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→ (3) Thymidine uptake and incorporation into DNA showed a biphasic response with an increase at 0.4 pg/ml and a reduction at 4 pg for uptake and 40 pg/ml for incorporation. (4) Intracellular LDH was reduced at 4 ng/ml. (5) Calcium efflux was reduced after 1-, 5-, and 15-min exposure to T-2 toxin in a concentration of 40 ng/ml. All of the changes noted, including protein synthesis inhibition, were present to a significant degree within 10 min of exposure to T-2 toxin. This time interval is too short to attribute all of these effects directly to protein synthesis inhibition since most short-lived proteins have half-lives measured in hours. In conclusion, T-2 toxin appears to have multiple effects on cell membrane function at very low concentrations (0.4 pg/ml to 4 ng/ml), which is independent of protein synthesis inhibition. These likely include effects on amino acid, nucleotide, and glucose transporters, as well as calcium and potassium (rubidium) channel activities. ←

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Alteration of Multiple Cell Membrane Functions in L-6 Myoblasts
by T-2 Toxin: An Important Mechanism of Action

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Alteration of Multiple Cell Membrane Functions in L-6 Myoblasts by T-2

Toxin: An Important Mechanism of Action. BUNNER, D.L. AND MORRIS, E.R.

(1987). Toxicol. Appl. Pharmacol. , - . Recent studies suggest that T-2 toxin interacts with cell membranes and alters membrane function. This study was done to assess the effect of T-2 toxin on a broad range of cell membrane functions in L-6 myoblasts. The following parameters were assessed after exposure to T-2 toxin for 10 min: (1) the uptake of calcium, rubidium, and glucose; (2) the uptake of leucine and tyrosine and incorporation into protein; (3) the uptake of thymidine and incorporation into DNA; and (4) residual cellular lactate dehydrogenase (LDH) as a measure of cell membrane integrity. The effects of T-2 toxin on these parameters were: (1) The minimal effective concentration (MEC) of T-2 toxin that caused a reduction in uptake of calcium and glucose was 4 pg/ml. The uptake of rubidium was increased at 0.4 pg/ml and then reduced at 4 pg/ml and higher concentrations. (2) The MEC for reduction of uptake of leucine and tyrosine and their incorporation into protein was 4 pg/ml. (3) Thymidine uptake and incorporation into DNA showed a biphasic response with an increase at 0.4 pg/ml and a reduction at 4 pg for uptake and 40 pg/ml for incorporation. (4) Intracellular LDH was reduced at 4 ng/ml. (5) Calcium efflux was reduced after 1-, 5-, and 15-min exposure to T-2 toxin in a concentration of 40 ng/ml. All of the changes noted, including protein synthesis inhibition, were present to a significant degree within 10 min of exposure to T-2 toxin. This time interval is too short to attribute all of these effects directly to protein synthesis inhibition since most short-lived proteins have half-lives measured in hours. In conclusion, T-2 toxin appears to have multiple effects on cell membrane function at very low concentrations (0.4 pg/ml to 4 ng/ml), which is independent of protein synthesis inhibition. These likely include

effects on amino acid, nucleotide, and glucose transporters, as well as calcium and potassium (rubidium) channel activities.

T-2 toxin is a potent protein synthesis inhibitor both in vitro (Ueno et al., 1973) and in vivo (Rosenstein and Lafarge-Frayssinet, 1983). It was also reported to be associated with alimentary toxic aleukia (Joffe, 1971). Toxic systemic effects have been reported for nearly every major organ system, including cardiovascular, central nervous system, bone marrow, liver, and intestine (Bunner et al., 1985; Lutsky et al., 1978; Weaver et al., 1978). In more recent years, a number of alterations in cell, organ, or whole animal measurements have been described. Altered prostaglandin release (Shohami and Feuerstein, 1986), mitochondrial function (Schiller and Yagen, 1981; Schappert and Khachatourians, 1986; Schappert and Khachatourians, 1983; Schappert and Khachatourians, 1984), platelet function (Yaron et al., 1984; Cosgriff et al., 1984), peripheral vascular responses (Wilson and Gentry, 1985), electrocardiographic changes, and serum potassium elevation (Bunner et al., 1985) have all been noted. The interrelationship of these many phenomena is not yet understood.

Although ribosomal binding (Bamburg, 1974; Cannon et al., 1976A; Cannon et al., 1976B; Cundliffe et al., 1974; Cundliffe and Davies, 1977) is the most plausible explanation for protein synthesis inhibition, a clear description of the cascade of events connecting protein synthesis inhibition to in vivo injury and death has not yet been published. Even early landmark publications in this area (Bamburg, 1972; Ueno et al., 1973) suggested that protein synthesis inhibition was not the only cellular mechanism of action. Bamburg (1972) noted that specific data comparing protein synthesis inhibition and cell growth and replication suggested that there was not a direct correlation in all instances and that other mechanisms of action were likely. Ueno, who originally described protein synthesis inhibition as a dominant effect of trichothecenes (Ueno et al., 1968), pointed out that T-2 toxin was a very weak

protein synthesis inhibitor in cell-free systems, but very potent in intact cells (Ueno et al., 1973). His hypothesis was that a cell membrane effect of T-2 toxin altered the uptake of precursors. More recently, Thompson and Wannemacher (1986) found that several trichothecenes were very weak in vitro protein synthesis inhibitors, but were very potent in vivo and postulated that protein synthesis inhibition might not be the sole explanation of in vivo toxicity. In a different model, Rotten et al. (1984), demonstrated that trichothecenes had no effect on protein, DNA, or RNA synthesis in Mycoplasma gallisepticum but still had profound effects on cell growth and impaired cellular uptake of several acid-soluble substrates. This study substantiated cell membrane effects in the absence of impaired protein synthesis.

Given the potential implications of the apparent cell membrane effects of T-2, our study was instituted to examine in greater detail any perturbations in cell membrane function that T-2 might induce. A special effort was made to address the concentration range of effects and time of onset in relationship to protein synthesis inhibition. We hoped that these data would contribute to a better understanding of the relationship of in vivo and in vitro effects of T-2 toxin.

METHODS

Cell cultures. L-6 myoblasts (Yaffe, 1973) (American Type Culture Collection, Rockville, Md.) were grown to confluence in 24-well plates in 1 ml of growth medium which was Dulbecco's Modified Eagle Medium (DMEM) with 20% Nu Serum (Collaborative Research, Lexington, Mass.), gentamicin sulfate, 200 µg/ml; penicillin, 50 units/ml; and streptomycin, 50 µg/ml. Cells were cultured under optimal growth conditions (37° C in 98-100% relative humidity, 5% CO₂, and 95% room air). Twenty-four hr prior to assay, medium was replaced with fresh.

Radiolabel uptake assays. Media were replaced at time 0 in each well of L-6 myoblasts with test media (growth media) with varied concentrations of T-2 toxin (Myco Labs, Inc., St. Louis, Mo.) as indicated in the figures. After 5-min incubation under optimal growth conditions, a pulse of 5 µCi per well of the appropriate radioisotope was added: ⁴⁵CaCl₂, D [1-³H] glucose, L-[4,5-³H] leucine, ⁸⁶Rubidium, L-[3,5-³H] tyrosine, or [methyl, 1', 2'-³H] thymidine (Amersham Corporation, Arlington Heights, Ill.). This was followed by an additional 5-min incubation. The pulse labeling was stopped by placing the culture plates in an ice water bath, quickly removing the media by aspiration, and washing three times with 1 ml of ice-cold Hanks' Balanced Salt Solution (HBSS) with HEPES. The wells were then air-dried and snap-frozen with liquid nitrogen. The cells were solubilized with 0.5% Triton X-100 in HBSS with HEPES over 60 min. After mixing, 75 µl was added to 6.5 ml of CytoScint™ (ICN Radiochemicals, Irvine, Calif.), and radioactivity determined via liquid scintillation counting in a Beckman LS6800 (Beckman Instruments, Irvine, Calif.). Total cell-associated activity was then referred to as the uptake. A screening assay was done with a 1-, 3-, and 5-min radiolabeled pulse after a

5-min incubation with T-2 toxin to show that the response was linear over these time intervals.

Initial screening assays showed that there was no significant breakdown of glucose or leucine for the time intervals tested. Tyrosine is not broken down by muscle cells (Goldberg and Chang, 1978).

Radiolabel incorporation into protein and DNA. A 0.5-ml aliquot from the same wells used to determine total cell-associated radioactivity was transferred to 1.5-ml Eppendorf tubes. Ice-cold (0.5 ml), 35% TCA was added to these samples. Each sample was mixed well and refrigerated for 2 hr. Samples were then centrifuged for 10 min at 4° C and 3000 rpm. The supernatants were aspirated and discarded. Pellets were resuspended and dissolved in 0.5 ml of 0.5 N NaOH; 0.075 ml of this solution was transferred to 6.5 ml of CytoScint™. Total acid-precipitable radioactivity was determined on a Beckman LS 6800 (Beckman Instruments, Irvine Calif.) as a measure of incorporated thymidine into DNA and leucine and tyrosine into protein.

⁴⁵CaCl₂ efflux. At ambient temperature, 5 µCi/ml of the labeled isotopes were added to three sets of four replicate wells and incubated under optimum growing conditions for 16 hr. Each set of replicates for control and treatment was then rinsed three times with 1 ml ice-cold HBSS plus HEPES. After rinsing, the first set (1-min efflux) was exposed to 1 ml of control or 40 ng/ml T-2 toxin for 1 min. Medium (75 µl) from each well was then transferred to 6.5 ml of CytoScint™. This was used to quantitate the ⁴⁵Ca efflux into the media. The remaining medium was aspirated and discarded and the monolayers treated as described in the radiolabel uptake assay. This

aliquot was used to measure total residual cellular ^{45}Ca . This process was then repeated for 5- and 15-min measurements from separate wells.

Residual cellular lactate dehydrogenase (LDH). Cells were exposed to T-2 toxin for a total of 10 min, as in the uptake assay, and similarly solubilized in Triton X-100. An aliquot was then used to determine total residual intracellular LDH activity using a COBAS Bio (Roche, Analytical Instruments, Nutley, NJ) with its standard reagents and procedures.

Statistics. Data were compared by using one-way analysis of variance.

Significance levels are indicated in the figures. Data are expressed as means and standard error in the figures. The standard error was usually < 1%. Some of the standard error bars are not visible because of their small value. Each of the treatments and control was done by using four replicate wells. All data were reproducible on repeat assays.

RESULTS

As noted in METHODS, the term uptake, as used in this paper, is the net-total, cell-associated radioactivity determined at the time points indicated. Incorporation of the amino acids and thymidine is defined as the total cell-associated trichloroacetic acid-insoluble radioactivity. Efflux measures the net change in both residual, intracellular radioactivity and total radioactivity in the media at given time points. The residual cellular LDH is the amount of enzyme activity left within the cells after exposure to T-2 for a given interval of time.

The response to a single concentration of T-2 toxin (40 ng/ml) was tested after a 5-min incubation with T-2 toxin. There was a linear response in uptake of calcium and glucose to a 1-, 3-, and 5-min radiolabeled pulse for both controls and treated wells. The response of tyrosine incorporation was also time related, although tyrosine uptake had plateaued by the 1-min time point (data not shown). Based on this information, all of the uptake studies were performed after a 5-min radiolabeled pulse, since they were more easily reproducible than after the shorter time intervals. The pre-exposure to toxin for 5 min was based on ease of reproducibility and knowledge from prior testing that clear-cut effects were noted by this duration of exposure.

Figures 1 and 2 demonstrate the effect of T-2 toxin on calcium, glucose, rubidium, leucine, and tyrosine uptake, and leucine and tyrosine incorporation 10 min after T-2 exposure, and 5 min after test radiolabels were added. Each parameter was significantly decreased. The MEC was 4 pg/ml with more substantial changes induced at 4 ng/ml, which is the approximate concentration at which protein synthesis is inhibited in L-6 cells by 50% (ED₅₀) in this laboratory. A definite but gradual response over a broad range of T-2 concentrations was present. Rubidium uptake was stimulated at 0.4 pg/ml, even

though it was reduced at higher doses. There does not appear to be a sudden threshold effect at any given concentration level.

Figure 3 demonstrates that there were easily measurable increases in thymidine uptake and incorporation at the 0.4 pg/ml concentration 10 min after T-2 exposure. The response was biphasic and we noted a reduction in uptake at 4 pg/ml and in incorporation at 40 pg/ml. Of note, the stimulation of thymidine uptake and incorporation occurred at concentrations lower than that required for depression of cytoplasmic protein synthesis (Fig. 2).

Table 1 summarizes the effect of higher concentrations of T-2 toxin on cell membrane functions. All are in a range that would in turn be expected to alter other cell functions.

Intracellular LDH was reduced 10 min after T-2 exposure as well, but required concentrations of 4 ng/ml for a significant effect (Fig. 5).

Calcium efflux (Fig. 4) decreased at each time point, including the 1-min time point, demonstrating that the onset of efflux impairment is similar to the other effects noted. This parameter was measured with no prior T-2 exposure, emphasizing the rapidity of onset of T-2 effects, i.e. 1 min.

In brief, nearly all of the cell membrane functions measured were altered at the earliest measured time points at the same MEC as protein synthesis inhibition. Cell membrane integrity, as measured by loss of intracellular LDH, did require a higher concentration.

DISCUSSION

Of particular relevance is a proposal by Pritchard (1979) that toxins interact with the protein or lipid component of cell membranes and induce changes in cell membrane function. Compounds which are lipophilic may specifically interact with cell membrane lipids and induce a shift in lipid-protein interactions (Korpela and Tahti, 1986) and a net change in membrane fluidity. The altered membrane fluidity then can induce changes in enzyme function (Korpela and Tahti, 1986) and could theoretically affect other proteins, such as ion channels, and amino acid and hexose transporters. Segal et al. (1983) reported that T-2 toxin induced hemolysis in red blood cells and Gyonggyossy-Issa et al. (1986) showed that T-2 distributed in the outer half of the phospholipid bilayer. More recently, increased species sensitivity to hemolysis induced by T-2 toxin was correlated with the presence of membrane phosphatidylcholine (DeLoach and Mollenhauer, 1987). Reduced freedom of motion of spin-labeled phospholipids in T-2-treated mycoplasma was also reported (Rottem et al., 1984). Schappert and Khachatourians (1984) also documented that membrane-active agents alter the toxic response of yeast to T-2 toxin.

Thus, the combination of T-2 toxin's known membrane interactions and the recent suggestion that many toxic compounds assert themselves by interacting with cell membrane lipids and cause secondary changes in membrane proteins, including transporters, lead us to evaluate broadly membrane functions after T-2 toxin exposure.

Data in this study document that T-2 toxin has a significant effect on all cell membrane functions tested in L-6 myoblasts. The onset of these effects was universally at the earliest time point (10 min) measured. Of particular

interest is the potency, with many effects starting at a concentration of 4 pg/ml (8.6 picomolar) or less, which is in the same molar range as cellular effects of steroid hormones. These cell membrane alterations occurred at nearly 5000-fold lower concentrations than the 200 µg/ml concentration reported (Gyonggyossy-Issa, et al., 1986) for red blood cell effects, and well within the range that one might expect tissue levels to be in intoxicated animals.

An earlier report (Gerberick, et al., 1984) implied that leucine uptake in macrophages was not impaired at concentrations that reduced protein synthesis, but concentrations of T-2 were not stated and α-amino isobutyric acid (AIB) was the actual radioisotope used. In our study, we found impairment of amino acid uptake at concentrations that impaired protein synthesis for both leucine and tyrosine as well as AIB (data for AIB not shown). Of interest, hyperaminoacidemia in vivo during T-2 intoxication has been reported (Wannemacher and Dinterman, 1983), although no data were available to explain its exact cause. In the L-6 myoblast model used, several amino acid transporters are likely affected by T-2 since all three amino acids tested showed depressed uptake. The relationship of depressed amino acid uptake to the in vivo hyperaminoacidemia is not known.

In a study reported by Schappert and Khachatourians (1983), toxicity of T-2 toxin to a yeast was variable, depending on the presence of different carbohydrates, and they postulated an alteration in hexose transport systems. The data in our study show that glucose transport is first affected at 4 pg/ml and is suppressed by 86% at 40 ng/ml (Fig. 5). This substantiates their hypothesis.

Calcium uptake was also reduced at a T-2 concentration of 4 pg/ml, and at 400 ng/ml was decreased by 71% compared to control (Table 1). Although only

tested at one concentration, calcium efflux was also reduced within 1 min of T-2 exposure. An earlier report (Yarom et al., 1986) showed that myocardial cells had a lessened ionotropic response to increased calcium concentrations in the bathing media after exposure to T-2 toxin. Impaired calcium transport could explain this finding.

Pavlovkin et al. (1986) reported that several trichothecene toxins impaired uptake of potassium in plant root hair membranes and depolarized the membranes. Our data showed impaired rubidium uptake and suggest that potassium transport in mammalian systems may be impaired as well since rubidium transport does correlate well with that of potassium (Dawson et al., 1986; Smith et al., 1986). The role that altered calcium and potassium transport might play in altered electrocardiograms (Bunner et al., 1985) and shock (Siren and Feuerstein, 1986) is not known. Note that rubidium uptake was stimulated in the L-6 myoblasts at a concentration that had no measurable effect on protein synthesis, suggesting that the alteration in rubidium uptake is independent of protein synthesis inhibition.

Gerberick et al. (1984) suggests that impaired DNA synthesis caused by T-2 exposure of macrophages is caused by impaired cytoplasmic protein synthesis; this is based solely on a delay in maximal impairment of thymidine incorporation for 2 hr. Even their data, however, showed reduced thymidine incorporation at the earliest time point measured, 1 hr. Bamburg (1972) argued just the opposite, however, that impairment of DNA synthesis is likely an independent process since he saw a rapid onset in mouse L-cells. Data in this study showed a 95% reduction in thymidine incorporation at 10-min post-T-2 exposure at a concentration of 0.4 ng/ml. In addition, thymidine uptake was reduced by 80% at the same time and concentration. This, in conjunction with stimulation of thymidine uptake and incorporation at a concentration that had

no effect on protein synthesis, suggests that they are independent effects. There have not been reports suggesting that proteins exist with a half-life so brief that a decrease in protein synthesis for 10 min would likely cause such a profound reduction in DNA synthesis. Degradation of protein in L-6 cells has been reported to be less than 1% per hr (Smith 1985). Whether T-2 mediates its effects on reduced transport of DNA precursors through the cell and nuclear membrane, or by a direct nuclear interaction, is unknown. The lowest concentration, 0.4 pg/ml, of T-2 tested induced both thymidine uptake and incorporation, raising the possibility that the altered thymidine uptake could cause the alteration in incorporation. This concentration caused no change in protein synthesis. An earlier argument by Ueno et al. (1973) that DNA polymerase was not affected by T-2 in a cell-free system certainly does not exclude the possibility that impaired DNA synthesis would be a direct effect. This is the case since there are many other steps in DNA synthesis starting with the uptake of thymidine itself.

Cell and/or organ injury by T-2 toxin sufficiently severe to cause leakage of intracellular enzymes in vitro (Tremel and Szinicz, 1984) and in vivo (Bunner et al., 1985) has been reported. We noted statistically significant reduction of intracellular LDH at 4 ng/ml 10 min after exposure to T-2 toxin. This concentration is well within the range that could occur in vivo. Its rapid onset also suggests a cause other than protein synthesis inhibition.

In conclusion, cell membrane effects of T-2 toxin occur at pg/ml concentrations within minutes of exposure in L-6 myoblasts. The uptake of all substances tested requiring membrane transport was altered. The higher concentrations of T-2 (Table 1) caused substantial alterations in cell membrane functions. These effects appear to be independent of protein

synthesis effects. Reports noted above (Korpela and Tahti, 1986; Gyonggyossy-Issa et al., 1986; DeLoach and Mollenhauer, 1987; Rottem et al., 1984) document that T-2 changes membrane fluidity and has an affinity for the phospholipid bilayer. The possibility exists then that secondary changes in membrane integral proteins, such as transporters, may occur because of T-2's effect on cell membrane fluidity.

Our data support the necessity of studying membrane-toxin interactions to better understand the mechanisms of action of T-2 toxin. The described effects on cell membrane function may also be applicable to other cellular membranes, such as nuclear and mitochondrial, since alteration of mitochondrial function and DNA synthesis have been documented. The mechanism of action of T-2 in mammalian cell systems and in vivo will likely be explained by multiple effects on cellular and subcellular structures. More detailed knowledge of T-2's direct biochemical interaction with cell membranes, ribosomes, mitochondria, DNA, and intracellular membrane systems will be required in this regard.

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TABLE 1

HIGH CONCENTRATION EFFECTS OF T-2 TOXIN ON CELL MEMBRANE FUNCTION

T-2 Concentration	a. Percent Reduction in Uptake				
	Calcium	Rubidium	Glucose	Leucine	Thymidine
40 ng/ml	69 ± 0.25%	60 ± 0.21%	75 ± 0.32%	61 ± 0.23%	83 ± 0.14%
400 ng/ml	71 ± 0.22%	41 ± 0.16%	81 ± 0.13%	59 ± 0.19%	85 ± 0.06%
	b. Percent Reduction in Incorporation				
	Leucine		Tyrosine		Thymidine
40 ng/ml	95 ± 0.10%		88 ± 0.05%		98 ± 0.14%
400 ng/ml	97 ± 0.10%		89 ± 0.11%		97 ± 0.03%

Percent reduction (mean ± SEM) from control for parameter indicated after a 10 min exposure to 40 or 400 ng/ml of T-2 toxin. The pulse of radiolabel was during the last 5 min of toxin exposure.

FIGURES

FIG. 1. Uptake of calcium, glucose, and rubidium in L-6 myoblasts. Percent change from control (mean $\pm$ SEM) plotted versus concentration of T-2 toxin in ng/ml. Toxin exposure time was 10 min. Standard error bars only shown if greater than 1%. Indicates $P < .001$ for significant difference between control and test concentration results.

FIG. 2. Uptake and incorporation of leucine and tyrosine in L-6 myoblasts. Percent change (mean $\pm$ SEM) from control plotted versus concentration of T-2 mycotoxin in ng/ml. Toxin exposure time was 10 min. Standard error bars only shown if greater than 1%. Indicates $P < .001$ for significant difference between control and test concentration results.

FIG. 3. Thymidine uptake and incorporation in L-6 myoblasts into DNA. Percent change (mean $\pm$ SEM) from control plotted versus concentration of T-2 mycotoxin in ng/ml. Toxin exposure time was 10 min. Standard error bars only shown if greater than 1%. Indicates $P < .001$ for significant difference between control and test concentration results.

FIG. 4. Calcium efflux from L-6 myoblasts. Percent change from control (mean $\pm$ SEM) at 1, 5, and 15 min after exposure to 40 ng/ml of T-2 mycotoxin. The radiolabel was added at time 0. L-6 cells were labeled with calcium-45 for 16 hr prior to toxin exposure. Standard error bars only shown if greater than 1%. Indicates $P < .001$ for significant difference between control and test concentration results.

FIG. 5. Residual cellular LDH in L-6 myoblasts after exposure to T-2 toxin. Percent change (mean $\pm$ SEM) from control plotted versus concentration of T-2 mycotoxin in ng/ml. Toxin exposure time was 10 min. Standard error bars only shown if greater than 1%. Indicates $P < .001$ for significant difference between control and test concentration results.









