In Vitro and In Vivo Toxicity of T-2 Toxin, a Fusarium Mycotoxin, to Mouse Peritoneal Macrophages

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The effects of T-2 toxin on mouse peritoneal macrophages were investigated. Scanning electron microscopy of macrophages treated in vitro with T-2 toxin revealed retraction of pseudopodia. Protein synthesis was inhibited after in vitro contact with T-2 toxin but was not affected 24 h after injection of a sublethal dose of toxin into mice. There was reduction in the phagocytosis of Pseudomonas aeruginosa when macrophages were exposed in vitro to T-2 toxin and when mice were injected with T-2 toxin. Clearance of colloidal carbon was not modified after T-2 toxin injection, whereas spleen weight was decreased 24 h after T-2 injection. T-2 toxin enhanced the mortality of mice infected with Salmonella typhimurium C58 when it was administered 24 h prior to oral challenge with the bacterium.

Trichothecenes are toxic secondary metabolites that are produced by Fusarium spp. (1). Trichothecene mycotoxins, particularly T-2 toxin, have attracted attention because of their possible use in chemical warfare as the agent "Yellow Rain" (15). T-2 toxin induces radiomimetic lesions in lymphoid, hematopoietic, and gastrointestinal tissues (18). T-2 toxin is cytostatic for unstimulated and mitogen-stimulated lymphocytes and induces depletion of splenic and thymic cells (11, 22). It inhibits the synthesis of anti-sheep erythrocyte antibodies and prolongs skin graft rejection (18). Protein synthesis and DNA synthesis in lymphocytes and macrophages are strongly inhibited by T-2 toxin (1, 11, 14, 25). Inhibition by T-2 toxin of isoprenoid synthesis in the murine macrophage cell line J774 is a sign of growth inhibition (14).

T-2 toxin is toxic to alveolar macrophage function in vitro, as demonstrated by Gerberick et al. (8, 9) and Sorenson et al. (24). T-2 toxin causes loss of cell viability, reduction of cell volume, release of 51Cr from the cells, and characteristic microscopic changes, such as cellular blebbing and smoothing of membranes (8); exposure of a macrophage to T-2 toxin inhibits incorporation of radiolabeled leucine and macrophage activation and decreases the capacity of the macrophage to phagocytize Saccharomyces cerevisiae and Staphylococcus aureus (9).

A single oral dose of T-2 toxin has no effect on the in vivo phagocytosis of sheep erythrocytes by peritoneal macrophages in nonsensitized mice (4). Experimental T-2 toxicity in swine following inhalation exposure is responsible for the reduction in phagocytosis by alveolar macrophages (20).

Recently, Niyo et al. (16) have reported that ingestion of T-2 toxin by rabbits for 3 weeks causes a decrease in alveolar macrophage number and in the ability of these macrophages to phagocytize Aspergillus fumigatus conidia in vitro. In vivo, defense mechanisms against A. fumigatus infection are compromised by treatment with T-2 toxin (17).

The purpose of this investigation was to examine the correlation between the in vitro and in vivo acute toxicity of T-2 toxin to mouse peritoneal macrophages and the effect of T-2 toxin on mice infected with Salmonella typhimurium.

Crystalline T-2 toxin (Sigma Chemical Co., St. Louis, Mo.) was dissolved in ethanol at a concentration of 10 mg/ml. For in vivo use, this solution was diluted in saline at 0.20 mg of toxin per ml; for in vitro use, it was diluted in sterile tissue culture medium at the desired concentration.

Resident peritoneal macrophages were harvested from BALB/c mice (IFAA-CREDO, Les Oncins, France) by peritoneal lavage with 5 ml of cold medium 199 (Boehringer Mannheim, Meylan, France) containing 2% heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). Cell suspensions from five animals were pooled and distributed among 12-well tissue culture plates (Falcon; Becton Dickinson Labware, Oxnard, Calif.) at a concentration of 2 × 10⁶ cells per well and then incubated at 37°C in a 5% CO₂ atmosphere for 1 h. Nonadherent cells were removed by washing the plates with isotonic saline solution. The adherent cells were counted, and cell viability was assessed by the trypan blue exclusion technique (21).

For scanning electron microscopy, the macrophage monolayers prepared on 15-mm plastic tissue culture dishes (Falcon) were exposed to 0.1 or 1 µM T-2 toxin for 48 h and were fixed for 48 h in a 5% buffered glutaraldehyde solution at room temperature. The cells were dehydrated by treating them with a graded series of alcohols and Freon 113, coated with palladium, and observed with a scanning electron microscope (JEOL 35 CF).

To assess protein synthesis, peritoneal macrophage monolayers were exposed to 10 µM T-2 toxin, with a contact time of 5, 15, 30, or 60 min, and then incubated in medium 199 with 0.1 µCi of L-[14C]leucine per ml (300 mCi/mM; Commissariat à l’Energie Atomique, Saclay, France) for 24 h at 37°C. The cells were washed with phosphate-buffered saline and solubilized with 1.0 ml of 10% trichloroacetic acid. The acid-precipitable proteins were dissolved in 0.5 ml of 1 M NaOH, and the radioactivity was counted in a liquid scintillation counter (26).

The effect of T-2 toxin on the phagocytosis of Pseudomonas aeruginosa [1H]DNA was evaluated. The bacteria were grown at 37°C for 18 h in broth medium containing 5% peptone (Institut Pasteur Production, Marnes la Coquette, France) and supplemented with [1H]thymidine (20 Ci/mmol; Commissariat à l’Energie Atomique). P. aeruginosa was killed by heating at 80°C for 15 min and then was opsonized by incubation in guinea pig complement (BioMérieux, Marcy l’Etoile, France) for 30 min at 37°C. Macrophage cultures

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were incubated for 6 h in medium 199 containing T-2 toxin at a final concentration of 0.001, 0.01, or 0.1 μM. The culture medium was removed, and the labeled P. aeruginosa in medium 199 were added to macrophage monolayers at a 100:1 ratio (100 bacteria to 1 macrophage). The culture plates were centrifuged at 1,800 × g for 5 min to layer the bacteria on the monolayer and then were incubated for 1 h. The peritoneal macrophage monolayers were rinsed with phosphate-buffered saline and solubilized for 10 min with 300 μl of 0.1% Triton X-100. The solubilized suspension was collected, and the radioactivity was counted in a liquid scintillation counter. The total inhibition of phagocytosis was assayed by preincubating the peritoneal macrophages with cytochalasin B (100 μg/ml; Sigma) for 15 min.

For the in vivo study, T-2 toxin was given to mice by intraperitoneal or subcutaneous injection in a single dose of 2 mg/kg of body weight (approximately one-half the 50% lethal dose). After 24 h, mice were anesthetized and killed to harvest macrophages. Protein synthesis and phagocytosis of P. aeruginosa were assessed with macrophages from treated mice; macrophages from untreated mice were used as a control.

Carbon clearance was tested in mice given T-2 toxin (2 mg/kg) subcutaneously 6 h, 24 h, 2 days, 3 days, 4 days, or 7 days before the test (2). The carbon (Gunter Wagner, Pelikan Werke, Hannover, Federal Republic of Germany) was diluted at 8 mg/ml in phosphate-buffered saline and injected into the upper tail vein (1 ml/100 g body weight). Serial blood samples (0.025 ml each) were removed from the retro-orbital venous plexus at intervals of 5, 10, and 15 min and transferred into tubes containing distilled water. The relative amounts of carbon were estimated in a colorimeter at 650 nm. The density readings were converted to a logarithmic scale and plotted against time. The slope of the line is called the phagocytic coefficient (K). The mice were killed, and the livers and spleens were removed and weighed individually. The corrected phagocytic index (α) is a measure of the phagocytic activity per unit weight of tissue and is given by the following formula: $\alpha = (W/WLS) \times \sqrt{K}$, where $W/WLS$ is the ratio of body weight (W) to the combined weight of liver and spleen (WLS).

S. typhimurium C55 was purchased from the Institut Pasteur (Paris, France). Suspensions for oral administration were prepared from a logarithmic-phase culture grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.). Plate counts were performed on agar-containing petri dishes. T-2 toxin (2 mg/kg) was injected intraperitoneally into mice (37 animals) 24 h before challenge with S. typhimurium C55. Control animals were given either T-2 toxin (20 animals) or S. typhimurium C55 (37 animals).

Student’s t tests were performed to analyze the results of in vitro and in vivo experiments, and the chi-square test was used to determine the mortality of animals infected with S. typhimurium.

On the basis of scanning electron micrographs of treated and control peritoneal macrophages, the macrophages treated for 48 h with 0.1 μM T-2 toxin had retracted pseudopodia and had become rounded (Fig. 1).

Protein synthesis was quickly inhibited, because $[^{14}\text{C}]$ leucine incorporation was decreased by 70% after macrophages were exposed for 5 min to 10 μM T-2 toxin (Fig. 2). After 1 h of exposure to 0.1 μM T-2 toxin, protein synthesis was not inhibited, but after exposure to 1 μM T-2 toxin it was inhibited by 80% (Table 1).

Phagocytosis of P. aeruginosa $[^{1}\text{H}]$DNA by macrophages exposed in vitro to 0.001 μM T-2 toxin was significantly inhibited (Fig. 3).

Injection of T-2 toxin into mice (2 mg/kg of body weight) did not modify significantly protein synthesis by peritoneal macrophages. After 24 h of incubation, the amount of $[^{14}\text{C}]$leucine incorporated was 47.3 ± 16.9 cpm/mg of protein in control macrophages, 49.1 ± 11.6 cpm/mg of protein in macrophages from T-2 toxin-treated mice (subcutaneously...
TABLE 1. Effect of T-2 toxin concentration on protein synthesis by peritoneal macrophages in vivo

<table>
<thead>
<tr>
<th>Treatment (µM T-2)</th>
<th>Cpm/mg of protein</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.2 ± 2.8</td>
<td>101.3</td>
</tr>
<tr>
<td>0.1</td>
<td>15.4 ± 2.3</td>
<td>107.9</td>
</tr>
<tr>
<td>1.0</td>
<td>3.3 ± 1.3d</td>
<td>20.4</td>
</tr>
<tr>
<td>10.0</td>
<td>1.6 ± 0.2d</td>
<td>10.5</td>
</tr>
</tbody>
</table>

a Macrophage cultures were treated for 1 h with T-2 toxin. 
b Cpm/mg of [3H]leucine incorporation into macrophage proteins was measured after incubation for 24 h.

The corrected phagocytic index of carbon from the bloodstream of mice was not modified significantly during the 7 days after injection of T-2 toxin at 2 mg/kg (Table 2). However, the spleen weight was decreased significantly (P < 0.01) at 24 and 48 h after T-2 toxin administration and then increased 96 h (4 days) postinjection.

The mortality of mice given T-2 toxin (2 mg/kg) 24 h before oral challenge with 107 S. typhimurium CSS increased significantly (P < 0.01), from 13.5% (without T-2 toxin) to 46% (Fig. 4). There was no mortality in the control group of mice given only T-2 toxin.

Submicromolar concentrations of T-2 toxin are cytotoxic to alveolar macrophages and inhibit macrophage activation (8, 9, 24). By suppressing macrophage activation, the cell becomes unable to function normally as an immunocompetent cell.

In rat alveolar macrophages, T-2 toxin inhibits both protein synthesis and phagocytosis of Staphylococcus aureus at 0.1 µM (9). In contrast, in our experiments phagocytosis of P. aeruginosa was inhibited significantly by a very low dose (0.001 µM) of T-2 toxin and protein synthesis was inhibited by a higher dose (1 µM). It has been shown that protein synthesis is not required during phagocytosis (13), and T-2 toxin may affect a cellular function other than protein synthesis, such as mitochondrial functions. It has been observed that T-2 toxin has antimitochondrial action in Saccharomyces carlsbergensis (23), inhibits oxygen consumption by rat liver mitochondria (19), and inhibits reduction of a tetrazolium salt (MTT) by mitochondrial enzymes of the respiratory chain (10). However, the ATP level is not decreased in T-2 toxin-treated macrophages (9).

We also observed that in vivo intraperitoneal or subcutaneous treatment of mice with T-2 toxin (2 mg/kg) did not affect incorporation of leucine into cellular proteins of macrophages but significantly decreased phagocytosis of P. aeruginosa. A single oral dose of T-2 toxin (4 mg/kg) has no effect on the viability or phagocytic activity of resident peritoneal macrophages in nonsensitized mice (4). It is likely that a 4-mg/kg oral dose of T-2 toxin is not fully absorbed, producing a lesser effect than a 2-mg/kg intraperitoneal dose.

Inhalation exposure of pigs to T-2 toxin at 9 mg/kg results in death, with clinical signs resembling those reported in pigs given T-2 toxin intravascularly at a dose of 1.2 mg/kg (20).

As we observed in vitro, the phagocytosis of P. aeruginosa was more sensitive to the effect of T-2 toxin than was protein.
synthesis after in vivo treatment with toxin. In contrast, carbon clearance was not affected during the 7 days after T-2 toxin administration, although spleen weight was significantly decreased 24 and 48 h post injection.

We chose S. typhimurium as a model to assess the effect of T-2 toxin, because these organisms are intracellular parasites of macrophages (12). Mice were challenged by oral administration of bacteria on day 1 after treatment with T-2 toxin, when the spleen weight was lowest and the phagocytic ability of peritoneal macrophages was inhibited. Treatment with T-2 toxin increased the mortality of mice challenged orally with S. typhimurium. The mortality was not a result of toxic effect but of a real infection because the mice died between day 6 and day 24 postinoculation, as shown in the mortality curve (Fig. 4).

T-2 toxin increases the mortality of chickens with Salmonella infections (3) and impairs the normal defense mechanisms against A. fumigatus infection (17). The effects of T-2 toxin on cell-mediated resistance to bacterial infection have been evaluated in mice exposed to Listeria monocytogenes by Corrier et al. (4-7) and Ziprin et al. (27-29). Treatment with T-2 toxin after challenge with L. monocytogenes increases mortality due to listeriosis in mice (7). The immunotoxicity of T-2 toxin is higher than that of cyclophosphamide and was attributed to the depletion of T lymphocytes and subsequently of T-cell-dependent activated macrophages (6). Pretreatment with T-2 toxin before exposure to L. monocytogenes enhances resistance to listeriosis (5) but does not increase the percentage of peritoneal macrophages with Ia surface antigen (28). Administration of T-2 toxin 4 days prior to infection with L. monocytogenes increases the acute phase of serum amyloid protein in response to infection, suggesting that the enhanced resistance to listeriosis that is induced by T-2 toxin is a result of increased intracellular killing of L. monocytogenes by macrophages, stimulated by serum amyloid protein (28). T-2 toxin given orally 4 days prior to intraperitoneal inoculation with S. typhimurium has no effect on infection (29). In contrast, diacetylscirpenol given 1 and 2 days before inoculation increases mortality in mice exposed to L. monocytogenes (27).

The results reported here show that T-2 toxin inhibits the in vitro function and alters the shape of peritoneal macrophages: in vivo, T-2 toxin inhibits phagocytosis but not protein synthesis. T-2 toxin clearly impairs the defense mechanisms of macrophages and decreases resistance to infection when given just before inoculation with the pathogen. However, increased mortality to S. typhimurium infection could be due to the immunotoxic effects of T-2 toxin on other cells, particularly on T lymphocytes (11, 22), and to the cytotoxic effect on mucosal epithelial cells because S. typhimurium was given orally.

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LITERATURE CITED

of mycotoxins on the protein synthesis in rabbit reticulocytes. J. Biochem. (Tokyo) 66:419-422.