Short communication

Thioridazine protects the mouse from a virulent infection by Salmonella enterica serovar Typhimurium 74

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ABSTRACT

When administered to mice at doses of 100 μg/mouse and 200 μg/mouse, thioridazine (TDZ) significantly protected animals from the lethality produced by a virulent strain of Salmonella enterica serovar Typhimurium and reduced the number of bacteria retrieved from the spleen, liver and heart blood. The protection conferred by TDZ against a virulent Salmonella infection is hypothesised to be due a reduction in the 55 kDa virulence protein of the outer membrane of the organism, as this protein is almost totally absent when the organism is exposed to the phenothiazine. It is further hypothesised that the reduction in the 55 kDa virulence factor renders the organism susceptible to the action of hydrolytic enzymes of the neutrophil phagolysosome, whereas in the absence of exposure to TDZ intracellular ingestion and localisation of the phagocytosed bacterium does not result in killing owing to rapid induction of the two-step PmrA/B regulation that results in the eventual synthesis and insertion of lipid A into the nascent lipopolysaccharide layer of the outer membrane.

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1. Introduction

The therapeutic effectiveness of antibiotics and antibacterial chemotherapeutics is becoming more and more limited due to increased emergence of resistance, especially in Gram-negative bacteria. The need for new antimicrobial agents is consensually accepted, however it is anticipated that regardless of how recently the agent is introduced, resistance to that agent will soon occur. The search for newer antimicrobials has prompted studies demonstrating that many medicinal compounds belonging to various pharmacological groups, such as tranquilisers, antihypertensives, antipsychotics, anti-inflammatory agents, cardiovascular drugs, proton pump inhibitors and even antispasmodic agents, have remarkable antimicrobial activities and have been named 'non-antibiotics' [1–3].

Thioridazine (TDZ) belongs to the class of phenothiazines and was originally introduced in 1959 as an antipsychotic drug. Because it has been shown to have antimicrobial activity against many bacteria, it is now recognised as a possible antimicrobial agent [2]. However, the in vitro activities of TDZ occur at concentrations that are clinically irrelevant as they exceed the maximum concentration that can be achieved in humans (0.5 mg/L). Phenothiazines such as chlorpromazine inhibit the growth of Salmonella during the first 8 h of in vitro exposure to the agent and during this period the 55 kDa virulence protein of the outer membrane is markedly decreased [4]. This study therefore suggests that early exposure to a phenothiazine such as TDZ may cause elimination of the virulence factor, rendering the organism susceptible to the immune system of the infected host.

The ability of Salmonella to cause infection in humans is due in part to its ability to survive within the phagolysosome of the neutrophil [5]. Survival within the phagolysosomal compartment results from activation of the two-step PmrA/B regulon, an operon that involves at least nine genes resulting in the synthesis and insertion of lipid A into the nascent lipopolysaccharide layer of the cell envelope [5]. These responses render the organism resistant to enzymatic digestion, to other antimicrobial proteins as well as to many antibiotics [5]. Mycobacterium tuberculosis [6] and Staphylococcus aureus [7] are able to survive the phagolysosomal environment of the human non-

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killing macrophage, the former for decades [6]. However, whereas the mechanism of survival by intraphagosomal Salmonella in neutrophils is due to the aforementioned genetic responses within an acidic milieu, the mechanism by which survival of M. tuberculosis takes place is due to the absence of acidification of the lysosome resulting from efflux of K+ from that structure [8]. However, the killing of intracellular mycobacteria [6] as well as that of intracellular staphylococci [7] by non-killing human macrophages can be enhanced by T9D, supposedly by inhibition of K+ efflux from the phagolysosomal unit containing the respective bacteria [8].

It is this mechanism that is believed to be the means by which T9D cures mice of antibiotic-susceptible M. tuberculosis infection [9].

Similar to the case with chlorpromazine [4], in vitro exposure of Salmonella strains to T9D also inhibits growth of the organism during the first 6–8 h as well as promoting disappearance of the 55 kDa virulence protein. Because T9D also cures the mouse of intracellular infection such as that produced by M. tuberculosis [9], we have studied the question of whether administration of T9D may affect the course of a highly virulent Salmonella infection in mice.

2. Materials and methods

2.1. Bacteria

Highly virulent Salmonella enterica serovar Typhimurium 74 was grown overnight at 37 °C in brain–heart infusion medium from Oxoid Ltd. (distributor, Fisher Scientific, Mumbai, India).

2.2. Determination of the minimum inhibitory concentration (MIC) of thiadizone

T9D was obtained in pure dry powder from Sigma (Copenhagen, Denmark). A stock solution was prepared by dissolving 25 mg of T9D in 5 mL of 5% dimethyl sulfoxide (DMSO), resulting in a concentration of T9D of 5 mg/mL. This stock solution was sterilized by filtration through a 0.25-mm sintered glass filter and stored at 4 °C. Determination of the MIC was conducted by the broth dilution method according to Clinical and Laboratory Standards Institute guidelines as described previously [10].

2.3. Animal experiments

In vivo experiments were performed with male Swiss white mice, each weighing 18–20 g. Animals were maintained under standard conditions of temperature (21 ± 1 °C) and relative humidity (50–60%) with a photoperiod of 14:10 h of light:dark. Water and a dry pellet diet were provided ad libitum. Determination of the virulence of infection produced by S. Typhimurium 74 has been previously described in detail [11]. Briefly, five groups of mice consisting of five animals each were injected with increasing concentrations of S. Typhimurium 74 and the number of mice that died was recorded for up to 100 h.

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (μg/mL)</th>
<th>No. of mice died (N=40)</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>34</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>20</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

3. Results

The MIC of T9D against S. Typhimurium 74 as determined from three separate broth dilution assays was 500 μg/mL.

The virulence of the infection produced by varying CFU of S. Typhimurium 74 is summarised in Table 1. Briefly, as the number of CFU of S. Typhimurium 74 injected intraperitoneally into mice increases, the percent mortality increases, reaching 100% with a dose of 2.2 × 10⁹ CFU.

As shown in Table 2, the protective effect conferred by T9D against the virulence of an S. Typhimurium 74 infection was complete at the highest dose level of T9D administered (200 μg/mouse).

<table>
<thead>
<tr>
<th>Control group (not receiving T9D)</th>
<th>Test groups (receiving T9D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (μL/mouse)</td>
<td>T9D (μg/mouse)</td>
</tr>
<tr>
<td></td>
<td>No. of mice died (N=20)</td>
</tr>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>200*</td>
</tr>
</tbody>
</table>

* Mice received a challenge of 0.95 × 10⁹ colony-forming units of S. Typhimurium NCTC 74 in 0.5 mL of brain–heart infusion medium.

P < 0.001 vs. controls (χ² test).

as none of the animals died during the 100 h post-infection. In contrast to the protective effect provided by TDZ at 30 mg/kg dose administered, 34 (85%) of the 40 mice that received saline in place of TDZ died within 100 h. We may conclude that since none of the mice treated with 200 mg/kg of TDZ died, TDZ does not produce any lethal toxicity, at least with 200 mg/kg of the agent, as also shown by others employing similar concentrations of the agent [9].

The effects of TDZ on the CFUs of S. Typhimurium 74 retrieved from the liver, spleen and heart blood are summarised in Fig. 1. Briefly, treatment of mice acutely infected with S. Typhimurium 74 significantly decreases equally the number of CFUs of the bacterium retrieved from the sources evaluated, but only after 4 h. These results show that within a few hours following infection, the bacterial load of the heart blood, spleen and liver is reduced to more than one-half of that present in the corresponding sources of the untreated mouse.

4. Discussion

The results obtained in this study show that administration of TDZ protects the mouse from the lethality produced by a virulent Salmonella infection. Moreover, the effects of the agent are evident within a few hours after the agent is administered given the marked and significant reduction in the number of CFUs of the organism retrieved from acutely infected mice. TDZ was devoid of any lethal toxicity since none of the 200 mg/kg TDZ-treated mice died during the 100 h of the experiment.

Introduction of Salmonella via the intraperitoneal route should very quickly result in their phagocytosis by neutrophils [5]. However, the organism survives the activity of hydrolases owing to induction of the two-step regulon PmrA/R that leads to resistance to the action of hydrolases [5]. As shown by this study, the MIC of TDZ against the virulent S. Typhimurium 74 is 500 μg/mL, and, if we assume that the weight of the mouse is equivalent to the weight of water, the concentration of TDZ in a mouse treated with 200 μg of the agent would be more or less equal to 10 μg/mL, which is 50-fold lower than the MIC. Therefore, why does such a low concentration of TDZ not only protect the mouse for 100 h from a virulent infection by S. Typhimurium 74 but also markedly reduces the number of CFU retrieved from sources expected to harbour the organism? Because phenothiazines are concentrated by macrophages as much as 100-fold over the concentration of the agent in the medium in which macrophages are maintained [7,8], and because the concentration takes place in the lysosome [3], one would suggest that this concentration effect produces a level of agent in the lysosome that is equal to the concentration that is bactericidal. However, given the fact that the phenothiazine may promote loss of the 55 kDa virulence protein [4], it is possible that what has taken place within the phagolysosome is a significant reduction of virulence of the organism and, consequently, although the presence of the infective agent continues as expected from the very high CFU retrieved from sources of the infective animal, the lethality associated with virulence is obliterated. Nevertheless, until there is a demonstration that the virulence protein of the phagocytosed S. Typhimurium 74 is lost after exposure to the phenothiazine, the mechanism by which the phenothiazine, and possibly other phenothiazines, protects the infected mouse from a lethal Salmonella infection remains highly speculative. Lastly, although we do not recommend that TDZ be used for the therapy of Salmonella infections, we do suggest that this agent be exploited as a ‘lead compound’ for the creation of new antimicrobial agents with significant activity against virulent Salmonella infections.

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Ethical approval: The guidelines of the University of Kolkata (India) for the care and use of laboratory animals were strictly followed.

References
