Effect of 2,7-Bis[2-diethylamino)ethoxy]fluoren-9-one dihydrochloride (Tilorone) upon Cell-mediated Immunity in Mice

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SUMMARY

Tilorone (2,7-bis[2-(diethylamino)ethoxy]fluoren-9-one dihydrochloride) was administered to CD-1 or C57L x A F1, mice alone or with allogeneic leukemia L1210 cells. Tilorone given alone elicited thymus-dependent lymphocytes in the spleen causing cell-mediated immunity (CMI) comparable to that of lymphocytes exposed to 5 x 10^4 L1210 cells. This occurred whether Tilorone was administered p.o. or i.p. When administered 24 hr after L1210 cells, an adjuvant effect of Tilorone upon CMI was not demonstrable unless glass-adherent spleen cells were removed before in vitro assay. In groups given Tilorone or L1210 alone, CMI was unchanged after removal of adherent cells. Release of lymphocyte-activating factor by the spleen was unaffected by Tilorone. It is suggested that Tilorone has a moderate direct stimulatory effect upon thymus-dependent lymphocytes but may elicit inhibitory effects mediated by adherent spleen cells (macrophages and/or sticky bursa-dependent lymphocytes) that have also been stimulated by antigen.

INTRODUCTION

Tilorone (2,7-bis[2-(diethylamino)ethoxy]fluoren-9-one dihydrochloride) and its related congeners are interferon-inducing agents that have shown to alter host immunity following p.o. or parenteral administration. In mice, Tilorone has caused increased phagocytosis, elevated 19 S antibody production, and induced the production of interferon (14, 16, 17). Tilorone has also evoked an antiviral (13, 14, 16, 17), antistaphylococcal (20, 23), and either an antitumor or a tumor-enhancing response, depending upon the timing of administration (1, 2, 5, 20, 24). No induction of interferon has yet been demonstrated in humans with Tilorone when given p.o. or topically (12). Recently, limited Phase I studies have been carried out in patients with a variety of cancers, and 2 of 10 patients, both with malignant melanoma, showed a partial subjective and objective response (23).

Because of its potential therapeutic usefulness as a stimulator of host defense mechanisms, especially of antitumor activity, much attention has been directed toward defining the nature and scope of Tilorone activity. It was the purpose of this study to quantitate the effect of Tilorone on lymphocyte-mediated cytotoxicity, CMI, in mice. Results indicate that at the regimens used in this study, Tilorone elicited a population of cytotoxic lymphocytes independently of antigen but did not exert a true adjuvant effect.

MATERIALS AND METHODS

Drug. Tilorone was supplied by Merrell-National Laboratories, Cincinnati, Ohio. The material was dissolved in sterile 0.9% NaCl solution at concentrations of 5 mg/ml for i.p. injection (50 mg/kg), and 12.5 mg/ml for force feeding (250 mg/kg).

Animals. Swiss CD-1 male mice (closed colony but noninbred) (Charles River Laboratories, Wilmington, Mass.), 8 to 10 weeks of age were used in most experiments. Weights varied from 25 to 38 g with a median of 30 g. C57L x A F1 (H-2*) mice (The Jackson Laboratories, Bar Harbor, Maine) were used in assays of LAF (see below) because of the uniformity of their in vitro production of this factor.

Immunization with Tumor Cells. Leukemia L1210 cells were carried as an ascitic tumor, transferred weekly in DBA/23 (H-2b) mice (The Jackson Laboratories). Dilutions were made in Fischer's medium following harvest and washing in the same solution.

Experimental Design. Experimental groups of mice were given 5 x 10^4 L1210 cells i.p. (on Day 0) and were given Tilorone 1 day later. Five x 10^4 cells were known to produce suboptimal CMI in these mice. Controls received Tilorone alone, 5 x 10^4 L1210 cells alone, or an optimal immunizing dose (5 x 10^6) of L1210 cells alone. Tilorone was administered by force feeding at 250 mg/kg, or was injected i.p. at 50 mg/kg, a parenteral dose comparable in toxicity. Spleen cells were obtained on Day 10 or 11 after administration of antigen or a 0.9% NaCl solution placebo. The dosage and timing of Tilorone selected for this study were adopted from earlier studies (2, 16) to try to augment host immunity and yet avoid lethal toxicity. The i.p. 50% lethal dose is 145 mg/kg, and p.o. 50% lethal dose is 959...
mg/kg (13). Preliminary experiments indicated that the level of CMI was similar whether Tilorone was administered the day preceding or the day following immunization with antigen. We chose to concentrate on a dosage schedule involving administration of Tilorone after immunization with tumor antigen in an effort to anticipate the therapeutic usefulness of the drug in established disease.

The total number of animals in each experimental group is indicated above the abscissa in Charts 1 and 2. This represents data collected from several experiments in which individual groups contained 3 to 6 CD-1 mice.

**Assay for CMI.** A modification of the in vitro assay for CMI described by Brunner et al. (4) was used. P815Y mastocytoma, a sensitive indicator of immunity to H-2a antigens carried by leukemia L1210, was the target tumor cell. Mastocytes were carried in tissue culture in Fischer's medium supplemented with 10% complement inactivated horse serum. Spleen cells (1 × 10⁷) were incubated with P815Y mastocytes (1 × 10⁶ cells) in a volume of 1 ml for 48 hr at 37°C, in an atmosphere of 5% CO₂ in air. Remaining mastocytes were counted in a hemocytometer. Results were expressed as percentage lysis or 100 × (1 - [number of tumor cells in experimental tube]/[number of tumor cells in tube with normal spleen cells]).

This comparison with the growth of mastocytes incubated with normal spleen cells, rather than with mastocytes alone, controls for such nonspecific effects as crowding or competition for nutrients by the spleen cells. Although Brunner et al. (4) found no difference in growth caused by normal controls for such nonspecific effects as crowding or competition for nutrients by the spleen cells, we have frequently noted 10 to 15% fewer mastocytes in their presence compared with tumor cells in medium alone. Thus the lytic effects caused by Tilorone are somewhat understated by this calculation but are more clearly attributable to the influence of the agent rather than the allogeneic nature of the effector lymphoid cells. Student's t test was used to evaluate statistical significance.

**Spleens used to obtain the results expressed in Chart 1 were assayed individually. Those for Chart 2 were pooled within each group, and weighted means were used for statistical analysis.**

**Removal of Macrophages.** Prior to final resuspension, a portion of spleen cells from each group was incubated serially on the 4 broad surfaces of two 1-liter glass medicine bottles for 30 min each at 37°C, in an atmosphere of 5% CO₂ in air. Each group began with 8 ml containing an average of 6.8 × 10⁷ cells/ml; approximately 6 ml were recovered following the 2-hr incubation, containing nearly 5% of the original number of cells. The concentration of macrophages was reduced from 11 to less than 2%, as judged by Giemsa staining. The relative percentage of lymphocytes and macrophages in Tilorone-treated animals before incubation was not significantly different from controls. B-lymphocytes, which are also adherent to glass (22), were also presumed to be significantly reduced.

**Assay for LAF.** The technique of Gery et al. (6, 7) was used to measure LAF. This mediator is released by splenic adherent cells (macrophages) and potentiates thymidine-³H incorporation into thymocytes suboptimally stimulated by PHA. In our assay, which was performed with the kind assistance of Dr. Gery, 6 × 10⁶ spleen cells were incubated for 24 hr and 0.5 ml of supernatant fluid, either undiluted or a 1:6 dilution, was added to 4.5 × 10⁶ thymocytes in a total volume of 1 ml. PHA was also added to each tube of thymocytes (0.3 μl/ml). Thymidine-³H incorporation was measured 48 hr later, after the addition of a pulse of 1 μCi at 24 hr.

**RESULTS**

**Effect of Tilorone on CMI.** Chart 1 indicates that Tilorone alone, 50 mg/kg i.p. or 250 mg/kg p.o., evoked a...
level of CMI similar in magnitude to that caused by $5 \times 10^4$ L1210 cells (approximately 30% lysis). Tilorone administered 1 day before or 1 day after $5 \times 10^4$ L1210 cells failed to augment CMI significantly above the level elicited by the antigen alone. The i.p. dose of Tilorone was near the maximal tolerated dose but failed to approximate the stimulatory effects of $5 \times 10^4$ L1210 cells (almost 90% lysis).

**Effect of Removal of Macrophages.** Spleen cells from mice treated with Tilorone failed to demonstrate any change in cytotoxicity after removal of adherent cells (macrophages) in groups treated with L1210 or Tilorone alone. However, a statistically significant increase ($p < 0.05$) in CMI was effected in the group given both Tilorone and L1210 cells (Chart 2).

**Assay for LAF.** Table 1 indicates that there was no difference in the amount of LAF liberated from spleen cells of Tilorone-treated mice compared with that from normal spleen cells. Additional stimulants (mitogens) failed to affect differently the production of LAF by the 2 populations of spleen cells.

**DISCUSSION**

Tilorone has been shown to be not only an interferon-inducing agent in mice, but also an effective stimulator of humoral (IgM) antibody against sheep erythrocytes (20). An antitumor effect of Tilorone or its congeners has also been demonstrated against Walker carcinosarcoma 256, reticulum cell sarcoma A-RCS, Friend leukemia virus-induced tumors, and Ehrlich adenocarcinoma E2 (1, 2, 20, 24), but the mechanism of effectiveness was not elucidated. Under different dosage schedules, enhancement of the growth of Moloney sarcoma virus-induced tumors has also been demonstrated (5), perhaps as a result of stimulation of humoral enhancing antibodies or blocking factors (3, 9–11). Since many interferon-inducing agents have been shown to have adjuvant effects upon CMI as well as antibody production (8), we have investigated whether Tilorone shared this property.

Our data indicate that Tilorone administered without antigen is capable of creating “killer” lymphocytes, probably T-lymphocytes which have been shown to be the effector cells in our in vitro assay (18, 19). However, Tilorone did not exert a truly adjuvant effect here, since it did not increase the response to concomitant antigen. These results are similar, although quantitatively inferior, to those obtained previously with BCG, also an interferon-inducing agent (8), which at the large dose administered created a high level of “pseudoimmune” T-lymphocytes (19). However, the failure of Tilorone to increase either the percentage of macrophages in the spleen or the production of LAF is in direct contrast to results with BCG, indicating that a direct effect upon T-lymphocytes was probably the sole mode of action of Tilorone in augmenting CMI. We have not established whether the killing effected by Tilorone was completely nonspecific, because no other tumor has yet grown consistently enough in our medium to allow a similar assay.

The removal of adherent cells (macrophages and sticky B-lymphocytes) (21) facilitated CMI only in the group given both Tilorone and L1210 cells, suggesting that if Tilorone had any effect upon the adherent cell population it was to facilitate the inhibition of cytotoxicity by that population. Inhibition of DNA synthesis in lymphocytes by adherent cells (or substances released by them) has been described previously (21, 25). In addition, Lonai and Feldman (15)

### Table 1

**LAF production by spleen cells**

Cultures used for production of supernatant fluid: $6 \times 10^4$ spleen cells in 4 ml of minimal essential medium with 5% human serum (see Ref. 7 for further details). The stimulants indicated were also added to certain cultures.

<table>
<thead>
<tr>
<th>Source of spleen cells yielding Sup*</th>
<th>Stimulant</th>
<th>Normal mice</th>
<th>Tilorone-treated mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sup dilution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1:1</td>
<td>214 ± 10</td>
<td>180 ± 10</td>
</tr>
<tr>
<td></td>
<td>1:6</td>
<td>172 ± 12</td>
<td>158 ± 13</td>
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<tr>
<td>LPS</td>
<td>1:1</td>
<td>938 ± 80</td>
<td>612 ± 13</td>
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<tr>
<td></td>
<td>1:6</td>
<td>361 ± 57</td>
<td>276 ± 4</td>
</tr>
<tr>
<td>Con A</td>
<td>1:1</td>
<td>18,878 ±194</td>
<td>14,554 ±798</td>
</tr>
<tr>
<td></td>
<td>1:6</td>
<td>771 ± 18</td>
<td>1,046 ± 59</td>
</tr>
</tbody>
</table>

* Sup, supernatant fluid obtained following incubation of $6 \times 10^4$ spleen cells for 24 hr; LPS, E. coli lipopolysaccharide (15 µg/ml); Con A, concanavalin A (2 µg/ml).

† Mean ± range, cpm of thymidine-$^3$H incorporated into $5 \times 10^4$ thymocytes incubated with supernatant fluid; 0.1 ml of a 1:200 dilution of PHA was added to all thymocyte cultures (see Ref. 6 for further details).

* Sup, supernatant fluid obtained following incubation of $6 \times 10^4$ spleen cells for 24 hr; LPS, E. coli lipopolysaccharide (15 µg/ml); Con A, concanavalin A (2 µg/ml).

† Mean ± range, cpm of thymidine-$^3$H incorporated into $5 \times 10^4$ thymocytes incubated with supernatant fluid; 0.1 ml of a 1:200 dilution of PHA was added to all thymocyte cultures (see Ref. 6 for further details).

* Tilorone, 250 mg/kg p.o., was administered 10 days prior to harvest.
have reported an inhibition of lymphocytotoxicity toward xenogeneic cells when an “excess” of adherent cells was present. Our failure to find evidence for inhibition of CM1 in other groups given Tilorone alone could be explained by a necessity for maximal stimulation of adherent spleen cells, by both antigen and Tilorone, to cause the inhibition. We have recently made other observations of this sort; mice given large doses of BCG had splenic lymphocytes that responded better to PHA or concanavalin A in vitro when adherent cells were removed (19). It should be emphasized, however, that our BCG-treated groups showed no change in CM1 after removal of adherent cells.

The net result of the stimulation of both humoral (potentially blocking) immunity and CM1 by Tilorone must be judged in each tumor system. While Tilorone does not have selective or strong stimulating effects on CM1, we are hopeful that a congener of this agent will have such desirable characteristics and thus will consistently favor rejection of tumors. Nevertheless, these data demonstrate that the creation of pseudoimmune T-lymphocytes is not a property of BCG alone, but is shared by another of the substances known to be adjuvants and stimulators of interferon.

ACKNOWLEDGMENTS

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REFERENCES

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