored, and when the optical density at 600 nm (OD490) indicated a cell 
centrifugation associated with log growth (based on previously constructed 
growth curves), the culture was diluted to the concentration desired for 
infection.

On Day 21 of treatment with Pyr, L. monocytogenes was administered to 
mice (14–18 per group) via injection in a 0.2-ml volume into the lateral tail 
vein. The infecting dose was 2, 4, or 8 × 10⁷ colony-forming units (CFU) of 
L. monocytogenes. For determination of the actual number of bacteria admin-
istered to the animals, the dosing solution was diluted and plated on tryptic soy 
agar with 5% defibrinated sheep blood (TSA-SB, No. 21261). Colonies were 
counted after a 24-h incubation period at 37°C.

Bacterial counts in liver and spleen. At 24 and 48 h after infection, L. 
monocytogenes colonies were quantitated in spleen and liver of mice. Whole 
organs were weighed and standardized portions of these tissues were removed, 
weighed, and homogenized in sterile saline. Homogenates were diluted serially 
and plated on duplicate on TSA-SB plates. The plates were incubated at 37°C 
for 24 h and colonies counted using a Model C-110 Bactronic colony counter 
(New Brunswick Scientific, New Brunswick, NJ).

Statistical analysis. Body weights and hematologic parameters were evalu-
ated by analysis of variance (ANOVA) followed by Dunnett’s test. These 
values were calculated by the Labcat (Innovative Programming Associates, 
Inc., Princeton, NJ) data capture software. Mortality data were evaluated using 
Fisher’s Exact test, and immunologic assays were analyzed using either Dun-
nett’s or Student’s t test.

RESULTS

Effect of Pyr on Immunologic Parameters

Treatment for 30 days by oral gavage with 60 mg/kg Pyr resulted in statistically 
significant decreases in peripheral blood WBC and lymphocyte counts (Figs. 1A and 1B); 30 mg/kg Pyr had no significant effect on WBC counts or other hematological parameters. Neither dose of Pyr caused significant changes from control values in neutrophils (Fig. 1C), RBC or platelet counts, or hemoglobin levels (data not shown).

Percentages of T cells (CD3⁺, CD4⁺, and CD8⁺), macrophages/monocytes, and B cells in spleens of Pyr-treated mice were determined using flow cytometry. Data are presented in

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WBC Count</th>
<th>Lymphocyte Count</th>
<th>Neutrophil Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>12.3 ± 0.5</td>
<td>8.6 ± 0.3</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td>Pyr 30</td>
<td>10.8 ± 0.3</td>
<td>7.2 ± 0.2</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>Pyr 60</td>
<td>9.4 ± 0.2</td>
<td>6.0 ± 0.1</td>
<td>3.0 ± 0.0</td>
</tr>
</tbody>
</table>

Table 1. No significant differences in total spleen cell recovery were observed in Pyr-treated mice compared to untreated or vehicle-treated mice. Treatment with CY did result in significant decreases in total spleen cell numbers (data not shown). Treatment with CY or with 60 mg/kg Pyr resulted in a statistically significant decrease (p <0.01) in the percentage of splenic B cells. Since total spleen cell numbers were not changed by treatment with Pyr, decreases in percentages also reflect decreases in total numbers of B cells. Increases in percentages of T cells that corresponded to the decreases in B cells were observed in Pyr- and CY-treated mice. Since a fixed number of cells are counted in each analysis, the decrease in B cells results in a corresponding apparent increase in T cells.

The percentage of CD3⁺ T cells (total T cells) that expressed CD4⁺ or CD8⁺ was determined by use of a T cell gating method. Treatment with either 30 or 60 mg/kg Pyr did not alter the percentage of total T cells that were CD4⁺ or CD8⁺; however, a statistically significant decrease in CD4⁺ cells was observed in CY-treated mice compared to untreated mice. In summary, treatment with the highest dose of Pyr decreased the percentage of B cells in spleens without causing major changes in percentages of T cells or macrophages.

No significant differences in T cell proliferation in response to Con A were observed when mice treated for 30 days with 30
TABLE 1
Effect of Pyrimethamine on Spleen Cell Phenotypes in Female BALB/c Mice

<table>
<thead>
<tr>
<th>Treatment group (mg/kg)</th>
<th>T cells</th>
<th>CD3+</th>
<th>CD4+</th>
<th>CD8+</th>
<th>Macrophages</th>
<th>B cells</th>
<th>T cell gate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| CY, 25*                | 32.2 ± 2.9
|                       |        | 21.6 ± 1.6 | 9.7 ± 0.6 | 6.6 ± 0.2 | 39.0 ± 0.9 | 61.2 ± 1.3 | 25.3 ± 0.9 |
| MC, 0.5%*              | 35.0 ± 2.0
|                       |        | 23.3 ± 1.5 | 10.4 ± 0.6 | 6.8 ± 0.3 | 38.7 ± 1.6 | 57.0 ± 1.3 | 27.8 ± 1.3 |
| Pyr, 30                | 38.1 ± 2.7
|                       |        | 25.8 ± 2.0 | 10.9 ± 0.9 | 6.9 ± 0.3 | 36.1 ± 2.1 | 60.9 ± 0.6 | 25.7 ± 0.5 |
| Pyr, 60                | 45.2 ± 3.9
|                       |        | 30.2 ± 2.6 | 13.4 ± 1.3 | 6.9 ± 0.3 | 27.1 ± 2.2 | 62.7 ± 0.6 | 24.3 ± 0.8 |

* N = 6.

b The percentage of spleen cells expressing CD4 or CD8 as a percentage of the CD3+ cells was determined by use of a T cell gating method. Forward light scatter and 90° light scatter were used to define the lymphocyte population.

c Mean percentage of spleen cells ± SE.

d Cyclophosphamide.

e Methylcellulose.

* Statistically significant (p < 0.05) as determined by Dunnett’s method when Pyr group was compared to 0.5% MC group and CY group was compared to untreated control group.

or 60 mg/kg Pyr were compared to mice treated with the vehicle (Table 2). A statistically significant decrease in B cell proliferation was observed after treatment with 60 mg/kg Pyr.

T cell proliferation in response to stimulation by allogeneic cells is presented in Table 3. Treatment with Pyr did not result in any decreases in T cell proliferation; in fact, statistically significant increases (p < 0.05) were observed.

The ability of T cells to lyse allogeneic targets after a 5-day incubation with irradiated targets was measured in a 4-h 51Cr-release cytotoxicity assay. Treatment with 30 and 60 mg/kg Pyr had no effect on T-cell-mediated cytotoxicity (data not shown).

Since the percentages of B cells were decreased in spleens, the ability of Pyr-treated mice to initiate an antigen-specific antibody response during Pyr treatment was evaluated. Mice were immunized with KLH/alum as described above, and KLH-specific IgM responses in the serum were measured on Day 7 after immunization (Table 4). KLH-specific IgG responses were measured on Day 14 (Table 5). Treatment with 60 mg/kg Pyr resulted in a 50% decrease in IgM OD490 values at three of the four serum dilutions, compared to mice treated with the vehicle (p < 0.05). No effect on anti-KLH IgG responses was observed after treatment with 30 or 60 mg/kg Pyr. OD490 values for IgG at the various serum dilutions were not statistically different between Pyr-treated and vehicle-treated mice.

TABLE 2
Effect of Pyrimethamine on Mitogen-Induced Lymphocyte Proliferation in Female BALB/c Mice

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Mean counts per minute ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>Con A (2.5 µg/ml)</td>
</tr>
<tr>
<td>Untreated</td>
<td>3,441 ± 242</td>
</tr>
<tr>
<td>CY, 25*</td>
<td>1,341 ± 193*</td>
</tr>
<tr>
<td>MC, 0.5%*</td>
<td>3,592 ± 156</td>
</tr>
<tr>
<td>Pyr, 30</td>
<td>3,524 ± 348</td>
</tr>
<tr>
<td>Pyr, 60</td>
<td>2,520 ± 323</td>
</tr>
</tbody>
</table>

* N = 6.

b Number in parentheses is Stimulation Index = (cpm of mitogen-induced cultures)/(cpm of cultures in medium alone).

c Cyclophosphamide.

d Methylcellulose.

* Statistically significant (p < 0.05) as determined by Dunnett’s method when Pyr group was compared to 0.5% MC group and CY group was compared to untreated control group.
PYRIMETHAMINE IMPAIRS HOST RESISTANCE

TABLE 3
Effect of Pyrimethamine on Alloantigen-Induced Lymphocyte Proliferation in Female BALB/c Mice

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Mean counts per minute ± SE</th>
<th>Responder</th>
<th>Responder + stimulator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>26,885 ± 379</td>
<td>5,339 ± 379</td>
<td>4,461 ± 334</td>
</tr>
<tr>
<td>CY, 25°</td>
<td>24,060 ± 1,772</td>
<td>2,811 ± 376*</td>
<td>24,060 ± 1,772 (5)</td>
</tr>
<tr>
<td>MC, 0.5%</td>
<td>5,069 ± 997</td>
<td>30,181 ± 2,080*</td>
<td>5,069 ± 997</td>
</tr>
<tr>
<td>Pyr, 30</td>
<td>4,663 ± 763</td>
<td>31,998 ± 2,541 (7)*</td>
<td>4,663 ± 763</td>
</tr>
<tr>
<td>Pyr, 60</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* N = 6.
* Number in parentheses is Stimulation Index = (cpm of responder + stimulator)/(cpm of responder cultures alone).
C Cyclophosphamide.
D Methylcellulose.
* Statistically significant (p < 0.05) as determined by Dunnett’s method when Pyr group was compared to 0.5% MC group and CY group was compared to untreated control group.

100% mortality at 8 days after infection, whereas a maximum of 7% mortality (1 of 15 mice) was observed in mice treated with the vehicle (Fig. 2). There was a dose-related increase in mortality after treatment with 30 and 60 mg/kg Pyr when mice were infected with the lowest dose of *L. monocytogenes*: treatment with 30 mg/kg resulted in 40% mortality and treatment with 60 mg/kg Pyr resulted in 87% mortality. No mortality was observed in the vehicle-treated mice infected with the lowest dose (2 × 10³ CFU) of *L. monocytogenes*. When mice were treated with CY and infected with the two highest doses of *L. monocytogenes*, mortality was 100% at 6 days after infection. The lowest dose of *L. monocytogenes* was not tested in these animals.

Figure 3 illustrates survival as a function of time after infection with 2 × 10³ CFU of *L. monocytogenes*. All mice treated with MC survived infection. Two of the mice treated with 30 mg/kg Pyr died 6 days after infection, and 3 more had died by Day 8. The last death in this group occurred on Day 10, and 60% of these mice were alive at study termination. Four of fifteen mice (27%) treated with 60 mg/kg Pyr were found dead 4 days after infection, and by Day 5 only four (27%) of the mice remained alive. At 9 days after infection, two mice (13%) had survived treatment with 60 mg/kg Pyr. No further deaths were observed after Day 9.

To determine whether deaths were accompanied by an increase in the numbers of replicating bacteria after Pyr treatment, *L. monocytogenes* counts in spleens and livers were determined at 24 and 48 h after infection. Data are presented in Table 6. CFU were expressed both per milligram of spleen or liver and per total organ weight. The first value was calculated to normalize CFU and allow comparison between treatment groups. The second value (CFU total organ weight) was calculated to account for effects of Pyr on spleen and liver weight. At both 24 and 48 h after infection, significantly more *L. monocytogenes* colonies were present in both spleens and livers of mice treated with 60 mg/kg Pyr than in vehicle-treated or untreated mice. The number of CFU/mg spleen increased threefold between 24 and 48 h in Pyr-treated mice but did not change significantly in the other two treatment groups. The average spleen weight was significantly less at 48 h in Pyr-treated mice than in the two control groups.

The CFU/mg tissue was much lower in livers than in spleens at both 24 and 48 h, suggesting that at these time points *L. monocytogenes* accumulated preferentially in the spleen. In contrast to spleens, livers did not show an increase in CFU between 24 and 48 h postinfection.

DISCUSSION

The clinical observation of increased mortality in AIDS patients treated with Pyr prompted this immunotoxicity screening...

TABLE 4
Effect of Pyrimethamine on Anti-KLH Response: IgM Production on Day 7 Postimmunization

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Serum dilution</th>
<th>1 × 10⁻²</th>
<th>5 × 10⁻²</th>
<th>1 × 10⁻³</th>
<th>5 × 10⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonimmunized</td>
<td></td>
<td>0.739 ± 0.174*</td>
<td>0.243 ± 0.042</td>
<td>0.094 ± 0.025</td>
<td>0.039 ± 0.016</td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td>1.528 ± 0.160</td>
<td>0.450 ± 0.050</td>
<td>0.235 ± 0.026</td>
<td>0.041 ± 0.008</td>
</tr>
<tr>
<td>CY, 25°</td>
<td></td>
<td>0.363 ± 0.025*</td>
<td>0.086 ± 0.011*</td>
<td>0.043 ± 0.017*</td>
<td>0.030 ± 0.012</td>
</tr>
<tr>
<td>MC, 0.5%</td>
<td></td>
<td>1.740 ± 0.190</td>
<td>0.574 ± 0.041</td>
<td>0.297 ± 0.043</td>
<td>0.078 ± 0.014</td>
</tr>
<tr>
<td>Pyr, 30</td>
<td></td>
<td>1.511 ± 0.191</td>
<td>0.400 ± 0.061</td>
<td>0.201 ± 0.040</td>
<td>0.025 ± 0.010*</td>
</tr>
<tr>
<td>Pyr, 60</td>
<td></td>
<td>0.896 ± 0.088</td>
<td>0.203 ± 0.020*</td>
<td>0.110 ± 0.008*</td>
<td>0.013 ± 0.004*</td>
</tr>
</tbody>
</table>

* N = 6.
* Average OD₄₉₀ ± SE.
C Cyclophosphamide.
D Methylcellulose.
* Statistically significant (p < 0.05) as determined by Dunnett’s method when Pyr group was compared to 0.5% MC group and CY group was compared to untreated control group.
TABLE 5
Effect of Pyrimethamine on Anti-KLH Response: IgG Production on Day 14 Postimmunization

<table>
<thead>
<tr>
<th>Treatmenta (mg/kg)</th>
<th>Serum dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 × 10⁻³</td>
</tr>
<tr>
<td>Nonimmunized</td>
<td>0.011 ± 0.006b</td>
</tr>
<tr>
<td>Untreated</td>
<td>&gt;4.000</td>
</tr>
<tr>
<td>CY, 25°C</td>
<td>1.240 ± 0.564</td>
</tr>
<tr>
<td>MC, 0.5%f</td>
<td>&gt;4.000</td>
</tr>
<tr>
<td>Pyr, 30</td>
<td>&gt;4.000</td>
</tr>
<tr>
<td>Pyr, 60</td>
<td>3.556 ± 0.082f</td>
</tr>
</tbody>
</table>

* N = 6.
b Average OD 490 ± SE.
c NT, not tested.
d Cyclophosphamide.
* Methylcellulose.
f N = 4; two other values were out of range.
* Statistically significant (p < 0.05) as determined by Dunnett’s method when Pyr group was compared to 0.5% MC group and CY group was compared to untreated control group.

study of the effect of Pyr on assays of T and B cell function and host resistance to L. monocytogenes. The doses of Pyr used to treat mice in this study were chosen to mimic the toxic effects observed when humans were treated with Pyr as prophylaxis for TE. Since the doses of Pyr that are used clinically to treat TE are near toxic levels and require supplementation with leucovorin to counter bone marrow toxicity ("Physician’s Desk Reference," 1994), mice in this study were treated with doses of Pyr that resulted in bone marrow toxicity, as assessed by decreases in WBC and lymphocyte counts in the peripheral blood.

Treatment with 60 mg/kg Pyr resulted in a significant decrease in the percentage of splenic B cells, accompanied by a decrease in

FIG. 2. Effect of Pyr on mortality of BALB/c mice after infection with L. monocytogenes. Mice were treated for 21 days with either 30 or 60 mg/kg Pyr or 0.5% MC. Positive control mice were injected ip with 200 mg/kg CY 24 h before L. monocytogenes infection. Mice injected ip with 0.9% saline were used as controls for CY. Mice were infected with 2 × 10³, 4 × 10³, or 7 × 10³ CFU of bacteria. N = at least 15 for all treatment groups. Mortality in mice treated with Pyr at 30 or 60 mg/kg was significantly higher (p < 0.01) than in vehicle-treated mice, and mortality in CY-treated mice was significantly higher (p < 0.01) than in saline-treated animals at all concentrations of L. monocytogenes. In three replicate experiments mortality in saline- or vehicle-treated mice ranged from 0 to 40% after infection with the highest challenge dose of L. monocytogenes. NT, not tested.
PYRIMETHAMINE IMPAIRS HOST RESISTANCE

FIG. 3. Survival time in mice treated with Pyr and infected with *L. monocytogenes*. Mice were treated for 21 days with 30 or 60 mg/kg Pyr or with vehicle (MC) and infected with $2 \times 10^3$ CFU of *L. monocytogenes*. N = 15 in each treatment group. The incidence of mortality was significantly different at the end of the study ($p < 0.01$) when mice treated with either dose of Pyr were compared to vehicle-treated mice.

Both splenic B cell proliferation and serum levels of anti-KLH IgM antibodies. No significant effects of Pyr were observed on anti-KLH IgG levels in the serum, measured 14 days after immunization with KLH. Since immunization occurred 4 days before termination of treatment with Pyr, and KLH was administered in alum, which acts as a depot for the antigen, it may be that there was a recovery in the ability of the animals to mount an antigen-specific antibody response after cessation of Pyr treatment. This recovery may not have been detected when IgM levels were assayed on Day 7 after immunization but was observable when IgG was measured on Day 14.

Treatment with Pyr caused no significant decreases in T cell or macrophage/monocyte percentages in the spleen or in T cell proliferation in response to mitogens or allogeneic cells. *Ex vivo* T-cell-mediated cytotoxicity was also unaffected. It is possible that the lack of effect on T-cell-mediated cytotoxicity was due to a recovery effect during the period of *in vitro* culture, since spleen cells from Pyr-treated animals were cultured for 5 days with allogeneic EL-4 cells in the absence of Pyr before cytotoxicity was assessed. However, since other assays of T cell function showed no effect of Pyr, it is more likely that this result is not due to recovery. In contrast to the lack of effect of Pyr on cell-mediated cytotoxicity, T cells showed an increased proliferation in response to allogeneic cells after treatment with Pyr. This result has been observed consistently in replicate experiments, and the cause of this increase is not currently understood.

Although few immunological changes were observed after treatment with Pyr, a dramatic increase in *L. monocytogenes*-induced mortality was noted. This was best illustrated when mice were infected with $2 \times 10^3$ CFU of *L. monocytogenes*, a concentration that did not cause death in untreated mice. When Pyr-treated mice were infected with this dose of bacteria, a clear dose-related increase in deaths and decrease in time until death were observed. The increased mortality is interesting in light of the fact that Pyr showed no effect on T cell function, since host response to *L. monocytogenes* is thought to be a T cell—rather than a B cell—mediated phenomenon.

### TABLE 6

**Effect of Pyrimethamine on Bacterial Growth in Spleen and Liver of Female BALB/c Mice after Infection with *L. monocytogenes***

<table>
<thead>
<tr>
<th>Treatment* (mg/kg)</th>
<th><strong>L. monocytogenes</strong> in spleen</th>
<th><strong>L. monocytogenes</strong> in liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFU/mg spleen</td>
<td>Total spleen weight (mg)</td>
</tr>
<tr>
<td></td>
<td>($\times 10^4$)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>($\times 10^4$)</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>$2,510 \pm 390^c$</td>
<td>$88 \pm 4$</td>
</tr>
<tr>
<td>MC, 0.5%*</td>
<td>$4,600 \pm 920$</td>
<td>$97 \pm 2$</td>
</tr>
<tr>
<td>Pyr, 60</td>
<td>$12,268 \pm 3,930^*$</td>
<td>$99 \pm 4$</td>
</tr>
<tr>
<td>48 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>$1,916 \pm 340$</td>
<td>$137 \pm 6$</td>
</tr>
<tr>
<td>MC, 0.5%</td>
<td>$5,303 \pm 690$</td>
<td>$134 \pm 7$</td>
</tr>
<tr>
<td>Pyr, 60</td>
<td>$36,855 \pm 8,660^{* * * * *}$</td>
<td>$117 \pm 56^{* * * *}$</td>
</tr>
</tbody>
</table>

* $N = 10$ unless otherwise noted.

* All statistical analyses on these data were performed with variance normalized using the natural log because of the large variance observed.

* Mean ± SE.

* $N = 9$.

* Methylcellulose.

* Statistically significant ($p < 0.05$) when compared to untreated control group at the corresponding time point, as determined using Dunnett’s method.

* * Statistically significant ($p < 0.05$) when compared to 0.5% MC group at the corresponding time point, as determined using Student’s $t$ test.

* * * Statistically significant ($p < 0.05$) when Pyr 24-h and Pyr 48-h treatment groups compared using Student’s $t$ test.
To obtain insight into the mechanism whereby Pyr causes increased mortality, bacterial burdens in spleen and liver were determined at 24 and 48 h after infection. Not only were bacterial burdens higher in both organs at both time points with Pyr treatment, but a comparison of numbers of organisms in spleens at 24 and 48 h revealed that replication was not being controlled in Pyr-treated mice to the same extent as in untreated or vehicle-treated mice.

The observation of effects of Pyr in the first day after infection and the lack of effects on T cell functions suggests that Pyr may be affecting mechanisms of innate immunity. The role of innate immunity in the resolution of L. monocytogenes infection has been increasingly appreciated in recent years (Rogers et al., 1995). Innate resistance to L. monocytogenes infection involves two components: inflammatory cells, particularly neutrophils that are recruited to the site of infection and T-cell-independent generation of interferon-γ (IFN-γ) by NK cells in the presence of macrophage-generated cytokines (Rogers et al., 1995). Production of IFN-γ further activates macrophages to antimicrobial activity, antigen presentation, and, ultimately, antigen-specific T cell activation. Neutrophils have been shown to kill L. monocytogenes in vitro by releasing oxygen intermediates and enzymes with anti-listerial activity (Czuprynski et al., 1984) and macrophages can kill the organism in part by production of nitric oxide from L-arginine (Beckerman et al., 1993; Hull, 1996). Although no changes were observed in serum neutrophil levels in this study, cytokine production by macrophages and NK cells or production of nitric oxide and oxygen free radicals may be altered by treatment with Pyr. Assays are currently in progress to determine the effect of Pyr on these components of the innate immune system.

Infection with L. monocytogenes generally causes little pathology in immunocompetent individuals. However, pathology resulting from infection with this organism has recently been reported to be increasing and to be 145 times higher in AIDS patients than in the normal population (Jurando et al., 1993). If the effects of Pyr to increase susceptibility to L. monocytogenes infection that have been observed in mice are also found in humans, treatment with Pyr during a prophylactic regime for TE may put individuals at risk for development of listeriosis. In light of the increase in L. monocytogenes infection in the AIDS population, it is important to determine the mechanism whereby Pyr acts in mice and to determine whether a parallel mechanism exists in humans.

ACKNOWLEDGMENTS

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REFERENCES


