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In Vivo Induction of Apoptosis in the Thymus by Administration of Mycobacterial Cord Factor (Trehalose 6,6′-Dimycolate)

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It is reported that some bacteria or bacterial components cause thymic atrophy via the apoptotic process. The present study demonstrated for the first time in vivo induction of apoptosis in the mouse thymus by mycobacterial cord factor (CF) (trehalose 6,6′-dimycolate). When 300 μg of purified CF from *Mycobacterium tuberculosis* was intravenously administered to BALB/c mice in the form of water-in-oil-in-water (w/o/w) emulsion, thymic atrophy and pulmonary granulomas were induced with a peak on day 7, whereas, in the form of liposomes, CF induced thymic atrophy on days 14 to 21 in parallel with the development of hepatic granulomas. Thymic atrophy resulted from the depletion of cortical lymphocytes via apoptosis as revealed by DNA fragmentation and karyorrhectic changes. In contrast, mycobacterial sulfatide (2,3,6,6′-tetraacyl trehalose 2′-sulfate) caused neither thymic atrophy nor granuloma formation. Compared to lipopolysaccharide-induced thymocyte apoptosis, CF (w/o/w)-induced thymocyte apoptosis developed more slowly, reached a maximum later, and lasted longer but was less intense. Although serum tumor necrosis factor alpha (TNF-α) levels in CF-treated mice were not significantly elevated, administration of anti-TNF-α antibody almost completely inhibited thymic atrophy and granuloma formation. Serum corticosterone levels were only slightly elevated by CF administration. The present results indicate that mycobacterial CF induces thymic atrophy via apoptosis, which is closely linked with granuloma formation.

It has been reported that various kinds of stimuli, such as glucocorticoids (22), anti-CD3 antibody (18), tumor necrosis factor alpha (TNF-α) (8), and irradiation (17) induce apoptosis, or programmed cell death, of thymocytes in vitro. Recently, the effects of bacteria or bacterial components on thymocytes have been examined with respect to the relationship of bacterial infection to T-cell response. Lipopolysaccharide (LPS) is the well-known bacterial substance which induces a loss in weight of the thymus and a decrease in the number of thymic lymphocytes (3, 16). It has been demonstrated that LPS-induced thymic atrophy is attributed to the apoptosis of thymocytes in the cortex and may be mediated by TNF-α and corticosterone (15, 25).

Wang et al. (21) observed that intraperitoneal (i.p.) injection of live gram-negative bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, induced thymocyte apoptosis not only in C3H/HeN mice, which are LPS responders, but also in C3H/HeJ mice, which are LPS nonresponders, while LPS induced thymocyte apoptosis only in LPS responder mice, suggesting that components of gram-negative bacteria other than LPS might be able to cause thymocyte apoptosis. They further demonstrated that gram-positive bacteria, such as *Streptococcus pneumoniae*, also induced in vivo thymocyte apoptosis, the kinetics of which was different from that of gram-negative bacterium-induced thymocyte apoptosis. Peptidoglycan, an elementary cell wall component of all bacteria, is therefore assumed to be involved in this phenomenon (21). In addition to the cell wall constituents, exotoxins like staphylococcal enterotoxin B induce in vivo apoptosis of thymocytes (13).

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Mycolic acid-containing glycolipids such as cord factor (CF) (trehalose 6,6′-dimycolate [TDM]) and trehalose trimycolate (20), as well as sulfatide (SL) (2,3,6,6′-tetraacyl trehalose 2′-sulfate), are virulent factors characteristic of acid-fast bacteria. Mycoloyl glycolipids exert various biological effects, including adjuvant activity and granuloma formation (9, 14, 24), via the activation of macrophages, natural killer (NK) cells, or extrathymic T cells (19). It is reported that cytokines, such as TNF (10, 11) and gamma interferon (IFN-γ) (2), are involved in the development of pulmonary or hepatic granulomas induced by acid-fast bacteria or purified mycoloyl glycolipids. On the other hand, SL contributes to the progress of tuberculosis by suppressing phagosome-lysosome fusion in phagocytic cells (7). In the present study, we compared the inducing roles of CF and SL in the development of thymic atrophy or granulomas in mice and reported for the first time that in vivo administration of mycobacterial CF induces marked thymic atrophy via the apoptotic process, whereas SL induces neither thymic atrophy nor granuloma formation.

MATERIALS AND METHODS

Mice. Male specific-pathogen-free BALB/c mice, 5 weeks old, were purchased from SLC (Shizuoka, Japan). Experiments were performed in accordance with the standard guidelines for animal experiments of the Osaka City University Medical School.

Preparation of CF and SL. *Mycobacterium tuberculosis* AOYAMA-B was cultivated in Sauton medium for 5 to 6 weeks at 37°C. The bacterial cells were autoclaved at 121°C for 30 min and harvested. Lipids were serially extracted with chloroform-methanol (4:1 [vol/vol]), chloroform-methanol (3:1 [vol/vol]), and chloroform-methanol (2:1 [vol/vol]). Purification of CF and SL was performed by developing the lipids on a thin-layer plate of silica gel with chloroform-methanol-acetone-acetic acid (90:10:6:1 [vol/vol/vol/vol]) and subsequently with chloroform-methanol-water (90:10:1 [vol/vol/vol]). This purification procedure was repeated until a single spot of each glycolipid was ultimately obtained on a thin-layer plate.

Preparation of w/o/w emulsion and liposomes. Purified CF or SL was emulsified with 0.2% Tween 80 and 3.2% Freund's incomplete adjuvant (Difco Lab-

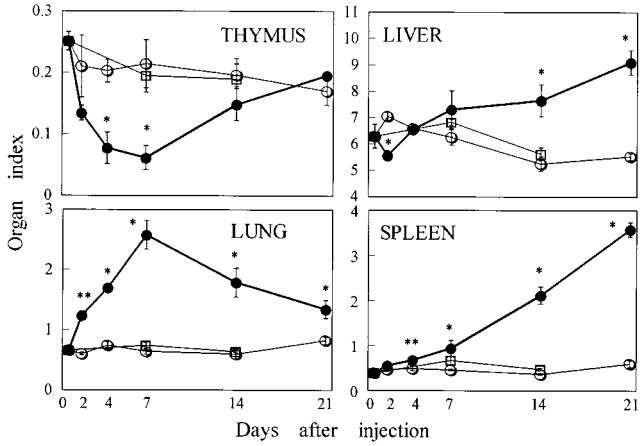

FIG. 1. Kinetics of organ indices after i.v. administration of 300 μg of CF (w/o/w) (●) or of SL (w/o/w) (□). As a control, w/o/w micelles alone were injected i.v. (○). Data represent mean values ± SD for three to four mice per group. *, $P < 0.01$ versus w/o/w micelles alone; **, $P < 0.05$ versus w/o/w micelles alone.

oratories, Detroit, Mich.) in 0.1 M phosphate-buffered saline (PBS) to form a water-in-oil-in-water (w/o/w) emulsion (23). As controls, w/o/w micelles without CF or SL were used. For the preparation of liposomes, mixtures of CF, dipalmitoyl phosphatidylcholine, and dicetyl phosphate (Sigma, St. Louis, Mo.) (1:9:0.1 [mol/mol/mol]) were dissolved in chloroform. They were evaporated and dried overnight. After PBS was added, the suspension was sonicated for 4 min at 60°C. For controls, glycolipid-free liposomes were prepared.

LPS. LPS extracted from *Salmonella enteritidis* by the phenol-water method was purchased from Difco Laboratories.

Administration of CF, SL, or LPS. Mice were intravenously (i.v.) injected with a single dose (300 μg per animal) of either CF in the form of a w/o/w emulsion [CF (w/o/w)] (19), SL in the form of a w/o/w emulsion [SL (w/o/w)], or CF in the form of liposomes [CF (liposome)]. They were sacrificed at different time points after injection. Some mice were subjected to i.p. administration of 100 μg of LPS.

Treatment of mice with anti-mouse TNF-α MAb. Mouse anti-mouse TNF-α monoclonal antibody (MAb) was prepared from the ascitic fluid in CD-1 nude mice injected with hybridoma MP6-XT3, a kind gift from Hiromi Fujiwara, Osaka University, Osaka, Japan. Mice were subjected to i.p. administration of 250 μg of anti-TNF-α MAb at 0, 24, and 48 h after i.v. injection of 300 μg of CF (w/o/w), and they were sacrificed at 65 h.

Organ index. Animals were anesthetized with ether, and their abdomens were opened. The blood was obtained via the inferior vena cava. Lungs, liver, spleen, and thymus were taken out and weighed. Thymus weight was measured by using a balance with an accuracy to 0.0001 g. In order to represent the degree of granuloma formation in each organ, the organ index was calculated as organ weight (in grams)/body weight (in grams) × 100.

Quantitation of serum TNF-α and corticosterone. At various intervals, serum was collected from each individual mouse and stored at −80°C until quantitated. For the bioassay of TNF-α, serially diluted sera were added to 4×10^4 L929 cells that had been incubated in microtiter plates for 5 h in a humidified atmosphere at 37°C with 5% CO_2. Plates were incubated in the presence of 1 μg of actinomycin D/ml for 20 h under the same conditions and then were stained with crystal violet. After the plates were washed with PBS, the dye was extracted with 0.1 M KH_2PO_4-ethanol (1:1 [vol/vol]). The optical absorbance of the solution at 550 nm was measured with a microplate reader. Serum corticosterone was measured with a radioimmunoassay kit (rat corticosterone [^{125}I] assay system; Amersham Life Science, Little Chalfont, Buckinghamshire, United Kingdom).

Histological examination. Organs taken out were fixed in 10% formalin, dehydrated, and embedded in paraffin. Sections were stained with hematoxylin and eosin (H-E). For electron microscopy, thymuses were fixed in 1% Karnovsky solution overnight at 4°C. They were cut into small blocks, postfixed in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4), dehydrated in ethanol series, and embedded in polybed (Polysciences Inc., Warrington, Pa.). Semithin sections (0.5 μm thick) were cut, stained with toluidine blue, and observed by light microscopy. Thin sections were stained with saturated uranyl acetate and lead citrate and were observed under a JEM 1200EX electron microscope (JEOL, Tokyo, Japan) at 100 kV.

For the morphometry of apoptotic cells in the thymus, three to six mice were used for each group. We observed toluidine blue-stained sections at a magnification of ×1,000 and counted the number of apoptotic thymocytes in five visual fields of the thymic cortex per animal. The density of apoptotic cells was expressed as the number of apoptotic cells per square millimeter of the cortex area.

DNA extraction and DNA electrophoresis. The thymocyte suspension (10^7 cells/ml in PBS) was centrifuged at 250 × *g* for 15 min. The pellet obtained was resuspended in lysing buffer (20 mM EDTA, 0.2% Triton X-100, 10 mM Tris-HCl [pH 8.0]). After centrifugation at 14,000 × *g* for 10 min at 4°C, the supernatant, which contained fragmented soluble DNA, was collected, incubated with 0.5 M EDTA and heat-treated RNase for 15 min at 37°C, and subsequently incubated with 10% sodium dodecyl sulfate for 10 min at 55°C. DNA was extracted from the supernatant with phenol-chloroform, precipitated by the addition of isopropanol containing 3 M sodium acetate (pH 5.2), and kept overnight at −20°C. After centrifugation at 14,000 × *g* for 10 min, it was suspended in 20 μl of a solution containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. Electrophoresis was performed at 100 V with 1.5% agarose gels in TAE buffer (40 mM Tris-HCl, 40 mM acetic acid, 1 mM EDTA [pH 8.0]). After electrophoresis, the gel was stained with ethidium bromide (1 μg/ml) and visualized with UV light.

Statistical analysis. The results were analyzed according to the unpaired Student *t* test. Data were expressed as mean values ± standard deviations (SD) or standard errors.

RESULTS

Induction of thymic atrophy and granuloma formation by CF. By i.v. administration of CF (w/o/w), the lung index began to increase on day 2, reaching a maximum on day 7, and then gradually decreased until day 21 (Fig. 1). Liver and spleen indices showed a slight increase on day 7, continued to go up by day 21 (Fig. 1), and then declined on day 28 (data not shown). Histological examination of H-E-stained sections revealed that organ indices were well correlated with the degree of granuloma formation (data not shown). The thymus index began to decrease on day 2, reached the lowest level on day 7, and returned to the value of w/o/w control mice on day 21 (Fig. 1). In contrast to CF (w/o/w), SL (w/o/w) induced neither granuloma formation nor thymic atrophy (Fig. 1). Administration of w/o/w emulsion alone did not cause any significant changes in organ indices.

On the other hand, when CF (liposome) was administered, there was no increase in the lung index throughout the experimental periods, whereas liver and spleen indices exhibited conspicuous increases on days 14 and 21 (Fig. 2). The thymus index remained unchanged until day 7 and then was significantly decreased on days 14 and 21 (Fig. 2).

By i.p. injection of LPS, thymus weight decreased. The kinetics and degree of thymic atrophy in LPS-treated mice were similar to those observed in CF (w/o/w)-treated mice, although the thymus weight loss on day 1 was much larger in LPS-

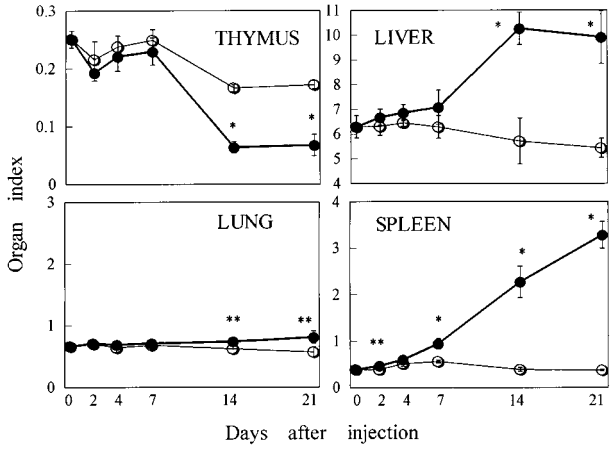

FIG. 2. Kinetics of organ indices after i.v. administration of 300 μg of CF (liposome) (●). As a control, liposomes alone were injected i.v. (○). Data represent mean values ± SD for three to four mice per group. *, $P < 0.01$ versus liposomes alone; **, $P < 0.05$ versus liposomes alone.

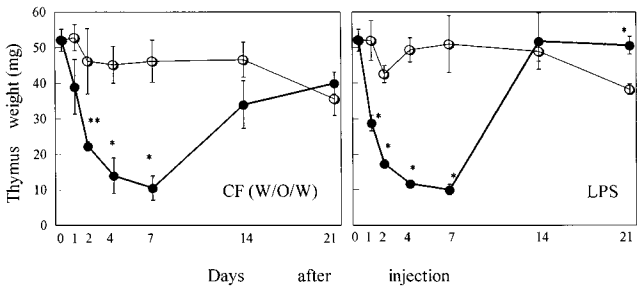

FIG. 3. (Left panel) Kinetics of thymus weight after i.v. administration of 300 μg of CF (w/o/w) (●) or w/o/w micelles alone (○). (Right panel) Kinetics of thymus weight after i.p. administration of 100 μg of LPS (●) or saline (○). Data represent mean values ± SD for three to five mice per group. *, $P < 0.01$ versus w/o/w micelles alone or saline; **, $P < 0.05$ versus w/o/w micelles alone or saline.

treated mice than that observed in CF (w/o/w)-treated mice (Fig. 3). LPS treatment did not induce massive granulomas in the lung, liver, and spleen.

Induction of thymocyte apoptosis by CF or LPS. To investigate the mechanism of thymic atrophy by CF and reasons for the difference in the kinetics of thymic atrophy among CF (w/o/w)-, CF (liposome)-, and LPS-treated mice, we examined histopathological changes in thymuses. In H-E-stained sections, cortical thymocytes began to decrease 6 h after LPS injection. The boundary between the cortex and medulla became obscure at 18 h and was diminished at 24 h (Fig. 4). The thymic cortex remained lost until day 7 but reappeared on day 14. On the other hand, CF (w/o/w)-treated mice exhibited the normal features of the thymic cortex at 18 h. The cortex was then profoundly decreased at 36 h and had entirely disappeared by 65 h (Fig. 4). When CF (liposome) was administered, the thymic cortex showed no changes in volume until day 7 but was completely diminished on day 14 and partially recovered on day 21 (Fig. 4).

We next examined the morphology and frequency of apoptotic thymocytes in the cortex at a higher magnification. By LPS injection, many thymocytes underwent apoptosis diffusely in the cortical area at 12 h, showing pyknotic and karyorrhectic changes (Fig. 5). Pyknotic foci were still extensive at 18 h and then were decreased in number at 24 h (Fig. 5 and Table 1). On the other hand, in CF (w/o/w)-treated mice, pyknotic thymocytes were sparse in the cortex at 18 h, were increased in number at 24 h, and reached a maximum at 36 to 48 h, although the frequency of apoptotic thymocytes in CF (w/o/w)-treated thymus at 36 to 48 h was less than that observed in LPS-treated thymus at 12 to 18 h (Fig. 5 and Table 1). They gradually decreased thereafter (Table 1). On the other hand, we could not detect the increase of apoptotic thymocytes on days 2, 4, 7, 14, and 21 in CF (liposome)-treated thymuses.

As observed by electron microscopy, the morphology of thymocyte apoptosis was similar in LPS- and CF (w/o/w)-treated thymuses (Fig. 6a and b). Thymocytes with condensed nuclear chromatin were phagocytosed by macrophages, which were distributed in the thymic cortex, particularly around the blood vessels (Fig. 6c). The nuclei of apoptotic thymocytes were fragmented into the debris and scattered in the cytoplasm of

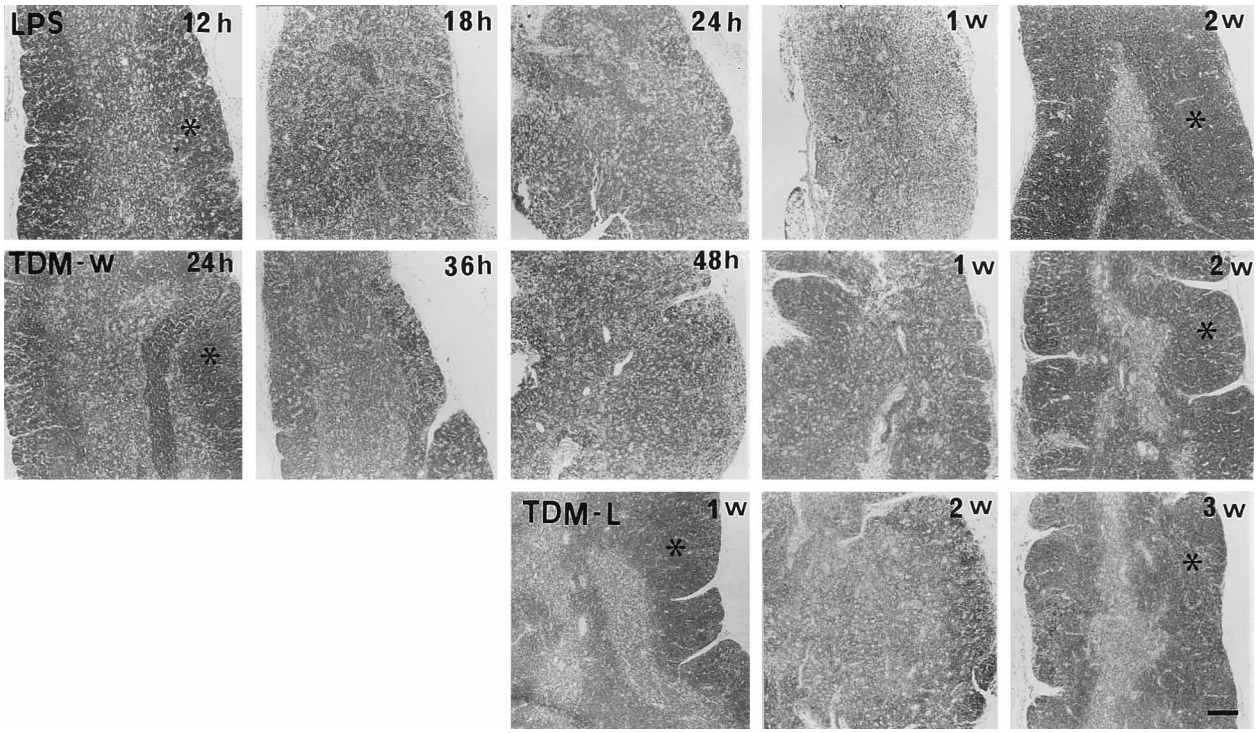

FIG. 4. Low-power light micrographs of thymuses at various intervals after i.p. administration of 100 μg of LPS (top panels), i.v. administration of 300 μg of CF (w/o/w) (middle panels), or i.v. administration of 300 μg of CF (liposome) (bottom panels). In LPS-treated mice, depletion of cortical lymphocytes (asterisks) begins at 12 h and is almost completed at 18 h, whereas in CF (w/o/w)-treated mice, the thymic cortex is still obvious at 24 h and is partially diminished at 36 h. In both cases, the thymic cortex is completely lost at 1 week (w) postinjection, followed by recovery to the normal state at 2 weeks, as indicated by asterisks. In CF (liposome)-treated mice, the thymic cortex (asterisks) shows no change at 1 week, disappears at 2 weeks, and is reconstructed at 3 weeks. H-E stain was used. Bar = 100 μm.

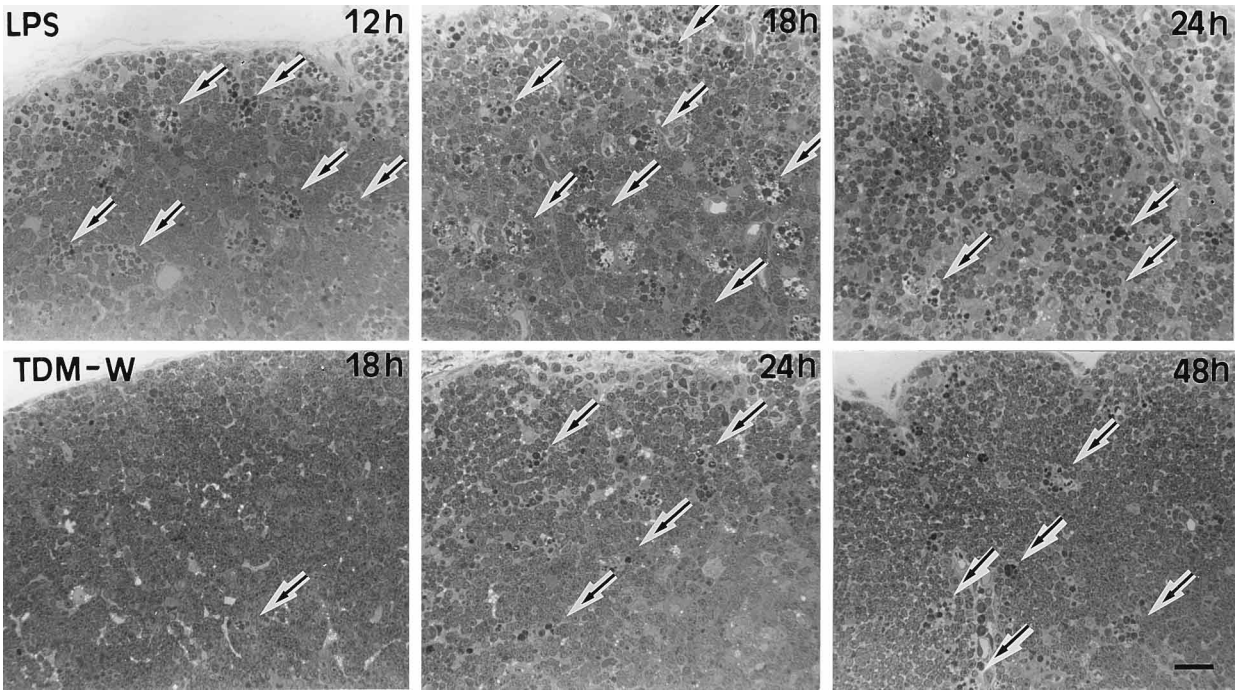

FIG. 5. High-power light micrographs of thymuses at various intervals after i.p. administration of 100 μg of LPS (upper panels) or i.v. administration of 300 μg of CF (w/o/w) (lower panels). In LPS-treated mice, foci of apoptotic lymphocytes (arrows) are increased in the thymic cortex 12 h after injection. They become more evident at 18 h and are decreased at 24 h. In CF (w/o/w)-treated mice, the frequency of apoptotic lymphocytes (arrows) is increased at 24 h and persists at a high level at 48 h. Toluidine blue stain was used. Bar = 20 μm.

macrophages. Some macrophages contained many lipid droplets in the cytoplasm (Fig. 6c).

By electrophoresis, DNA extracted from CF (w/o/w)-treated thymocytes showed fragmentation in ladder form, although the bands were less intense than those obtained from LPS-challenged thymocytes (Fig. 7).

Involvement of TNF-α and corticosterone in CF-induced thymic atrophy. Serum TNF-α levels were significantly increased at 1 and 2 h after LPS injection, whereas no elevation of TNF-α was detected in the sera of CF (w/o/w)-treated mice at any time between 1 and 36 h after injection (Fig. 8). Control mice treated with w/o/w micelles alone or saline did not show any elevation of TNF-α at any time point (data not shown). Administration of anti-mouse TNF-α MAb, however, almost completely inhibited thymic atrophy and pulmonary granuloma formation 3 days after CF (w/o/w) injection (Table 2). Increases in the number of apoptotic thymocytes and loss of the thymic cortex were not found in anti-TNF-α MAb-treated mice on day 3 (data not shown).

Serum corticosterone levels were profoundly elevated at 2 to 6 h after LPS injection, while CF (w/o/w)-treated mice showed only a slight elevation of corticosterone at 1 to 6 h, compared to w/o/w micelle-treated mice (Fig. 9). In CF (liposome)-treated mice, we could not detect elevation of serum corticosterone on days 2, 4, and 7, prior to the thymic atrophy observed on day 14 (Fig. 9).

DISCUSSION

The present study has revealed that mycobacterial CF has a potential for the induction of thymic atrophy via the apoptotic process. Light and electron microscopic analyses demonstrated typical features of apoptotic thymocytes in the cortex area of CF-treated thymus as reported for LPS- or *E. coli*-induced thymic atrophy (4, 21). The localization of apoptotic foci around the blood vessels seen here is also observed in LPS-treated thymuses (4).

Thymic atrophy induced by CF was closely related to granuloma formation. The kinetic study demonstrated that the time of maximal thymic atrophy coincided exactly with that of the peak of granuloma formation. When CF (w/o/w) was administered via the tail vein, micelles were trapped in the alveolar capillaries, as revealed by electron microscopy (data not shown), and developed pulmonary granulomas with a peak on day 7, accompanied by marked thymic atrophy. On the other hand, CF (liposome), which is much smaller in size than micelles and passes through the alveolar capillaries, did not in-

TABLE 1. Density of apoptotic thymocytes in the cortex areas of thymuses after CF (w/o/w) or LPS injection[a]

Time after injection	No. of apoptotic thymocytes/mm^2 (mean ± SD) after injection with:		
	w/o/w micelles alone	CF (w/o/w)	LPS
0 h	574 ± 96	574 ± 96	574 ± 96
12 h	NT[b]	NT	5,451 ± 180
18 h	NT	856 ± 64	6,670 ± 614
24 h	752 ± 52	1,083 ± 85	1,184 ± 274
36 h	NT	1,459 ± 212	NT
48 h	836 ± 51	1,451 ± 212	950 ± 59
65 h	NT	1,211 ± 83	NT
4 d[c]	791 ± 38	902 ± 26	877 ± 70
7 d	641 ± 64	911 ± 36	854 ± 85
14 d	574 ± 96	695 ± 72	NT

[a] Mice were subjected to i.v. administration of 300 μg of CF (w/o/w) or i.p. administration of 100 μg of LPS. Three to six mice were used for each group.
[b] NT, not tested.
[c] d, days.

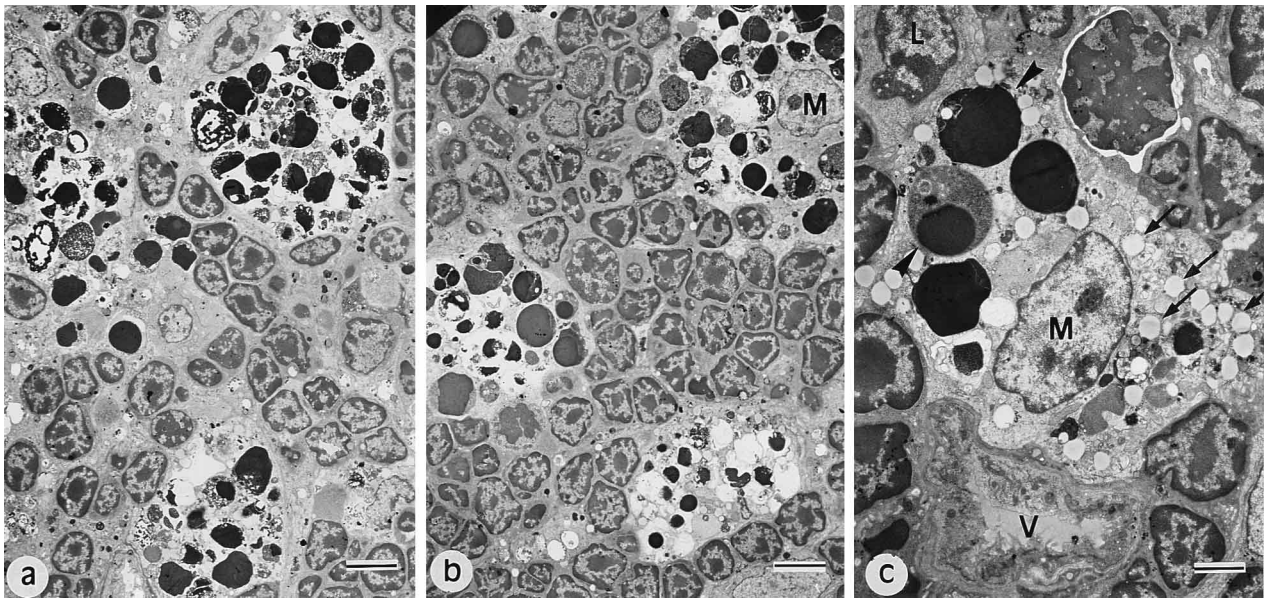

FIG. 6. Electron micrographs of the thymic cortex 18 h after i.p. injection of 100 μg of LPS (a) or 36 h after i.v. injection of 300 μg of CF (w/o/w) (b and c). Macrophages (M) which have phagocytosed many apoptotic lymphocytes are found among intact cortical lymphocytes. (c) A macrophage which ingests several apoptotic lymphocytes (arrowheads) is located near the blood vessel (V). It contains many lipid droplets (arrows) in the cytoplasm. (a and b) Bars = 5 μm. (c) Bar = 2 μm.

duce pulmonary granulomas but did induce prominent hepatic granulomas observed on days 14 and 21, with marked thymic involution at these time points. Experiments using different doses of CF or different analogs of mycoloyl glycolipids, such as trehalose trimycolate from *Gordona aurantiaca* (20), have demonstrated that there is a good correlation between the degree of thymic atrophy and the extent of granuloma formation (data not shown). Furthermore, mycobacterial SL, which did not induce granuloma formation, did not cause thymic atrophy. Both CF and SL contain trehalose as a carbohydrate moiety, so the different biological effects of these two substances observed here must be derived from the difference in the fatty acid moieties; mycobacterial CF contains C_{76-86} mycolic acids with a long alkyl branched chain at the α position, while SL has shorter-chain (C_{38-42}) phthioceranic acids. Mycolic acids occupy a larger part of the host cell membrane than phthioceranic acids do and thereby cause more profound perturbation of the membrane.

Thymic atrophy was invariably induced independently of the kinds of organs in which granulomas were formed; namely, it was seen during CF (liposome)-induced hepatic granuloma formation as well as during CF (w/o/w)-induced pulmonary granuloma formation. In either case, loss of the thymic cortex was transient and cortical lymphocytes were restored within a week after the peak of thymic atrophy. In CF (w/o/w)-treated mice, pulmonary granuloma formation was followed by hepatic and splenic granulomas, although the latter did not prolong the thymic atrophy induced by the former, suggesting that granuloma formation may not affect the process of cortical restoration, which includes the entry of thymocyte precursors into thymuses and the subcapsular proliferation of thymoblasts.

Although thymic atrophy was maximum on day 7 both in CF (w/o/w)- and LPS-treated mice, the onset of thymic atrophy in the former was apparently behind that in the latter. The time period and duration of the augmented thymocyte apoptosis were also different, i.e., 12 to 24 h in LPS-treated mice and 24 to 65 h in CF (w/o/w)-treated mice. Our present results, together with the previous studies by others (13, 21), indicate that the kinetics of thymocyte apoptosis is characteristic to each bacterial component.

It has been considered that TNF-α may play a central role in the in vivo induction of thymocyte apoptosis by LPS (15), *E. coli* (21), or *S. pneumoniae* (21) in light of the facts that the serum TNF-α level is elevated 1 h, 1 h, or 6 to 9 h, respectively, after injections and that anti-TNF-α MAb treatment suppresses the induction of thymocyte apoptosis by these substances. It has also been demonstrated that TNF-α directly acts on thymocytes and induces apoptosis in vitro (8). In the present study, administration of anti-TNF-α MAb prevented mice from developing CF (w/o/w)-induced thymic atrophy, indicating the involvement of TNF-α. However, no significant amount of TNF-α was detected in the sera of CF (w/o/w)-treated mice; this finding is consistent with our previous data

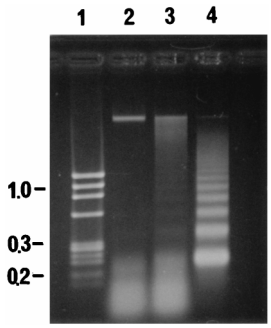

FIG. 7. DNA fragmentation of LPS-treated or CF (w/o/w)-treated mice. DNA was extracted from the thymocytes and electrophoresed in agarose gel. Lane 1, molecular-size marker of *Hae*III-digested ϕX174 RF DNA, in kilobases; lane 2, mice treated with w/o/w micelles alone at 24 h; lane 3, mice treated with 300 μg of CF (w/o/w) at 24 h; lane 4, mice treated with 100 μg of LPS at 24 h.

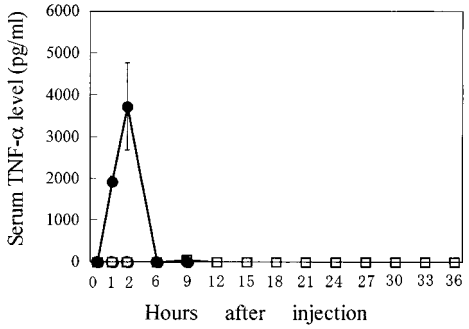

FIG. 8. Time course of the serum TNF-α level after i.p. administration of 100 μg of LPS (●) or i.v. administration of 300 μg of CF (w/o/w) (□) or saline (○). Data represent mean values ± standard errors for three mice.

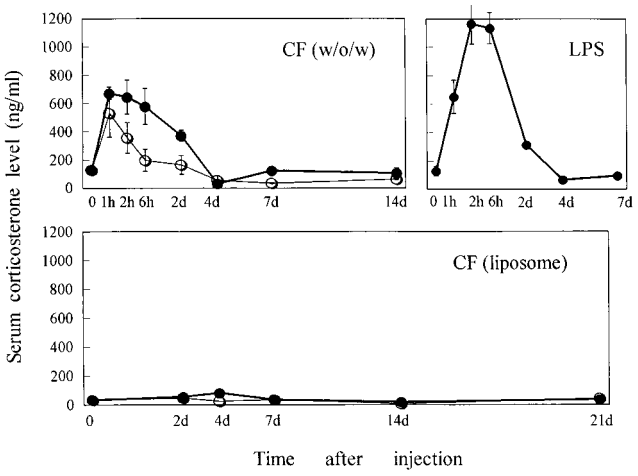

FIG. 9. Time course of the serum corticosterone level after i.v. administration of 300 μg of CF (w/o/w), i.v. administration of 300 μg of CF (liposome), or i.p. administration of 100 μg of LPS (all shown by ●). As controls, w/o/w micelles alone (○) or liposomes alone (○) were injected i.v. Data represent mean values ± standard errors for three to four mice per group.

showing that mycoloyl glycolipids do not induce TNF-α production even after multiple injections but show TNF-priming activity (14). There is a report that TNF-α is synthesized locally in *Mycobacterium bovis* BCG-induced granulomas and contributes to the development of granulomas in an autocrine or paracrine manner (11). In this study, CF (w/o/w)-induced pulmonary granuloma formation was significantly suppressed by anti-TNF-α MAb. These data suggest that CF (w/o/w)-induced thymic atrophy is not the result of direct action of serum TNF-α on thymocytes but occurs in association with TNF-α-dependent growth of granulomas and that anti-TNF-α MAb may inhibit thymic atrophy by interfering with granuloma formation.

Possible mediators which affect thymocytes during CF-induced granuloma formation are essentially unknown. However, several cytokines or substances released from macrophages, i.e., interleukin-1 (IL-1) (12), TNF-β (12), granulocyte-macrophage colony-stimulating factor (6), and nitric oxide (NO) (5), have been reported to induce thymocyte apoptosis, and we have observed increased mRNA expression for IL-1, inducible NO synthase, TNF, and IFN-γ in the lung 1 day after CF (w/o/w) administration (15a). Corticosterone is also reported to participate in LPS-induced thymocyte apoptosis (15, 25). In the present study, corticosterone levels were elevated slightly in the sera of CF (w/o/w)-treated mice. Abo et al. (1) reported that *E. coli*-induced thymic atrophy may result from the inactivation of intrathymic T cells that occurs reciprocally under the condition of extrathymic T-cell activation induced by bacterial stimulation. We have also observed in CF (w/o/w)-treated mice that NK cells and extrathymic T cells ($CD3^{+/-}$ IL-$2R\beta^{+}$ and γδ T cells) increase in pulmonary granulomas, while thymus-derived T cells ($CD3^{+}$ IL-$2R\beta^{-}$ cells) decrease (19).

TABLE 2. Effect of anti-TNF-α MAb on CF (w/o/w)-induced thymic atrophy and pulmonary granuloma formation 65 h after CF injection[a]

Substance(s) injected	Wt (mean ± SD) of:		
	Body (g)	Thymus (mg)	Lung (g)
w/o/w micelles alone	22.21 ± 1.25	46.2 ± 9.2	0.13 ± 0.01
CF (w/o/w)	15.05 ± 0.52*[b]	24.1 ± 1.5*	0.27 ± 0.02*
CF (w/o/w) + anti-TNF-α MAb	20.85 ± 0.36	43.2 ± 3.8	0.17 ± 0.00

[a] Mice were subjected to i.v. administration of 300 μg of CF (w/o/w) or w/o/w micelles alone. Anti-TNF-α MAb was administered i.p. at a dose of 250 μg at 0, 24, and 48 h after CF injection. Three mice were used for each group.

[b] *, $P < 0.01$ versus w/o/w micelles alone.

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