Interstitial and Hemorrhagic Pneumonitis Induced by Mycobacterial Trehalose Dimycolate

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Intraperitoneal injection of cord factor (trehalose dimycolate, TDM) provides a model for interstitial and hemorrhagic lung disease that is produced by a chemically defined substance. A single injection of 10 μg of TDM, in light mineral oil or hexadecane, into C57BL/6 mice produces interstitial and hemorrhagic pneumonitis. Following the injection of TDM the pulmonary lesions increase gradually and become maximal by the seventh to ninth day, at which time 70% of the mice show both gross hemorrhages and dense mononuclear infiltrates; an additional 20% of the mice show only microscopic lesions. From day 14 onward the incidence and severity of the lesions decrease, and by day 28 the lungs are normal by both gross and light-microscopic examination. Only 5% of the mice succumb. Except for peritonitis other organs are not affected.

Doses of 3.3 and 10 μg of TDM are equally effective in producing the lesions, but a dose of 1.0 μg of TDM causes only mild interstitial inflammation and lesser doses do not induce lesions. A single subcutaneous injection of 10 μg of TDM causes lesions in only 20% of mice. Vehicle-injected mice do not develop lesions. Electron microscopy revealed that the majority of the infiltrating cells are monocytes and macrophages and that extensive interstitial damage is produced. The mechanism of the effects of TDM are unknown and is currently under study. Our preliminary data suggest that the phenomenon is dependent upon T-lymphocytes. (Am J Pathol 1982, 106:348–355)

CORD FACTOR (trehalose dimycolate, TDM) is one of the glycolipid constituents of the cell walls of several strains of bacteria, including *Mycobacteria*, *Nocardia*, and *Corynebacteria*.[1] In the early fifties Hubert Bloch[2,3] reported that repeated intraperitoneal injections of partially purified mycobacterial cord factor in light mineral oil into susceptible mice caused weight loss and death. At autopsy the lungs were grossly hemorrhagic. Single injections, either intraperitoneally or subcutaneously, were alleged to have no effect, but repeated subcutaneous injections of TDM produced hemorrhages. Confirmation of Bloch's experiments has not been reported during the ensuing years. However, several groups have reported production of pulmonary granulomas after intravenous administration of TDM into laboratory animals,[4–7] and there is a report the MER (methanol extractable residue of *Mycobacteria*), used in immunotherapy of malignant melanoma, was associated with the development of pulmonary granulomas in man.[8]

We describe herein our observations on the pathogenesis and histologic features of an interstitial and hemorrhagic pneumonitis produced with a single injection of TDM in C57BL/6 mice. Nearly all of the mice develop lesions within 1 week, and the lesions slowly subside over 28 days, at which time essentially all mice are healthy and have histologically normal lungs.

Materials and Methods

Mice

Male C57BL/6 mice of varying ages (4–12 weeks) were obtained from the National Jewish Hospital animal care facility and from the Jackson Labora-

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tory, Bar Harbor, Maine. Most experiments were conducted with mice between 8 and 12 weeks of age, because the preliminary studies showed that within this age range there were no age-dependent factors associated with TDM-induced lung injury.

Cord Factor

Pure cord factor (TDM) was prepared from *M tuberculosis peurois* by the late Dr. R. Toubiana as described by Noll.[9] Homogeneity of the bacterially derived TDM after purification was established by its behavior in thin-layer chromatography (TLC) and by its characteristic infrared spectrum.[10] The latter showed it to be entirely free of the usual principal contaminant, mycolic acid, and the pattern was in accord with that described earlier.[10] Synthetic pseudo-cord factors DOA-TDA (α-D-glucopyranuronosyl [1-1] α-D-glucopyranuronoside, bis-N-1-octadecylamide) and C-76 amide (6,6′-di-(corynemycoloylamino)-6,6′-dideoxy-α,α-trehalose) were synthesized as described earlier.[11,12] The identity and purity of the synthetic pseudo-cord factors were established by TLC, by infrared and nuclear magnetic resonance spectrometry, and by elemental analyses.[11,12]

Protocols

In most experiments 10 μg of TDM in 0.1 ml of vehicle were injected intraperitoneally. In the initial experiments the vehicle was light mineral oil (Bayol F or Marcol 52); later experiments used hexadecane (Eastman Kodak, Rochester, NY). The pseudo-cord factors were tested in doses of 10 and 50 μg in Marcol 52 and were also injected intraperitoneally. Control animals received 0.1 ml of the appropriate vehicle intraperitoneally.

We studied the evolution of the lesions by sacrificing mice at times ranging from 6 hours to 28 days after injection of cord factor. The lungs, livers, spleens, and in some experiments kidneys, were removed for gross and histologic examination.

Dose-Response Relationships of the Cord Factor-Induced Pulmonary Lesions

TDM in doses of 0.01–10 μg was dissolved in 0.1 ml of hexadecane and given intraperitoneally. The mice were sacrificed on Day 7.

Effect of the Vehicle

To further study the role of the vehicle, 10 μg of TDM was administered intraperitoneally in either 0.01, 0.03, or 0.10 ml of hexadecane. The mice were sacrificed, and the lungs were examined on Day 7.

We examined the effect of the route of administration in animals by injecting TDM either intraperitoneally or subcutaneously.

At least 10 mice were studied in every experiment except for Days 3 and 28 of the time course study; there were 5 mice in these groups.

Preparation of Tissues

Livers, spleens, and kidneys were cut into 1-mm strips, fixed in 10% buffered formalin, sectioned, and stained with hematoxylin and eosin. After careful removal from the animals, the lungs were inflated with 10% buffered formalin under a pressure of 25 cm H_2O. When the lungs were fully inflated, the trachea was tied and the inflated lungs were submerged in 10% formalin solution overnight. Horizontal sections 1–2 mm thick were taken from whole lung. Sections of all lobes were taken for embedding and sectioning.

All sections were stained with hematoxylin and eosin. In certain experiments the lungs were stained with Masson's trichrome stain for connective tissue or for iron.

Electron Microscopy of Mouse Lungs

After careful removal from the animals, the lungs from control and TDM-treated mice were inflated at a pressure of 25 cm H_2O with 1.5% glutaraldehyde in 0.1 M cacodylate buffer (330 mOsm) at pH 7.3. The trachea was tied, and the lungs were left in fixative solution overnight. The tissue was postfixed in 1.0% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.3, at 4 C for 1 hour. *En bloc* uranyl acetate staining was performed; then the tissues were dehydrated in alcohol, cleared in propylene oxide, and infiltrated with Embed 812 resin overnight. The tissues were cured for 3 days at 70 C. Sections were cut on an LKB Ultratome III and stained with 2% aqueous uranyl acetate and Reynold's lead stain. The sections were examined with a Philips 201 electron microscope at an accelerating voltage of 60 kv.

At least 3 sections from each lung of 5 mice were examined for each time point. Infiltrating cells were identified by their ultrastructural criteria, as described by Cline.[13] Briefly, monocytes were characterized by a centrally located nucleus, often seen in horseshoe shape, with fine lacy nuclear chromatin. Generally, they had more cytoplasm than lymphocytes and very few vacuoles in cytoplasm. There were no secondary lysosomes. Macrophages were much

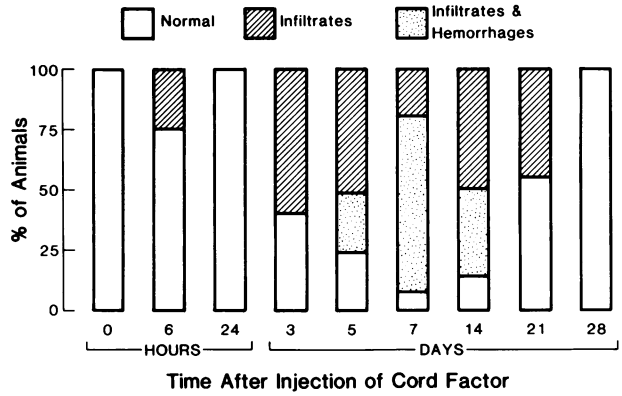

Figure 1 — Time course of the evolution of lesions in C57BL/6 mice given injections of 10 μg TDM in hexadecane. Note that the percentage of mice as well as the severity of the lesions increases to a maximum by Day 7. From Day 14 onward both incidence and severity of lesions decrease, so that by Day 28 all mice are normal. The bar for Day 7 includes data from 80 mice sacrificed on Days 7 and 9 combined. Other time points include data from 5-17 mice.

larger cells with abundant cytoplasm, which contained many granules and inclusions. The centrosomal region contained many dense bodies. They have a prominent Golgi apparatus and numerous mitochondria. There were many cytoplasmic digestive vacuoles and moderately abundant cisternae with rough endoplasmic reticulum. Cells identified as lymphocytes had high nuclear to cytoplasmic ratios, small numbers of mitochondria, and only occasional vesicles. There was no well-defined endoplasmic reticulum and only isolated ribosomes.

Scoring

Gross pulmonary changes were scored from 0 (normal-appearing lungs) to 4+ (confluent hemorrhages). A score of 1+ was given to multiple tiny macroscopic hemorrhages and 3+ to rather large hemorrhages, which were interspersed with normal-appearing areas. Lesions of intermediate severity were scored 2+. Microscopic changes were also graded with an arbitrary scale ranging from 0 (normal) to 4+ (marked increase in interstitial infiltrates, granuloma formation and hemorrhages). A questionable infiltrate (1+) was considered negative and in most instances was assigned to poorly inflated lungs without obvious pathologic features. Slight but definite focal mononuclear cell infiltrates, at times associated with thickening of alveolar walls and intraalveolar macrophages, were given a score of 2+. Changes scored 3+ consisted of dense focal or diffuse mononuclear cell infiltrates with or without granuloma formation, but without hemorrhages. The tissues were scored independently by two observers, and in every experiment at least one observer was unaware of the protocol employed or the treatment given to the mice.

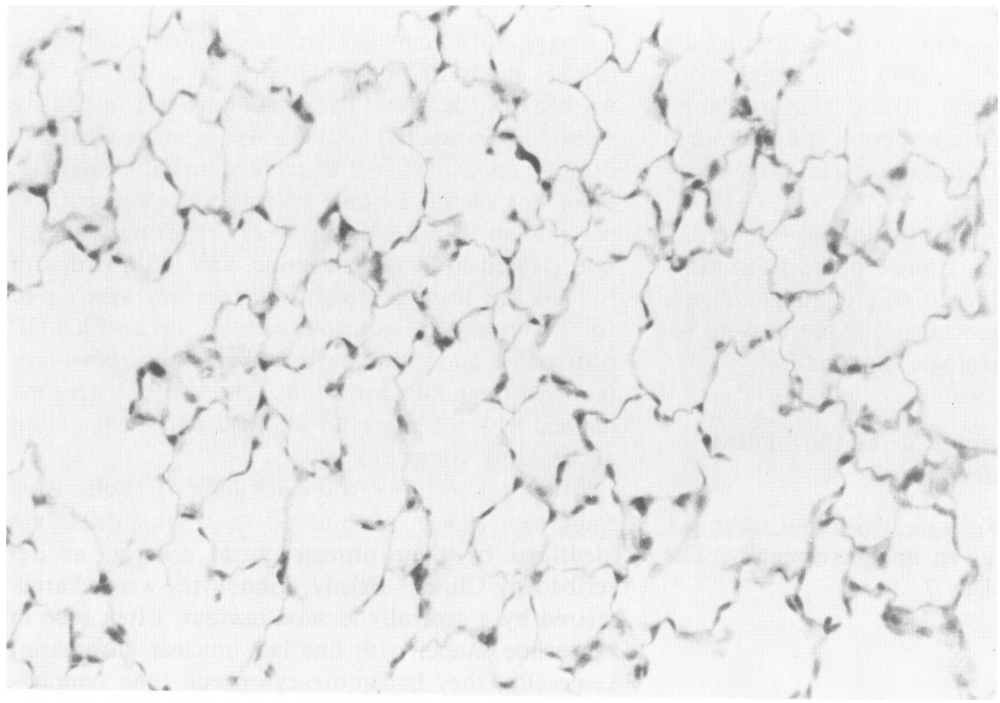

Figure 2 — Normal mouse lung. Note the thin alveolar walls, sparse cellularity, and absence of intraalveolar macrophages. (H&E, ×250)

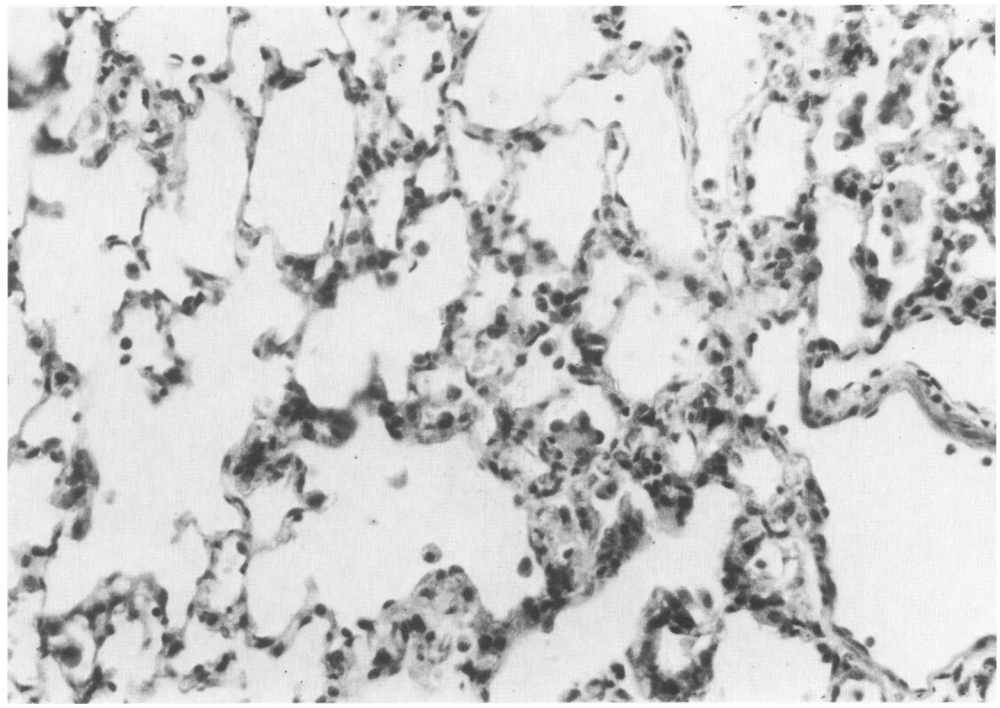

Figure 3—Mouse lung 5 days after intraperitoneal injection of TDM. The alveolar walls are thickened, and there is a dense mononuclear infiltrate. Many intra-alveolar macrophages are also present. These lesions were scored as 3+. (H&E, ×250)

Results

Time Course of Pulmonary Lesions Produced by Intraperitoneal Injection of TDM

The natural history of the pulmonary lesions is summarized in Figure 1. Within 6 hours of intraperitoneal injection of 10 μg of TDM in mineral oil or hexadecane, 25% of mice showed mild mononuclear cell infiltrates, which subsided by 24 hours. By Day 3, only 40% of the mice had normal lungs (Figure 2); whereas 60% of the animals had interstitial infiltrates with mononuclear cells (scored 2–3+). The lesions became even more striking by Day 5, at which time 52% of the animals showed extensive interstitial infiltrations (2–3+) (Figure 3), and an additional 25% showed infiltrates with intra-alveolar hemorrhages (4+). By Days 7 and 9 70% of mice showed hemorrhages and interstitial infiltrations (4+) (Figure 4), another 20% had interstitial infiltrations (2–3+) alone, and only 8% had normal-appearing lungs. In some fields the infiltrations had granuloma-like patterns (Figure 4). The lesions were bilateral and were evenly distributed throughout all lung fields.

By Day 14 the pulmonary lesions began to subside. Only 33% of the mice had gross hemorrhages, while microscopic lesions consisting of mononuclear infiltrates, with or without associated hemorrhages, were still present in most animals. By Day 21 most mice had normal-appearing lungs, but about 40% of the animals had yellowish lungs that contained mononuclear cell infiltrates and hemosiderin-laden alveolar macrophages. By Day 28 the lungs were normal to both gross and light-microscopic examination, and trichrome stains of these tissues did not reveal fibrin deposition or increases in collagen.

In spite of these extensive changes, only about 5% of the mice died.

Intraperitoneal injections of 10 and 50 μg of the highly purified preparations of the synthetic pseudocord factors, DOA-TDA and C-76 amide, in Marcol 52 produced identical lesions in the mice.

Control mice that were given intraperitoneal injections of oil or hexadecane alone had normal lungs as seen by both gross and light-microscopic examination.

Electron-Microscopic Findings

At 6 hours after the injection of TDM there was a slight increase in neutrophils in the pulmonary capillaries and mild interstitial damage. The time course of these lesions was essentially the same as that of those observed by light microscopy. By the third day interstitial damage was more marked, consisting of edema and disorderly fibers of collagen. These changes became even more marked by Days 5–9.

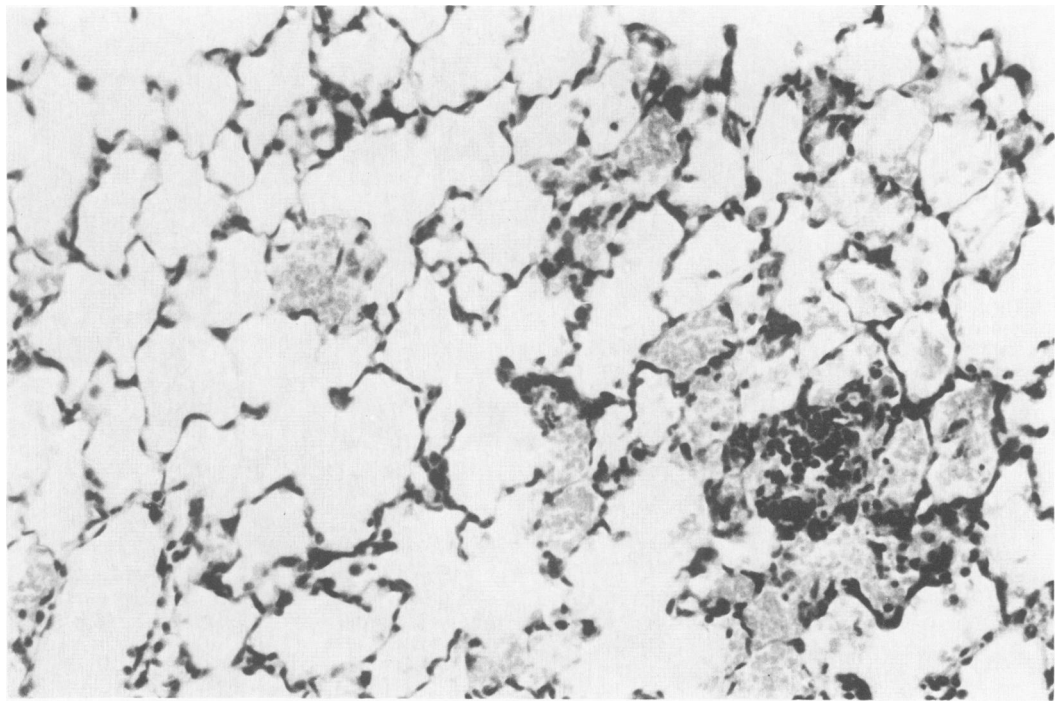

Figure 4 – Mouse lung 9 days after intraperitoneal injection of TDM. Note the focal intraalveolar hemorrhages and focal mononuclear infiltrate with granuloma-like formation; these are 4+ lesions. These are interspersed with normal appearing alveoli (H&E, × 250)

From Day 5 infiltrating cells consisting of monocytes and macrophages, including transitional forms, were present. Some macrophages contained ingested erythrocytes (Figure 5). A few animals showed deposition of fibrin as well as blebbing in endothelial cells. At Day 14, the number of infiltrating cells declined, although destructive changes in the interstitium were still marked. In contrast to the light-microscopic findings, ultrastructural abnormalities were still present on Day 28, at which time severe interstitial damage was interspersed with areas of normal-appearing lung. Recovery from the pulmonary lesions was not accompanied by hyperplasia of Type II alveolar cells.

Mice given injections of oil showed only mild edema in the interstitium (Figure 6), with occasional disorderly collagen. There were no infiltrating cells or hemorrhages.

Effects on Other Organs

At all time periods, the kidneys from mice treated with cord were normal by gross examination and by light microscopy. The livers showed occasional clusters of cells, but similar foci of mixed neutrophilic and mononuclear infiltrates were also found in mice given oil and even in untreated mice. Occasional giant cells were noted in the spleens of mice given cord factor, mice given oil, and mice that were left untreated.

Mice treated with trehalose dimycolate had marked peritonitis with formation of adhesions, but these changes were not found in vehicle-treated control mice. This inflammation subsided in parallel with the pulmonary lesions.

Dose-Response Curve Relationships

Doses of 3.3 and 10 μg produced both interstitial pulmonary infiltrates and hemorrhages ranging from 2+ to 4+, whereas 1.0 μg produced only diffuse interstitial inflammation (0–2+) but no hemorrhages. TDM in doses of 0.33 μg or 0.1 μg did not produce lesions (Table 1).

Effects of the Vehicle

Animals that received 10 μg of TDM in either 0.03 or 0.10 ml of vehicle had similar histologic changes, but recipients of the same dose of TDM in only 0.01 ml of vehicle did not develop pulmonary lesions. (Table 2).

Subcutaneous Injection of TDM

We also tried to produce pulmonary lesions by subcutaneous administration of the same dose of TDM (10 μg in 0.1 ml of oil). In the initial experiment 2 of the 4 mice developed gross hemorrhages, but in

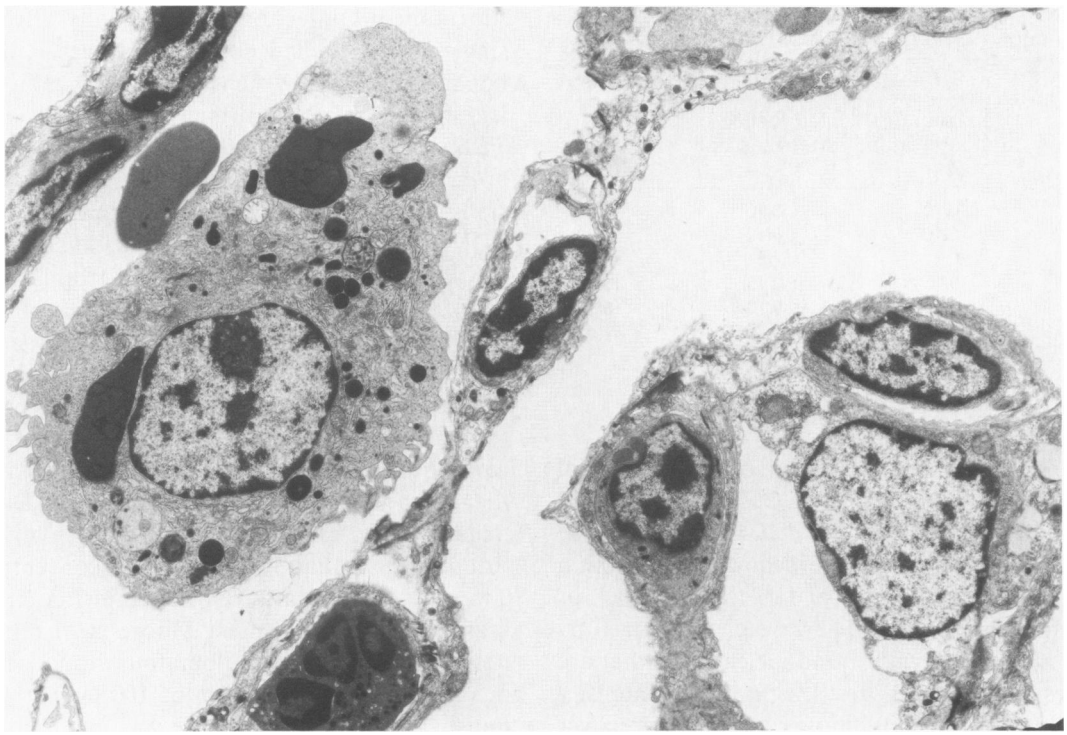

Figure 5 – Electron micrograph of mouse lung 9 days after intraperitoneal injection of TDM in 0.1 ml hexadecane. There is marked interstitial damage with thickening and blebbing. An intraalveolar macrophage that has ingested erythrocytes is to the left. (× 4500)

subsequent experiments we succeeded in producing pulmonary infiltrates and/or hemorrhagic lesions only in approximately 20% of mice 7 to 9 days after injection. Intraperitoneal injections of the same lot of TDM consistently produced lesions at this time. A few recipients of subcutaneous TDM were sacrificed on Day 28. The incidence and extent of lesions were the same as on Day 7.

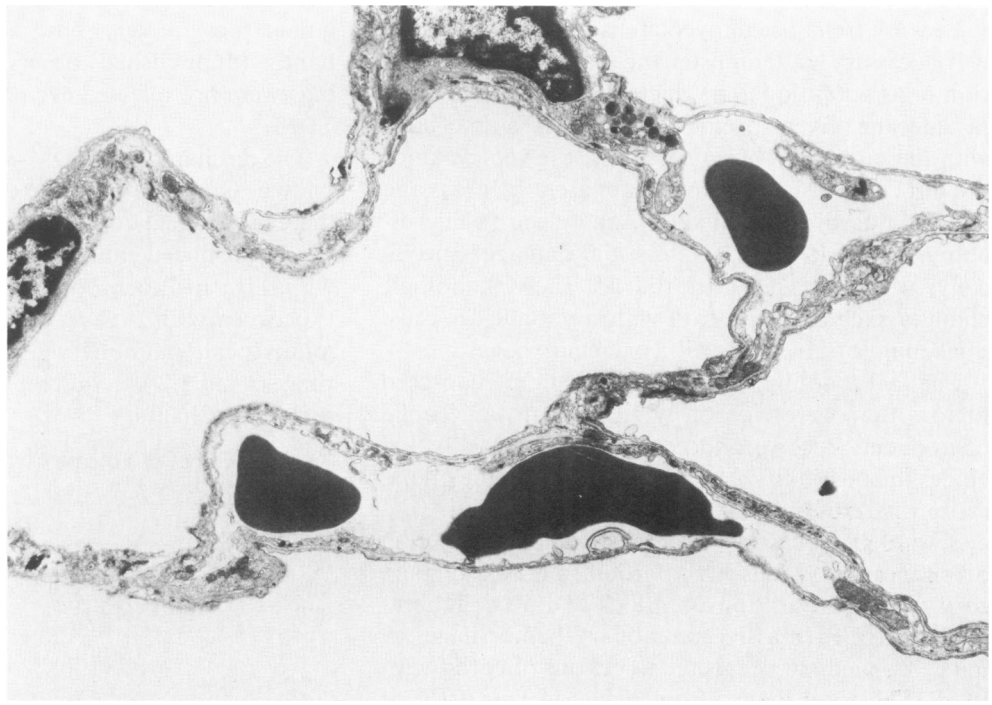

Figure 6 – Electron micrograph from a mouse 7 days after injection of 0.1 ml hexadecane. Only slight interstitial damage and edema are present. (× 4500)

Table 1—Dose-Response Relationship of TDM-Induced Pulmonary Injury

Dose of TDM (g)	Mice* showing: Hemorrhages and infiltrates (4+)	Microscopic infiltrates only (2-3+)	No lesions (0-1+)
10.0	6/10	3/10	1/10
3.3	5/10	5/10	0/10
1.0	0/8	4/8	4/8
0.33	0/15	0/15	15/15
0.10	0/10	0/10	10/10

* All mice were sacrificed on Day 7.

Discussion

We herein describe production of an interstitial and hemorrhagic pneumonitis in C57BL/6 mice by a single intraperitoneal injection of 10 μg of TDM. The lesions are apparent by the third day after injection and become maximal by the end of the first week or early in the second week. They are characterized by extensive infiltration with mononuclear cells that are monocytes and macrophages. In spite of the intensity of the pulmonary lesions, most of the mice recover and are free of gross and microscopic lesions by the twenty-eighth day.

We have not conducted detailed studies of long-term effects of TDM on the lungs of surviving animals. However, at 28 days after administration of TDM there was little evidence of the previous inflammatory reaction, and there was no evidence of fibrin deposition, proliferation of connective tissue, or hyperplasia of Type II alveolar cells.

Because trehalose dimycolate is insoluble in water, it is necessary to administer the material as an emulsion or as a solution in a vehicle such as mineral oil or hexadecane. Even in emulsions, TDM is associated with the oil phase.[14] In our studies, the vehicle alone did not cause lesions that were apparent by gross and light-microscopic examination, and electron microscopy revealed only mild interstitial damage. Specifically, vehicle-treated mice did not show pathologic changes such as infiltration with mononuclear cells, thickening of alveolar walls, or hemorrhages.

The fact that highly purified synthetic pseudo-cord factors that were rigorously free of any microbial components also produced lesions essentially excludes the possibility that a contaminant in the preparations is responsible for the lesions.

Several groups have previously studied production of pulmonary lesions with partially purified or pure cord factor preparations.[2-7] Bloch and his collaborators[2] described massive pulmonary hemorrhages in mice that died after repeated injections of cord factor in oil. The doses ranged from 20 μg to 20 mg of crude material[2] and 5 μg to 1 mg of partially purified TDM.[3] In their experience,[2,3] intraperitoneal and subcutaneous injections were equally effective, but single injections had no effect. However, mice were not sacrificed if they survived. As shown in our studies, mortality is low in mice treated only once with TDM.

Other investigators have produced pulmonary lesions by intravenous or intraperitoneal injection of TDM in oil-saline-Tween 80 emulsions.[4-7] The doses of intravenous TDM have ranged from 1 to 150 μg, and the ratios of oil and Tween 80 were also varied. Bekierkunst et al[4] found maximal granulomatous responses at Day 7 and noted that intraperitoneal administration was less effective than the intravenous route. McLaughlin et al[5] also reported that the granulomatous response was maximal at Day 7 and found that the lesions had nearly resolved by Day 28. In mice that were sacrificed on Day 4, Yarkoni and Rapp[6] found that increasing the concentration of Tween-80 or sonication of the emulsion reduced the granulomatous responses. Moore et al reported intense microscopic granulomatous responses in rabbits 3 weeks after injection of 100 μg TDM intravenously although the response was much milder than that elicited by BCG.[7]

It is noteworthy that no pulmonary hemorrhages were reported in any of these studies. A possible explanation might be the differences in sensitivity to the effects of TDM among various species and mouse strains. Sensitivity to the effects of TDM may correlate with sensitivity to experimental tuberculous infection.[2] However, guinea pigs and rats were reported to be resistant to the effects of TDM;[15,16] yet guinea pigs are very sensitive to tuberculosis. In our hands (unpublished observations) Hartley guinea pigs were not affected even by multiple injections of TDM.

The mechanism of TDM-induced lung injury is unknown. Our dose-response experiments indicate that there is a critical dose of TDM below which no damage is produced, and these findings are in keeping with those of Bloch.[2,3] This point is supported by our experiments with 1.0 μg of TDM, which caused only mild, focal, pulmonary infiltration, but no hemorrhages, and 3.3 μg, which produced both infiltration and hemorrhages.

Table 2—Effect of Volume of Vehicle on TDM-Induced Pulmonary Injury

Volume of hexadecane injected (ml)	Mice* showing: Hemorrhages and infiltrates (4+)	Infiltrates only (2-3+)	No lesions (0-1+)
0.1	2/10	5/10	3/10
0.03	6/15	7/15	2/15
0.01	0/15	0/15	15/15

* All mice were injected with 10 μg of TDM and were sacrificed on Day 7.

A direct toxic effect of TDM has been suggested as a mechanism for the production of pulmonary lesions. This possibility is supported by the experiments of Kato,[17] who reported that 50 μg of TDM produced disturbances of oxidative metabolism and caused swelling of liver mitochondria. Conceivably this could explain why mice recover from the effects of one injection but not from the effects of multiple injections of TDM. However, this effect would not explain the production of inflammation that is limited to the lung.

A second possible mechanism has an essential role for the vehicle. Single intraperitoneal injections of mineral oil or hexadecane produce only mild changes in the pulmonary interstitium, but it is possible that repeated injections of oil or TDM in oil could cause severe pulmonary damage. In our hands, however, repeated injections of oil do not cause lesions that are similar to those in TDM-treated mice. Thus, while oil alone is not injurious, it may have a permissive role in the pathogenesis of TDM-induced lung damage. This possibility is suggested by our finding that 10 μg of TDM, a dose that regularly produces lesions when it is administered in 0.03 ml or more vehicle, was ineffective when it was incorporated in only 0.01 ml of hexadecane.

Thus, while the mechanism of TDM-induced lung injury is unknown, several reports indicate effects of TDM on immunologic and inflammatory responses. For example, TDM may activate peritoneal macrophages,[18] promote chemotaxis of macrophages, monocytes, and leukocytes of several species including mice,[19] stimulate the release of thymocyte mitogenic protein (TMP) from macrophages *in vitro*,[20] and activate the alternative pathway of complement.[21] Thus far, we have conducted preliminary studies of possible immune mechanisms in the pathogenesis of TDM-induced lung injury, and these experiments suggested a requirement for T-lymphocytes.[22]

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