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The Effects of T-2 Toxin on Alveolar Macrophage Function *in Vitro*

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T-2 toxin, a metabolite of several *Fusarium* species, is a mycotoxin of the trichothecene family which occurs in a variety of grains. Previous work in our laboratory showed that T-2 toxin is highly toxic to rat alveolar macrophages *in vitro* at submicromolar concentrations. The present investigation was undertaken to study the basis of the cytotoxic effects observed. The following parameters of macrophage function were measured: macromolecular synthesis, release of ^{51}Cr, cellular ATP, phagocytosis, and alveolar macrophage "activation." The incorporation of radiolabeled leucine into acid-precipitable molecules was significantly inhibited within 1 hr of treatment at sublethal concentrations, although amino acid uptake was unaffected. Cell volume and release of ^{51}Cr was unaffected by 0.1 μM T-2 toxin after 6 hr but evidence of significant leakage was seen after 18 hr treatment. The capacity of alveolar macrophages to phagocytize *Saccharomyces cerevisiae* and ^{3}H-*Staphylococcus aureus* was significantly reduced whereas binding of ^{3}H-*S. aureus* to the macrophage was not. Macrophage activation with endotoxin (*Escherichia coli* lipopolysaccharide) and mitogen-generated lymphokines, as monitored by incorporation of [^{14}C]glucosamine, was significantly altered at 0.01 μM T-2 toxin. Thus, the data clearly demonstrate that T-2 is toxic to alveolar macrophage function *in vitro* and suggest that the primary mechanism of this toxicity is related to the inhibition of protein synthesis.

INTRODUCTION

Several previous studies have shown that exposure to grain dust can lead to various respiratory disorders (Schilling, 1980; DoPico *et al.*, 1980), yet much remains to be learned of the role of various grain dust components in pulmonary disease. Grain dust is a heterogeneous mixture which is often contaminated by silica, fungi and their metabolites, bacterial endotoxins, insects, mites, mammalian debris, and various chemical additives such as pesticides and herbicides. Mycotoxin contamination of various grain products has been well documented (Hsu *et al.*, 1972; Morooka *et al.*, 1972; Lee and Chu, 1981) and recently aflatoxin has been shown to occur in the respiratory fraction of airborne corn dust (Sorenson *et al.*, 1981; Burg *et al.*, 1982).

T-2 toxin is a product of several *Fusarium* species and is widely distributed in nature. *Fusarium* species are common contaminants of such field crops as corn, wheat, and oats (Ueno, 1976; Lee and Chu, 1981). T-2 toxin is a member of the trichothecene family of mycotoxins, which are known to be cytostatic to a variety

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of mammalian cell types (Morel-Chany *et al.*, 1980; Oldham *et al.*, 1980), to strongly inhibit protein and DNA synthesis in eucaryotic cells (Ueno, 1977), and to cause immunosuppressant effects in rats (Rosenstein *et al.*, 1979; Lafarge-Frayssinet *et al.*, 1979). In a previous study we have shown that T-2 toxin is highly toxic to rat alveolar macrophages *in vitro* (Gerberick and Sorenson, 1983). Submicromolar concentrations of T-2 toxin caused loss of viability, reduction of cell volume, release of ^{51}Cr from the cells, and characteristic microscopic changes. The purpose of the present report is to elucidate the basis of the cytotoxic effects of T-2 toxin on cultured rat alveolar macrophages. Pulmonary macrophages perform several important functions in the lung. These functions include phagocytosis of living and nonliving foreign particles, regulation of T-lymphocyte proliferation, provision of T-helper activity for antibody production, and production of mediators of cellular immunity. Thus, cytotoxic damage to alveolar macrophages could lead to serious pulmonary and/or systemic damage.

MATERIALS AND METHODS

T-2 toxin preparation. The mycotoxin T-2 (3-hydroxy-4,15-diacetoxy-8-[3-methyl-butyryloxy]-12,13-epoxy-Δ^9-trichothecene) was dissolved in 100% dimethylsulfoxide (DMSO, Pierce Chem. Co., Rockford, Ill.) at a concentration of 10 mM and stored at −20°C. Substocks were prepared by performing 10-fold dilutions in 10% DMSO. Samples were examined by thin-layer chromatography after extended storage to assure that the stability of T-2 toxin was maintained (Gilman *et al.*, 1966). The final concentration of DMSO in all experimental cultures, including controls, was 0.1%. T-2 toxin was purchased from Calbiochem, La Jolla, California.

Alveolar macrophage isolation and culture. Alveolar macrophages were harvested from male Long–Evans Hooded rats by tracheal lavage according to the method of Myrvik *et al.* (1961). Rats were anesthetized by intraperitoneal injection with sodium pentobarbital and exsanguinated by cutting the abdominal aorta. The lungs from each rat were lavaged with a total of 60 ml of prewarmed Hanks' calcium–magnesium-free balanced salt solution (HBSS). The cells from several animals were pooled, centrifuged at 50*g* for 10 min, and washed with phosphate-buffered saline (PBS). Supplements added to the medium included; 2% heat-inactivated fetal bovine serum (HI-FBS), penicillin (P), 100 units/ml; streptomycin (S), 100 μg/ml; and heparin (H), 200 units/ml. Medium and supplements were obtained from Grand Island Biological Company, Grand Island, New York. A Coulter counter (Coulter Instrument Co., Hialeah, Fla.) was employed to determine the cell concentration. Cell purity, as determined by cell volume distribution, was approximately 95%.

Alveolar macrophage (AM) suspensions were cultured at a cell concentration of 2.5×10^5 cells/cm^2 in either 35-mm tissue-culture plates (Falcon, Oxford, Calif.), or Linbro tissue-culture plates (Linbro, Hamden, Co.). AM monolayers, in Medium 199 (2% HI-PBS, H, PS), were incubated for 2 hr at 37°C in 5% CO_2 to allow for adherence of AM, rinsed with PBS to remove nonadherent cells, and

incubated with Medium 199 (10% HI-FBS, PS) until needed for experiments. The viability of the cultured AM was routinely greater than 95% as determined by trypan blue exclusion (Phillips, 1973). Also, greater than 98% of the cultured AM were esterase positive, suggesting that the culture cells are a homogeneous population of AM.

Macromolecular synthesis. For protein-synthesis studies AM monolayers were incubated in Hanks' balanced salt solution containing vitamins (Gibco), antibiotics (Gibco), glutamine (Calbiochem), and 19 amino acids (Mann Research Laboratories, New York, NY). Leucine was omitted from the tissue-culture medium. The AM monolayers were incubated with T-2 toxin and 1 μCi/ml [^{3}H]leucine (110 Ci/mmole; New England Nuclear, Boston, Mass.) at 37°C in 5% CO_2 for up to 6 hr (Varesio and Eva, 1980). After the desired incubation time the cultured medium was discarded and the cells were washed twice with PBS. The monolayers were solubilized with 0.5 ml of 7 M guanidine–HCl (Fisher Chemical Co., Pittsburgh, Pa.), and acid precipitated with 2.0 ml of 10% trichloacetic acid (TCA) and 150 μl of 1% bovine serum albumin (BSA). The acid-precipitable material was collected on glass-fiber filters, washed with cold TCA, and placed in scintillation vials with 10 ml Omnifluor (New England Nuclear). The radioactivity of the precipitates was counted in a liquid scintillation counter.

For DNA-synthesis studies (Tanaka *et al.*, 1980), cells were treated as described above except that the cells were incubated with medium 199 (10% HI-FBS, PS) containing 2 μCi/ml of [^{3}H]thymidine (20 Ci/mmole; New England Nuclear). For RNA-synthesis studies the treated and untreated AM cultures were incubated with 2 μCi/ml [^{3}H]uridine (29.3 Ci/mmole; New England Nuclear). After the desired incubation time the monolayers were rinsed with PBS containing 10 μM cold uridine and then solubilized for 20 min with 0.25 ml of 0.1 M KOH. The solubilized suspension was added to Omniflour and counted with a scintillation counter.

Determination of cell volume distribution. AM monolayers in Linbro tissue-culture plates were incubated with various concentrations of T-2 toxin at 37°C in 5% CO_2 for 6 hr. Following incubation the cultured medium was aspirated and 250 μl of 0.25% trypsin–EDTA (Gibco) containing 0.06 mg/ml DNase (Sigma) was added to each culture for 5 min at 37°C. Each well was carefully scraped with a plastic policeman. The cell suspension was harvested and added to 10 ml of Isoton. Cell volume determinations for both control and T-2 toxin-treated cultures were made using a Coulter counter with channelizer and are the results of three experiments each done in triplicate. To determine the mean cell volume (MCV) values the following equation was employed: MCV = (channel No. + base channel threshold)/(threshold factor) (Castranova *et al.*, 1979). By assigning an arbitrary distribution for normal macrophage volume of 600–2500 μm^3 the percentage of both control and T-2 toxin-treated AM which fell into this assigned normal volume distribution (NVD) was determined.

Chromium-release assay. Freshly isolated AM at a cell concentration of 1.0×10^7 cells/ml were incubated with labeled sodium chromate (New England Nuclear) at a 100 $\mu Ci/10^7$ cell ratio in Medium 199 (10% HI-FBS, PS) for 45 min at 37°C in 5% CO_2. The specific activity of the sodium chromate was 296.3 μCi/mg.

Labeled AM were washed three times with HBSS and resuspended in Medium 199 at a cell concentration of 2.5×10^6 cells/ml. Next, 0.1 ml of the labeled cell suspension was added to a series of test tubes along with 0.1 ml of Medium 199 containing the appropriate concentration of T-2 toxin or medium only. Control and treated AM were incubated for 6 hr at 37°C in 5% CO_2 on a rotary shaker. Total release of chromium from AM was obtained using 0.1% Triton X-100. After an appropriate incubation period the cells were pelleted by centrifugation and 0.1 ml of the supernatant counted in a γ-scintillation counter. The percentage chromium release equals the experimental release value divided by the total release value. Both values were corrected for spontaneous release.

Adenosine triphosphate determination. ATP levels in treated and untreated monolayer cultures were determined by the luciferin–luciferase assay. At the time of treatment, the medium was removed by aspiration and replaced with fresh medium containing T-2 toxin. Sodium iodoacetate was used as a positive control. After treatment, the medium was removed by aspiration, the cells were washed with PBS, and the cells were removed from the plates by trypsinization (150 μl of 0.25% trypsin for 5 min) and by gentle scraping with a plastic policeman. ATP was extracted from the cells with releasing reagent (Diagnostic Sciences, Inc., San Diego, Calif.) according to the recommendations of the manufacturer. Buffered firefly lantern extract (Sigma FLE-50, Sigma Chemical Co., St. Louis, Mo.) was rehydrated, stored overnight at 4°C, and used as a source of luciferin–luciferase. ATP determinations were done in a darkened laboratory with an ATP photometer (SAI Technology Co., San Diego, Calif.) using dark-adapted scintillation vials to avoid interference due to chemiluminescence. Quantitation was done by the method of internal standardization.

Phagocytosis of Saccharomyces cerevisiae. AM monolayers prepared on glass cover slips in Leighton tissue-culture tubes (Bellco Glass Inc., Vineland, N.J.) were incubated with 0.01 and 0.05 μM T-2 toxin for 20 hr. After incubation the monolayers were rinsed with PBS and exposed to opsonized *Saccharomyces cerevisiae* (Simpson *et al.*, 1979). The yeast was opsonized by incubation in 60% FBS (diluted in HBSS) for 30 min at 37°C on a rotary shaker. The yeast preparation was added to the macrophage monolayers at a 10:1 yeast to AM ratio and incubated with the test mixture containing the opsonized *S. cerevisiae* for 30 min at 37°C in 5% CO_2. The final serum concentration was 20%. Following incubation, the AM monolayers were rinsed twice with PBS, fixed with methanol, and stained with Wright's stain for microscopic examination.

At least 150 cells were counted for each culture tested. The parameters measured were phagocytic capacity and phagocytic activity. Phagocytic capacity equals the total number of yeast cells phagocytized per the total number of macrophages. The phagocytic activity equals the number of macrophages containing two or more yeast cells per macrophage.

Phagocytosis of Staphylococcus aureus. AM monolayers were prepared in 20 $\times$ 65-mm flat-bottomed glass vials at a concentration of 3.0×10^5 cells/cm^2 in Medium 199 (10% HI-FBS, PS). The monolayers were incubated for 48 hr at 37°C in 5% CO_2. The medium was aspirated and 1 ml Medium 199 (10% HI-FBS, PS) containing T-2 concentrations of 0.01 and 0.1 μM was added to the appropriate

vials. The control and T-2-treated cultures were incubated for 6 hr. The cultured medium was aspirated and the cultures assayed for their ability to phagocytize *Staphylococcus aureus* (Cowan strain) labeled with [^{3}H]thymidine (20 Ci/mmole; New England Nuclear) and then killed by heating for 15 min at 80°C. The ^{3}H-*S. aureus* had a specific activity of 3.5×10^{-4} cpm/*S. aureus* cell. The ^{3}H-*S. aureus* was first opsonized by incubating the bacteria in guinea pig complement (Gibco) for 30 min at 37°C (Murphey *et al.*, 1979). After opsonization the ^{3}H-*S. aureus* was added to AM monolayers at a 100:1 bacteria to AM ratio. The culture tubes were centrifuged for 5 min at 1800*g* to layer the bacteria on the monolayer and then incubated for 1 hr. The AM monolayers were rinsed with PBS and solubilized for 10 min with 300 μl of 0.1% Triton X-100. The solubilized suspension was collected and counted in a liquid scintillation counter. To assay AM binding of the ^{3}H-*S. aureus* the macrophages were first preincubated with 100 μg/ml cytochalasin B (Sigma) for 15 min to inhibit phagocytosis (Malavista *et al.*, 1971).

Activation of alveolar macrophage by lipopolysaccharide. AM monolayers were prepared as described above and incubated in 1 ml Medium 199 (10% HI-FBS, PS) containing 10 μg/ml lipopolysaccharide (LPS) W *Escherichia coli* 0111:B4 (Difco) for 15 hr at 37°C in 5% CO_2 (Wilton *et al.*, 1975). Control cultures received no LPS. After 15 hr incubation the medium was aspirated; 1 ml Medium 199 (10% Hi-FBS, PS) containing 10 μg/ml LPS, 1.0 μCi/ml [^{14}C]glucosamine (250 mCi/mmol, New England Nuclear), and various T-2 toxin concentrations was added to each well and incubated for 6 hours. The monolayers were rinsed with PBS, solubilized with 250 μl of 3% Triton X-100 for 20 min, and counted in a liquid scintillation counter. The stimulation index (SI) equals the cpm from experimental cultures divided by cpm from control cultures.

Lymphokine activation of alveolar macrophages. AM activation with lymphokines was assayed as has been described for LPS activation. Lymphokines were prepared from spleen cells removed aseptically from male LEH rats. The spleens were teased apart in HBSS and sieved through a nylon mesh. The spleen cells were layered onto Ficoll–Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden), centrifuged at 200*g* for 10 min, and the interface cells collected. The cells were placed in RPMI medium (RPMI 1640 containing 2% HI-FBS, glutamine, and Hepes (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid) and counted, and the cell suspension was adjusted to 1.0×10^7 cells/ml. The lymphocyte preparation was incubated at 37°C in 5% CO_2 for 48 hr in Falcon 25-cm^2 flasks with a total volume of 5 ml containing either 50 or 100 μg PHA (Pharmacia)/ml. Control lymphocyte cultures received no mitogen. The reconstitution controls were prepared by adding the same concentration of the mitogens to the control media at the conclusion of the incubation period. After incubation the supernatant media were centrifuged at 200*g* for 10 min, dialyzed against saline (0.9% NaCl), and concentrated five fold by ultrafiltration using an AM 10 membrane (Amicon, Danvers, Mass.). The crude lymphokine preparations were passed through a 0.45-μm filter (Gelman Inc., Ann Arbor, Mich.) and stored at −70°C. For AM stimulation 10 μl of the crude lymphokine preparation was added to each well of the AM culture plate, and [^{14}C]glucosamine uptake was monitored as described above.

RESULTS

Figure 1 illustrates the effect of T-2 toxin on synthesis of protein (Fig. 1A), DNA (Fig. 1B), and RNA (Fig. 1C) in cultured rat AM. The results indicate that control cultures incorporated labeled precursors at a nearly linear rate whereas incorporation of label into macromolecules was reduced in the presence of T-2 toxin. The inhibitory effect was most remarkable with respect to protein synthesis. For example, leucine incorporation ceased immediately in cultures containing 0.1 μM T-2 toxin, and terminated after 2 hr incubation in cultures containing 0.01 μM T-2. On the other hand, thymidine incorporation was reduced but not totally inhibited at 0.01 μM T-2 toxin and terminated after 2 hr incubation at 0.1 μM. RNA synthesis was affected the least and was terminated after 4 hr at 0.1 μM T-2 toxin.

Cell volume and chromium release determinations in AM cultures after 6 hr of incubation in the presence or absence of T-2 toxin are shown in Table 1. Treatment with 0.001 and 0.01 μM T-2 toxin produced no appreciable change in their mean cell volume (MCV) values after 6 hr, although a trend toward decreasing MCV was evident with increasing concentration of T-2 toxin. The percentage of treated macrophages falling into the normal volume distribution (NVD) (Fig. 2) was not significantly different from control cells after 6 hr of incubation at T-2 toxin concentrations of 0.001–0.1 μM. The assigned NVD is 600–2500 μm^3. Thus, 91.8% of the control cells fell into the assigned NVC (Table 1). When cultures were treated with 1 and 10 μM T-2 toxin, the percentage NVD values were significantly different after 6 hr of incubation. The percentage NVD values of these cultures were 85.8 and 82.9, respectively.

Incubation of AM with 0.001–1.0 μM T-2 toxin for 6 hr showed no significant differences in the percentage of chromium released from the labeled AM. However, cultures containing 10 μM T-2 toxin demonstrated release of a significant amount of ^{51}Cr from the AM as compared to control cultures.

To determine whether T-2 toxin inhibits ATP production, ATP measurements were made in treated and untreated AM cultures. The results are summarized in Table 2. Sodium iodoacetate (positive control) produced approximately a 90% decrease in ATP as compared to untreated cultures. On the other hand, the ATP levels in cultures treated with T-2 toxin showed a slight but not significant ($P < 0.05$) elevation in ATP.

The effect of T-2 toxin on phagocytosis of serum-opsonized yeast cells is shown in Table 3. T-2 toxin concentrations of 0.01 and 0.05 μM inhibited the phagocytic capacity to 76.9 and 16.8% of controls, respectively. Phagocytic activity was also affected and AM cultures containing 0.01 and 0.05 μM T-2 toxin had phagocytic activity values of 47.9 and 7.8%, respectively, whereas 63.3% of control macrophages contained two or more yeast cells.

The results of phagocytosis studies with ^{3}H-*S. aureus* indicate that the phagocytic activity was significantly inhibited at 0.1 μM T-2 toxin but not at 0.01 μM (Table 4). In two identical experiments the phagocytic activity of AM cultures treated with 0.1 μM T-2 toxin was reduced to 83.5 and 82.1% of control cultures. When AM were preincubated with cytochalasin B to inhibit phagocytosis, but

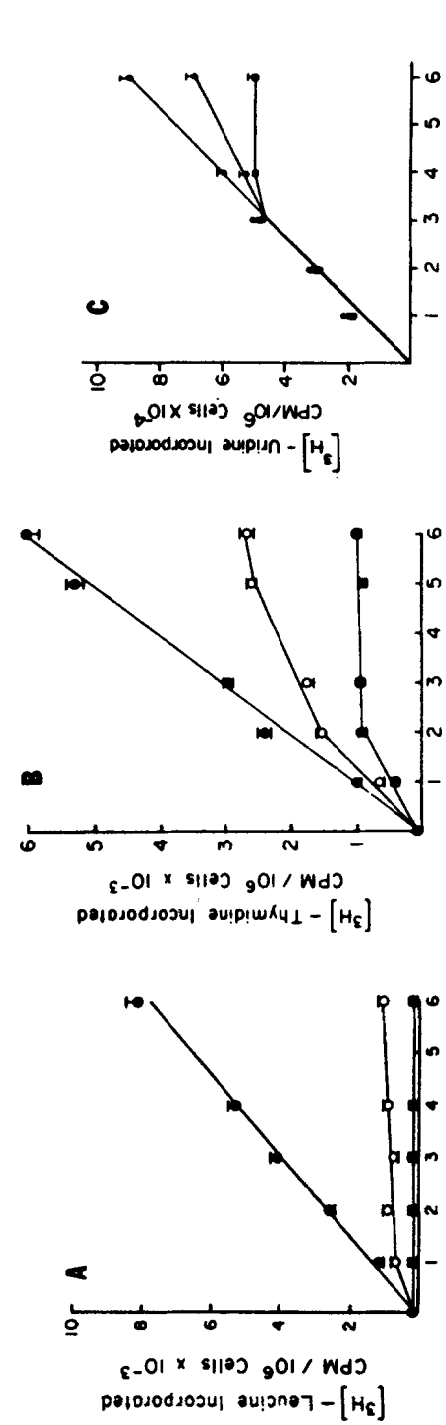

FIG. 1. Effect of T-2 toxin on synthesis of protein (A), DNA (B), and RNA (C) by rat alveolar macrophages *in vitro*. The amount of ^{3}H-precursor incorporated into macrophage macromolecules was monitored for 6 hr. The results are expressed as the mean cpm for quadruplicate cultures (± standard deviation). Symbols are controls (●), 0.01 μM T-2 toxin (○), and 0.1 μM T-2 toxin (■).

TABLE 1

CELL VOLUME AND ^{51}Cr RELEASE CHANGES FOLLOWING 6 hr INCUBATION OF ALVEOLAR MACROPHAGES WITH T-2 TOXIN

Treatment (μM T-2)	Cell volume		^{51}Cr release	
	Cell volume MCV (μm^3)[a]	NVD (%)[b]	Cpm	%[c]
Control	1278.4 (±13.3)[d]	91.8 (±16.6)	1986.3 (±168.5)[d]	—
0.001	1281.7 (±21.2)	92.4 (±1.06)	2252.0 (±201.1)	0.95
0.01	1279.5 (±50.6)	91.4 (±1.44)	2280.1 (±404.0)	1.05
0.1	1264.6 (±23.8)	88.0 (±0.93)	2390.6 (±291.6)	1.40
1.0	1259.0 (±21.0)	85.8 (±0.83)[e]	2783.1 (±460.1)	2.85
10.0	1244.1 (±19.9)	82.9 (±1.73)[e]	2813.9 (±293.3)	3.00[e]

[a] Mean cell volume in cubic micrometers.

[b] Normal volume distribution, percent of AM falling in NVD of 600–2500 μm^3.

[c] Total release equals 29860.0 (±1096) cpm.

[d] Values shown represent the mean ± standard deviation.

[e] Values are significantly different from controls, $P < 0.005$.

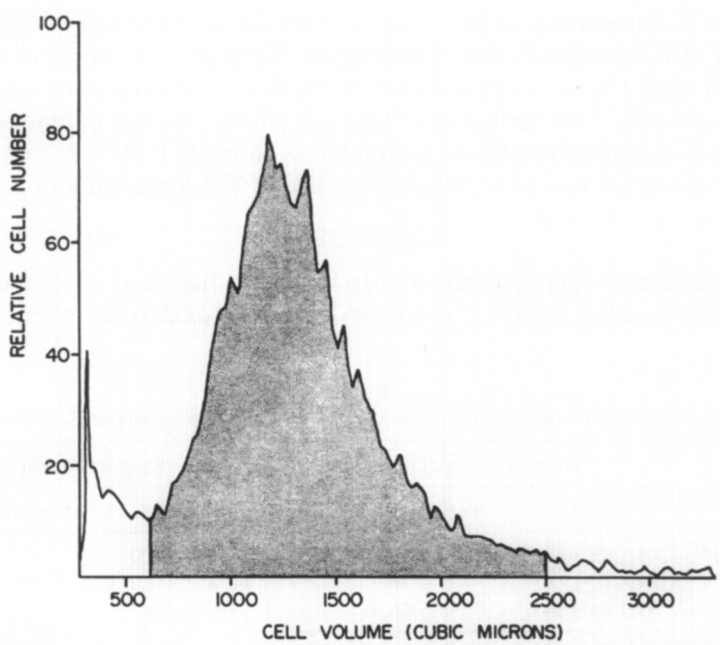

FIG. 2. Distribution of cell volumes for alveolar macrophages obtained with a Coulter Model Z_B electronic cell sizing unit. The shaded area represents the assigned normal volume distribution (NVD) of 600–2500 μm^3. The mean cell volume (±standard deviation) of control AM from nine experiments was 1278.4 ± 13.3 μm^3.

TABLE 2
EFFECT OF T-2 TOXIN AND SODIUM IODOACETATE ON ATP LEVELS IN RAT ALVEOLAR MACROPHAGE CULTURES[a]

Treatment	Concentration	Percentage control
Untreated, 6 hr	—	100.0
Sodium iodoacetate, 1 hr	0.3 mM	9.2
T-2 toxin, 6 hr	0.1 μM	116.6[b]
T-2 toxin, 6 hr	0.01 μM	107.0[b]

[a] Result of three experiments performed in triplicate.
[b] Not significantly different from untreated cultures ($P < 0.05$).

not binding of the ^{3}H-*S. aureus*, the results indicated that 0.01 and 0.1 μM T-2 toxin did not inhibit binding of the bacteria (data not shown).

The effect of T-2 toxin on AM macrophage activation (assessed by [^{14}C]glucosamine uptake) was determined by incubating AM with either LPS (10 μg/ml) or lymphokines generated by mitogen-stimulated rat lymphocytes. As shown in Table 5, LPS markedly enhanced [^{14}C]glucosamine uptake (stimulation index [SI] = 10.3). However, 0.01 or 0.1 μM toxin caused significant inhibition of [^{14}C]glucosamine uptake in LPS-stimulated cultures. Similarly, lymphokines derived from phytohemagglutinin (PHA)-stimulated rat lymphocyte cultures promoted [^{14}C]glucosamine uptake (SI = 22.6 for PHA-derived lymphokine). The addition of T-2 toxin at 0.01 or 0.1 μM concentrations inhibited [^{14}C]glucosamine uptake by AM stimulated with lymphokines (Table 6). The identical experiment, performed with Con-A-stimulated rat lymphocyte cultures gave similar results (data not shown). The uptake of [^{14}C]glucosamine was not completely blocked by T-2 toxin in either the LPS- or lymphokine-stimulated AM cultures, suggesting that AM activation was underway at the time of T-2 toxin addition.

DISCUSSION

The objective of this research was to elucidate the basis of AM cytotoxicity caused by T-2 toxin. Since it is known that T-2 toxin is a potent inhibitor of

TABLE 3
EFFECT OF T-2 TOXIN ON ALVEOLAR MACROPHAGE PHAGOCYTOSIS OF *S. cerevisiae*[a]

Treatment (μM T-2)	Phagocytic capacity[b]		Phagocytic activity[c]	
	Mean	Percentage control	Mean	Percentage control
Control	245.1 (±10.1)[d]	—	63.3 (±3.23)	—
0.01	184.4 (±15.4)[e]	76.9	47.9 (±1.13)[e]	75.7
0.05	41.1 (±4.24)[e]	16.8	7.8 (±3.61)[e]	12.4

[a] Macrophages were treated 20 hr with T-2 toxin.
[b] Phagocytic capacity equals the total number of intracellular yeast cells per the total number of macrophages.
[c] Phagocytic activity equals the percentage of macrophages containing two or more yeast cells.
[d] Values shown represent the means ± standard deviation.
[e] Values are significantly different from the controls, $P < 0.005$.

TABLE 4
EFFECT OF T-2 TOXIN ON PHAGOCYTOSIS OF ^{3}H-*Staphylococcus aureus* BY ALVEOLAR MACROPHAGES[a]

Concentration (μM T-2)	Experiment No. 1		Experiment No. 2	
	Cpm	Percentage of control	Cpm	Percentage of control
Control	1643.8 (±214.1)[b]	—	1868.1 (±216.4)	—
10^{-2}	1635.1 (±101.7)	99.5	1846.6 (±121.5)	98.8
10^{-1}	1367.4 (±232.8)[c]	83.5	1533.3 (±79.7)[c]	82.1

[a] Macrophages were treated 6 hr with T-2 toxin.
[b] The values shown represent the means ± standard deviation (cpm/3.0 × 10^5 cells).
[c] Values are significantly different from controls, $P < 0.05$.

protein synthesis (Smith *et al.*, 1975), we first investigated the effects of T-2 toxin on AM protein and other macromolecular synthesis. T-2 toxin at a 0.1 μM concentration caused immediate cessation of protein synthesis in rat AM, whereas synthesis of DNA and RNA did not cease until 2 and 4 hr of incubation, respectively. In addition, protein synthesis was completely inhibited after 2 hr with 0.01 μM T-2 toxin. At this concentration, DNA and RNA synthesis were reduced but not terminated. Thus, inhibition of protein synthesis occurred at an earlier time and with lower concentrations of T-2 than DNA or RNA. The inhibition of DNA and RNA synthesis observed may be secondary to the initial inhibition of protein synthesis and may be caused by the depletion of specific protein(s) required for DNA and RNA synthesis.

Measurements of ATP levels in treated cells suggest that the inhibition of protein synthesis observed cannot be explained by depletion of ATP levels in the cells. Although there was a 90% decrease in ATP levels in the positive control (sodium iodoacetate), there was no decrease in cultures treated with T-2 toxin. In fact, the results suggest a concentration-dependent increase in ATP levels in

TABLE 5
INHIBITION OF ALVEOLAR MACROPHAGE ACTIVATION BY T-2 TOXIN[a]

Cultures (μM T-2)	[^{14}C]Glucosamine incorporation	
	Cpm/culture	Stimulation index
Negative control[b]	131.4 (±12.4)[c]	—
LPS control	1347.0 (±101.8)	10.3
10^{-3}	1321.4 (±97.7)	10.1
10^{-2}	737.0 (±82.3)[d]	5.6
10^{-1}	720.2 (±78.4)[d]	5.5

[a] Alveolar macrophage cultures were activated with *E. coli* lipopolysaccharide (10 μg/ml) for 15 hr and then treated for 6 hr with T-2 toxin.
[b] Negative control cultures received no lipopolysaccharide.
[c] Mean counts per minute (cpm) of eight cultures ± standard deviation.
[d] Values are significantly different from the controls, $P < 0.001$.

TABLE 6
T-2 TOXIN INHIBITION OF ALVEOLAR MACROPHAGE ACTIVATION BY PHA-GENERATED LYMPHOKINES

Treatment[a]	[^{14}C]Glucosamine incorporation	
	Cpm	SI[b]
Negative control	70.8 (±15.7)[c]	—
10 μg/ml LPS	1686.8 (±166.3)	23.8
Control supernatant[d]	112.1 (±8.63)	1.6
Reconstitution control[e]	135.4 (±10.4)	1.9
50 μg/ml PHA[f]	1308.9 (±108.2)	18.5
50 μg/ml + 10^{-2} μM T-2	688.5 (±69.9)[g]	9.7
50 μg/ml + 10^{-1} μM T-2	684.6 (±20.4)[g]	9.7
100 μg/ml PHA[f]	1559.0 (±87.4)	22.6
100 μg/ml + 10^{-2} μM T-2	473.9 (±20.0)[g]	6.7
100 μg/ml + 10^{-1} μM T-2	468.5 (±89.2)[g]	6.6

[a] Macrophages were stimulated for 15 hr with lymphokines and then treated 6 hr with T-2 toxin.
[b] Stimulation index.
[c] Mean values ± standard deviation.
[d] Supernatant from mitogen-free lymphocytes; no PHA added.
[e] PHA was added to supernates of control lymphocyte cultures at the conclusion of the incubation period to control for the carryover of the mitogens into macrophage culture.
[f] Supernatant from lymphocytes incubated with 50 or 100 μg/ml PHA. 10 μl of the lymphokine preparation was added to the macrophage culture.
[g] Values are significantly different from controls at $P < 0.005$.

T-2-treated cultures (Table 2). Since macromolecular synthesis is a major consumer of ATP in the cell, it is possible that ATP levels could increase slightly if protein, DNA, and RNA synthesis were blocked. The levels of T-2 toxin employed in these experiments were previously shown to significantly inhibit macromolecular synthesis (Fig. 1) without killing the cells (Gerberick and Sorenson, 1983).

One possible explanation for the observed decrease in leucine incorporation is depletion of intracellular amino acid pools. AM were exposed to labeled aminoisobutyric acid (α-AIB) at concentrations of T-2 toxin capable of inhibiting protein synthesis. α-AIB is an analog of leucine which may be transported across the cell membrane by the same carrier system as leucine, but is not incorporated into protein (Brown *et al.*, 1980). No change in labeled α-AIB uptake was observed even after complete cessation of protein synthesis had occurred (data not shown). Thus it is unlikely that inhibition of protein synthesis was the result of amino acid depletion.

Chromium release and cell volume studies were performed after 6 hr of incubation to study the effect of T-2 toxin on membrane integrity. At low concentrations known to inhibit protein, DNA, and RNA synthesis (Fig. 1), mean cell volume values were unchanged. At even higher concentrations (1 and 10 μM T-2 toxin) the cell volume values were still not significantly different than controls, but a decreasing trend in cell volume values was evident with increasing T-2 toxin concentrations. The NVD values of cultures treated with 1 and 10 μM T-2 toxin were significantly different than controls. Chromium release studies indicate that

nonlethal concentrations of T-2 toxin, which are capable of inhibiting AM protein synthesis, had no effect on the amount of chromium released during 6 hr of incubation. However, with 10 μM T-2 toxin a significant amount of chromium was released in comparison to control cultures. It is unlikely that membrane damage plays a significant role in cytotoxicity at low concentrations of T-2 toxin since sublethal concentrations of T-2 toxin significantly inhibited protein synthesis without a deleterious effect on AM membrane integrity as measured by the chromium release assay and the cell volume assay. However, cell volume and chromium release changes were evident after 6 hr of incubation with 10 μM T-2 toxin. The possible mode by which this high concentration of T-2 toxin is acting is unclear, but it could conceivably result from the inability of the cell to replace essential membrane proteins lost through normal attrition. Ueno *et al.* (1977) have suggested that T-2 toxin may be cytotoxic by interacting with SH-containing proteins and enzymes. Thus, high concentrations of T-2 toxin may cause cellular damage by affecting various cellular components needed to maintain the life of the cell.

T-2 toxin had a significant effect on phagocytosis of serum-opsonized *S. cerevisiae* (20 hr incubation) and *S. aureus* (6 hr incubation). Massaro *et al.* (1970) have shown that protein synthesis is not required during phagocytosis and, in fact, a depression of protein synthesis occurs during phagocytosis. It is possible, however, that T-2 toxin inhibits phagocytosis by inhibiting the synthesis of proteins needed for the phagocytosis process, but not the endocytosis process per se. Investigators have demonstrated that fibronectin is a protein required for enhancement of phagocytosis of gelatin-coated particles, gelatin-coated latex, and *S. aureus* (Dorean *et al.*, 1980; Gudewicz *et al.*, 1980). Investigators have suggested that AM are capable of synthesizing fibronectin, thus inhibition of fibronectin biosynthesis would cause an inhibitory effect on AM phagocytosis (Rennard *et al.*, 1981). Other proteins, e.g., actin and myosin, are essential for phagocytosis. Prevention of synthesis of such critical components could explain why AM incubated for 20 hr with T-2 toxin show striking inhibition, whereas AM incubated 6 hr show a significant but modest inhibition of phagocytosis. T-2 toxin had no effect on the ability of AM to bind the labeled *S. aureus* (data not shown).

The final aim of this research was to investigate the ability of T-2 toxin to inhibit AM activation by various known stimulants. The activated AM is a critical component of the immune response. For example, activated macrophages demonstrate an increased capacity for phagocytosis and increased production of various monokines involved in regulation of both T- and B-cell function (North, 1978). Thus, in the immune defense against foreign substances, activated macrophages play at least two important roles: they collaborate with lymphocytes in antigen recognition and serve as effector cells in antigen disposal. By means of ultrathin autoradiograph sections and cell fractination procedures, Hammond and Dvorak (1972) have observed that activated macrophages preferentially incorporate glucosamine into their cell membrane. Our results indicate that T-2 toxin significantly inhibited AM incorporation of labeled glucosamine (Tables 5 and 6). A possible reason that glucosamine incorporation is not totally inhibited may be that the macrophages contained some of the cellular components needed for the activation process at the time of the addition of T-2 toxin.

Macrophage activation was further investigated by stimulating the cells with mitogen-generated lymphokines in the presence and absence of T-2 toxin. Mitogen-induced lymphocytes produce a lymphokine called macrophage activating factor. Crude lymphokine preparations were prepared from rat spleen and placed in contact with AM for 15 hr. The macrophages were then incubated with T-2 toxin in the presence of labeled glucosamine to assay for macrophage activation. These results clearly demonstrate that macrophage activation is due to the lymphocyte mediators and not due to the mitogens. AM activation by LPS and mitogens, as assayed by glucosamine incorporation, is also significantly inhibited by T-2 toxin. By suppressing macrophage activation the cell becomes unable to function normally as an immunologically competent cell. Possible functions affected could include regulation of T-lymphocyte proliferation, provision of T-helper activity for antibody production, activation of phagocytosis, and production of mediators of cellular immunity. The means by which T-2 toxin inhibits AM activation is probably due to the ability of the toxin to inhibit protein synthesis. Obviously, activation would require an intact protein synthesis apparatus. Inhibition of macrophage monokines such as lymphocyte activating factor (Interleukin I), which are known to help regulate the immune response to foreign antigens, would be quite detrimental to the immunological state of an individual. Also, inhibition of AM phagocytosis would result in an individual becoming increasingly susceptible to opportunist infections. Since there is abundant evidence that macrophages in collaboration with T- and B-lymphocytes can destroy neoplastic cells *in vivo* (Adams and Snyderman, 1979; Levy and Wheelock, 1974), the possibility that exposure to T-2 toxin could lead to increased risk for cancer should be considered. In this context, Schoental *et al.* (1979) have reported induction of tumors of the digestive tract and the brain in rats given T-2 toxin by intragastric administration.

T-2 toxin may be an environmental hazard for agricultural workers exposed to mycotoxin-contaminated grain dust. It is also possible that grain workers and others might consume T-2-contaminated foodstuffs by ingestion. Alimentary toxic aleukia (ATA) was a major problem in the USSR during World War II (Joffe, 1978) and with the present worldwide economic depression ATA could become a problem again. We have demonstrated that submicromolar concentrations of T-2 toxin are cytotoxic for rat AM. Microscopic examination of AM treated with T-2 toxin demonstrated detachment of pseudopodia, cellular blebbing, smoothing of the membrane processes, and finally cell lysis. In this investigation we have demonstrated that the cytotoxicity observed is most probably due to the ability of the toxin to inhibit AM protein synthesis. By inhibiting AM protein synthesis T-2 toxin was shown to inhibit critical functional processes of macrophages such as phagocytosis and macrophage activation. Thus, inhalation of grain aerosols contaminated with T-2 toxin could have major deleterious effects on normal macrophage function.

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