Mechanism of Cellular Suppression Induced by Oral Tilorone Treatment of Mice

FRANK M. COLLINS
Trudeau Institute, Inc., Saranac Lake, New York 12983

Specific-pathogen-free B6D2 F1 hybrid mice were treated orally with tilorone hydrochloride (100 mg/kg of body weight per day) and infected with sublethal doses of Listeria monocytogenes, Salmonella enteritidis, Mycobacterium bovis (BCG Pasteur), or M. tuberculosis Erdman. Daily tilorone treatment inhibited the cell-mediated response to all of the intracellular parasites, and most of the mice succumbed to the challenge. Tilorone suppressed delayed hypersensitivity responses to the microbial sensitins as well as to sheep erythrocytes. However, humoral responses (immediate hypersensitivity reactions) were stimulated. The types of growth curves obtained in the tilorone-treated mice were quite different from those observed in T-cell-depleted mice and tended to resemble those seen in sublethally irradiated (400 rads) animals. Leukocyte counts were depressed 10-fold by daily tilorone treatment. Both monocyte and granulocyte (but not large-lymphocyte) counts were depressed. There was an initial drop in small-lymphocyte counts with a later recovery phase. Tilorone treatment reduced the granulomatous response within the Salmonella-infected liver, suggesting that the drug interferes with the mobilization of the mononuclear defenses within the normal host.

Tilorone is a fluorenone derivative with antiviral activity and an ability to induce interferon production in mice and rats exposed to oral doses of the drug (8). Although tilorone increases resistance to a number of viral infections, it can potentiate microbial infections by interfering with the induction of the normal T-cell-mediated host response (3, 10). Repeated daily dosage of this drug is associated with reduced tuberculin hypersensitivity, experimental allergic encephalomyelitis, and graft-versus-host, and adjuvant arthritis responses in appropriately sensitized rats (12, 13). Histological examination of the tilorone-treated host reveals a striking cellular depletion within the thymus-dependent areas of the spleen and lymph node (11). No such effect occurred within the spleens of athymic mice treated with tilorone (9).

Acquired resistance to facultative intracellular parasites depends upon both immunocompetent T-lymphocytes and monocytes entering the developing focal lesions within the liver and spleen (4). Earlier studies with Listeria and Salmonella-infected mice which had been treated with multiple oral doses of tilorone suggested that the normal cellular defense system had been compromised in some way (3, 10). However, the shape of the resulting growth curves was quite different from those observed in athymic nude mice (1) or in adult thymectomized, irradiated, bone marrow-reconstituted mice (2). This strongly suggests the involvement of other cells in the tilorone response; to explore this possibility further, a number of growth studies were carried out in mice in which the cell-mediated defenses had been modified by several different procedures and then compared with the effect of daily oral tilorone treatments.

MATERIALS AND METHODS

Animals. Specific-pathogen-free C57BL/6 × DBA-2 (B6D2) F1 hybrid mice were bred and maintained at the Trudeau Animal Breeding Facility under conditions described previously (5). Adult thymectomized, lethally irradiated, bone marrow-reconstituted (THXB) B6D2 mice, together with a small number of sham-thymectomized controls, were prepared as described elsewhere (5). Sublethally irradiated mice were exposed to 400 rads of whole-body gamma irradiation 24 h before challenge (19).

Organisms. Listeria monocytogenes RGE (intravenous [i.v.] 50% lethal dose [LD50], 1 × 104 to 2 × 104), Salmonella enteritidis NCTC 5694 (i.v. LD50, 8 × 103), Mycobacterium tuberculosis Erdman, TMC 107 (i.v. LD50, 5 × 105), and Mycobacterium bovis BCG Pasteur, TMC 1011 (i.v. LD50, >5 × 106) were grown and maintained at −70°C in 1-ml ampoules as described earlier (3, 7).

Enumeration of bacteria in vivo. Groups of five randomly selected mice were sacrificed at intervals, and the lungs, livers, and spleens were homogenized separately in sterile saline (7). The homogenates were diluted in 10-fold steps and plated onto tryptic soy agar or Middlebrook 7H10 agar (Difco, Detroit, Mich.). The mycobacterial plates were placed in sealed plastic bags and incubated at 37°C for 4 weeks before
Delayed hypersensitivity responses. Infected mice were footpad tested by injecting 2.5 µg of purified protein antigen (PPD; Connaught Laboratories, Toronto, Ont.) or the corresponding Listeria or Salmonella footpad test antigens prepared from culture filtrates of the respective organisms as described elsewhere (6). Increased footpad thickness measurements were made at 3, 6, 24, and 48 h by using a dial-gauge calipers (7). An increase of 0.2 mm (2 Schnelltaster units) or more was statistically significant at the 1% level (6).

Sheep erythrocytes. Groups of 10 normal and 10 tilorone-treated mice were sensitized subcutaneously with 10⁶ sheep erythrocytes and footpad tested 4 days later with 10⁷ sheep erythrocytes, as described elsewhere (14).

Drug treatment regimen. Tilorone hydrochloride (Merrill-National Laboratories, Cincinnati, Ohio) was dissolved in sterile distilled water at a concentration of 10 mg/ml and administered orally by gavage needle at a dosage of 100 mg of drug per kg of body weight, beginning 24 h before infection and continuing daily until the conclusion of the experiment (3). Control mice received 0.2 ml of sterile distilled water given orally each day. Drug was administered every other day during the more prolonged mycobacterial infection studies.

Leukocyte counts. Groups of five randomly selected mice were bled by heart puncture at daily intervals after the inception of the tilorone treatment regimen. Blood samples were diluted in saline, and the total number of leukocytes was determined by using a Coulter Counter model B (Coulter Electronics, Hialeah, Fla.). Air-dried smears of whole blood were stained with May-Grinwald Giemsa stain for the differential counts. Leukocyte concentrates were prepared where necessary, as described elsewhere (19).

T-lymphocytes. T-lymphocytes were counted after exposure of the leukocyte concentrates to fluoresceinated anti-thy 1.2 mouse antiserum (Accurate Chemical and Scientific Corp., Hicksville, N.Y.) and examined with a Zeiss epifluorescence microscope as described by Raff (17).

B-lymphocytes. The leukocyte concentrates were treated with fluoresceinated rabbit anti-mouse immunoglobulin G serum (Microbiological Associates, Bethesda, Md.) and examined under ultraviolet illumination (18).

RESULTS

Growth of L. monocytogenes in tilorone-treated B6D2 mice. Normal B6D2 mice were treated orally with 100 mg of tilorone per kg 24 h before intravenous challenge with a sublethal dose of L. monocytogenes (2 x 10⁴ to 5 x 10³ viable bacteria suspended in 0.2 ml of saline). The mice continued to receive daily dosages of tilorone until the completion of the experiment. The Listeria challenge multiplied logarithmically in the liver and spleen for 2 to 3 days, at which time the normal controls developed an immune response which limited the further systemic growth of the organisms so that none of the mice died as a result of the challenge infection (Fig. 1). The tilorone-treated mice showed continued logarithmic growth in both the liver and spleen until the counts reached lethal proportions (usually about day 5). None of the tilorone-treated mice exhibited significant levels of delayed hypersensitivity to the footpad test antigen (Fig. 1). Normal controls developed highly significant 24-h footpad responses to the Listeria test antigen by day 5 of the infection.

The growth curves that developed in the tilorone-treated mice were quite different from those observed when the same inoculum was introduced into T-cell-depleted mice (Fig. 1).
The *Listeria* growth curves in the THXB mice exhibited a characteristic slow increase in viable counts for both the liver and spleen. None of the THXB mice developed hypersensitivity to the footpad test antigen. The uncharacteristic *Listeria* growth pattern in the T-cell-depleted spleen has been explained by the presence of a stimulated population of macrophages within that organ (1). The nonspecifically activated phagocytic cells limited the growth of the bacterial population to spite the absence of a T-cell-mediated immune response (2).

Mice exposed to a sublethal dose (400 rads) of whole-body irradiation 24 h before the *Listeria* challenge showed a lack of resistance similar to that seen in the tilorone-treated mice (Fig. 1). Irradiation of the host before the normally sublethal challenge potentiated the lethality of this organism so that 100% of the irradiated, infected mice died within 5 days. None of the irradiated mice developed detectable levels of delayed hypersensitivity.

Tilorone treatment of T-cell-depleted mice. A group of THXB and sham-thymectomized B6D2 mice were treated orally with 100 mg of tilorone per kg and infected 24 h later with 2 × 10⁵ viable *L. monocytogenes*. The combined liver and spleen counts observed in the T-cell-depleted and control mice treated with the drug both increased at essentially the same rate, resulting in a 100% mortality rate for both groups by day 5. On the other hand, none of the untreated THXB mice or the normal controls died as a result of the *Listeria* challenge (Fig. 2).

Growth of *S. enteritidis* in tilorone-treated mice. Normal mice were treated with tilorone and 24 h later were challenged with about 10⁵ viable *Salmonella* (0.1 LD₅₀). The viability of the challenge inoculum increased by about 5 logs over a 4-day period, resulting in 100% mortality in the treated mice. Control mice showed a 1,000-fold increase in viability over the first 5 days of the experiment, followed by the normal immune decline (Fig. 3). The tilorone-treated mice failed to develop a delayed hypersensitivity response to the *Salmonella* test antigen, whereas the controls showed excellent footpad responses by day 4.

Growth curves for *S. enteritidis* introduced into T-cell-depleted mice were very different from those seen in the tilorone-treated mice. The slowed growth rate in vivo was consistent with the presence of activated macrophages within the T-cell-depleted host. On the other hand, sublethally irradiated mice provided a *Salmonella* growth curve and mortality rate similar to those seen in the tilorone-treated host (Fig. 3).

Histologically, the *S. enteritidis*-infected mice (examined after four oral doses of tilorone) showed an almost complete absence of granuloma formation within the heavily infected livers and spleens. A clear-cut cellular response was observed in the livers of the *Salmonella*-infected control mice (Fig. 4). Evidence of tissue toxicity was found in the tilorone-treated infected mice, and this was presumed to be due to endotoxin.

![Fig. 2. Effect of daily oral tilorone (100 mg/kg) treatment on the growth of *L. monocytogenes* in T-cell-depleted (T-Til) or sham-thymectomized (N-Til) B6D2 mice. THXB and normal, Controls received distilled water instead of tilorone.](image)

![Fig. 3. Growth of *S. enteritidis* in tilorone-treated (A) THXB (B), or sublethally irradiated (C) B6D2 mice. See legend for Fig. 1 for further details.](image)
release by the rapidly increasing *Salmonella* population. None of the drug-treated uninfected controls showed signs of liver damage, and the single-dose LD$_{50}$ for tilorone in normal B6D2 mice was approximately 850 mg/kg of body weight.

Growth of BCG Pasteur in tilorone-treated mice. *M. bovis* BCG multiplied slowly within the lungs, livers, and spleens of intravenously infected mice for 10 to 14 days before the immune response, which was mediated by a population of immunocompetent T-cells, grad-
usually eliminated the organisms from the tissues (Fig. 5). If the mice were treated daily with tilorone (100 mg/kg), early growth was not affected but the antituberculous response was largely ablated (Fig. 5), as was the development of tuberculin hypersensitivity. A substantial immediate (3-h) hypersensitivity to the PPD injection was seen in the tilorone-treated mice, however.

In a subsequent study, mice were treated daily with tilorone for increasing time intervals ranging from 1 to 28 days, and the effect of the various regimen was compared in terms of the BCG growth curves (Fig. 6). Mice receiving a single oral dose of 100 mg/kg, as well as those with seven daily treatments, showed little change in the BCG growth pattern compared to that observed in untreated controls. As the treatment period was prolonged, the viable counts for the spleen and lung homogenates tended to remain constant with little sign of any immune decline (Fig. 6). However, after a period of 2 weeks without further tilorone treatment, the BCG viable counts began to decline slowly, suggesting that the suppressive effect of the drug was quite short-lived (Fig. 6).

Effect of tilorone on *M. tuberculosis* Erdman infections in vivo. Normal B6D2 mice infected with a sublethal dose of *M. tuberculosis* Erdman (3 × 10⁸ viable units, representing 0.01 LD₅₀) failed to develop detectable disease even after 3 or 4 months of infection. The organisms were not eliminated from the tissues, and viable counts carried out after 60 days showed a 10,000-fold increase within the lungs (Fig. 7). Growth in the liver and spleen was much less prolific, and after an early phase of growth, the counts remained relatively constant for many weeks with little sign of any immune response. These mice developed an early tuberculin hypersensitivity which was later supplanted by a characteristic tuberculin anergy which continued throughout the remainder of the study.

Daily treatment of the Erdman-challenged mice with tilorone resulted in an ablation of the immune response in the liver and spleen, and virtually all of the mice died before day 90. None of the treated mice developed detectable levels of tuberculin hypersensitivity, although they did exhibit a substantial immediate (3-h) hypersensitivity response to the PPD (Fig. 7).
Effect of tilorone treatment on responses to sheep erythrocytes in vivo. Oral treatment of the mice with 100 mg of tilorone per kg 24 h before sheep erythrocyte sensitization almost completely suppressed the 24-h footpad swelling response regardless of whether the mice were sensitized by the subcutaneous or the intravenous route (Table 1). The immediate (3-h) hypersensitivity response was not greatly affected, however.

Effect of tilorone on peripheral leukocyte counts. Daily oral treatment of normal B6D2 mice with 100 mg of tilorone per kg resulted in a 10-fold drop in the total leukocyte count within 24 h. Some counts represented only 5% of the control values. However, of these cells, 80 to 85% morphologically resembled small lymphocytes (Fig. 8). Mice receiving only one dose of drug showed a substantial recovery in leukocyte counts within 3 days. However, continued daily treatment resulted in continued depression in total leukocytes for up to 7 days. Most of the cells present in the circulation after six daily doses of tilorone appeared to be small lymphocytes (Fig. 8). Examination of these cells by appropriate fluorescence staining indicated that they consisted almost entirely of B-cells (86%); in the untreated animals, on the other hand, approximately one third of the small-lymphocyte population carried the immunoglobulin G surface marker (Table 2). The T-cell population declined to virtually undetectable numbers over the 6-day treatment period. Over this same treatment period, the number of large and medium lymphocytes in the blood showed little change. Circulating monocyte and polymorphonuclear leukocyte counts both declined sharply in the drug-treated mice and fell to only a few hundred cells per cubic millimeter representing less than 5% of control values. The effect of this daily tilorone treatment bore a number of similarities to that seen after a sublethal dose of irradiation (Fig. 8). However, the extent of the drop in all of the peripheral leukocytes was greater and more prolonged than that seen in the tilorone-treated animals.

**TABLE 1. Effect of oral tilorone treatment on foot swelling responses to subcutaneous sheep erythrocyte sensitization**

<table>
<thead>
<tr>
<th>Sensitizing dose*</th>
<th>Expt no.</th>
<th>0 mg/kg</th>
<th>100 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3 h</td>
<td>24 h</td>
</tr>
<tr>
<td>10^6 SRBC s.c.</td>
<td>1</td>
<td>6.8 ± 0.4</td>
<td>14.8 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8.6 ± 0.2</td>
<td>13.2 ± 1.4</td>
</tr>
<tr>
<td>10^6 SRBC i.v.</td>
<td>3</td>
<td>3.8 ± 0.8</td>
<td>4.5 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5.2 ± 1.8</td>
<td>3.8 ± 0.4</td>
</tr>
</tbody>
</table>

* SRBC, Sheep erythrocytes; s.c., subcutaneous dose.

* Mean of five determinations (Schnelltaster units ± standard error of the mean).
nonreplicating antigens. However, it is interesting that the cell-mediated immune responses are generally depressed, whereas the humoral responsiveness is often increased (12). Multiple doses of tilorone will increase skin and organ graft retention (20), will suppress graft-versus-host reactions (13), and may affect the expression of an antitumor immunity (16). Tilorone also markedly suppressed the T-cell-mediated anti-Listeria responses in singly dosed mice in which a 1,000-fold increase in Listeria virulence was observed (10). This increase was largely ascribed to a T-cell depletive effect seen in multiply treated rats and mice (11, 12). However, this drug not only brought about a substantial drop in the peripheral blood small-lymphocyte counts, but also resulted in a significant drop (P < 0.01) in both monocyte and polymorphonuclear phagocyte counts (Fig. 8).

Acquired resistance to Listeria and Salmonella infections depends upon the entry of large numbers of immunologically activated macrophages into the developing lesion (4). Activation may be prevented if the mobilization of either T-cells or blood monocytes is temporarily blocked by the tilorone treatment. The shape of the growth curves obtained in the tilorone-treated mice (Fig. 1 and 3) was quite different from those observed in the T-cell-depleted mice. The tilorone-treated hosts were unable to survive a normally sublethal challenge inoculum which the THXB mice had no difficulty in limiting to nonlethal proportions (Fig. 1). However, the tilorone-treated, T-cell-depleted mice challenged with this same dose of viable L. monocytogenes quickly succumbed to the infection (Fig. 2). Since T-cell-depleted mice are known to have enhanced macrophage activity (1), it seems reasonable to assume that the daily tilorone treatment somehow interfered with the production or deployment of both of these partially activated macrophages.

Histological examination of the tilorone-treated, Salmonella-infected mice revealed an almost complete lack of cellular infiltration within the heavily infected liver (Fig. 4). Studies with BCG-infected, T-cell-depleted mice indicated that although there was an almost complete absence of cells morphologically resembling small lymphocytes in the lung tubercles, the lesions contained increasing numbers of monocytes and foamy macrophages (15). This was in sharp contrast to the tilorone-treated, BCG-infected mouse lungs which exhibited a

![FIG. 8. Variations in leukocyte counts from mice exposed to 400 rads of whole-body irradiation (A), one oral dose of tilorone (B), or daily tilorone doses (C).](http://iai.asm.org/)

<table>
<thead>
<tr>
<th>TABLE 2. Effect of oral tilorone (100 mg/kg) on circulating lymphocytes in the peripheral blood of normal B6D2 mice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. of daily doses</strong></td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>6</td>
</tr>
</tbody>
</table>

*SE, Standard error.

*Parentheses indicate percentage of small-lymphocyte count.
minimal cellular infiltration (Collins, unpublished data) despite the presence of a large viable population of BCG in vivo (Fig. 5). The reduced mononuclear cell response in the drug-treated host was also responsible for the almost complete lack of delayed hypersensitivity shown by these animals (Fig. 1, 3, and 5). This is consistent with the complete absence of T-lymphocytes from the peripheral blood of multiply treated mice (Table 2). This reduced number of T-cells in the blood of tilorone-treated mice was offset by a greatly increased B-cell count, which rose to 85 to 90% of the small-lymphocyte population in mice receiving six daily doses of tilorone. These changes are in agreement with earlier studies by Raychaudhuri and Megel (18). Presumably, there were still sufficient residual helper T-cells present in the host to bring about the observed enhanced humoral (immediate hypersensitivity) response (Fig. 1 and 3). The present study indicates that tilorone affects other cells besides the T-lymphocyte population, and further detailed studies of the kinetics of the mononuclear cell responses after repeated tilorone treatments will be needed before the cellular responses to this drug can be fully evaluated.

ACKNOWLEDGMENTS

I wish to thank A. Richardson, Merrill-National Laboratories, Cincinnati, Ohio for the generous gift of tilorone used in these studies. Excellent technical assistance was provided by William Woodruff, Joyce Reome, and Linda Auclair.

The work was supported by Public Health Service grants AI-14065, administered by the U.S.-Japan Cooperative Medical Sciences Program, HL-19774, from the National Heart, Lung and Blood Institute, and (Biomedical Research Support Grant) RR-06705 from the General Research Support Branch, National Institutes of Health.

LITERATURE CITED