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Activation of Protein Kinase C by Mycobacterial Cord Factor, Trehalose 6-Monomycolate, Resulting in Tumor Necrosis Factor-α Release in Mouse Lung Tissues

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Cord factors are mycoloyl glycolipids in cell walls of bacteria belonging to *Actinomycetales*, such as *Mycobacterium*, *Nocardia* and *Rhodococcus*. They induce granuloma formation in the lung and interstitial pneumonitis, associated with production of macrophage-derived cytokines. We studied how cord factors induce biological activities in the cells. Cord factors isolated from *M. tuberculosis*, trehalose 6-monomycolate (mTMM) and trehalose 6,6′-dimycolate (mTDM), enhanced protein kinase C (PKC) activation in the presence of phosphatidylserine (PtdSer), diacylglycerol and Ca^{2+}, and mTMM activated PKCα more strongly than PKCβ or γ under the same assay conditions. Kinetic studies of mTMM in response to PKC activation revealed that mTMM increased the apparent affinity of PKC to Ca^{2+} in the presence of both PtdSer and diolein. Although this is similar to observations with unsaturated fatty acids, such as arachidonic acid, mTMM was synergistic with PtdSer for PKC activation, but arachidonic acid was not. mTMM was also different as regards PKC activation, as phorbol ester was. A single i.p. administration of mTMM to mouse induced tumor necrosis factor-α (TNF-α) in serum and in the lung, which is a unique target tissue of cord factors. Based on our recent finding that TNF-α is an endogenous tumor promoter, the correlation between lung cancer and pulmonary tuberculosis is discussed.

Key words: Cord factor — Pulmonary tuberculosis — Lung cancer — Protein kinase C — Tumor necrosis factor-α

Epidemiological studies have revealed that lung cancer frequently occurs among populations with a history of pulmonary tuberculosis, at rates 5 to 10 times higher than those in the normal population.[1-3] Causative factors to support the correlation between lung cancer and pulmonary tuberculosis have not yet been fully elucidated. *Mycobacterium tuberculosis* has unique components in the cell wall, so-called cord factors, trehalose 6-monomycolate (mTMM) and trehalose 6,6′-dimycolate (mTDM), which are glycolipids containing mycolic acids[4] (Fig. 1). Mycolic acid is a 3-hydroxy fatty acid with a C_{80-86} long-chain alkyl branch at the 2-position. Cord factors have various biological activities, such as granuloma-forming, anti-tumor and immunoadjuvant activities,[5-10] although their mechanisms of action are not known. Arachidonic acid and other unsaturated fatty acids stimulate protein kinase C (PKC) activation synergistically with diacylglycerol in the presence of phosphatidylserine (PtdSer).[11] The evidence suggested to us that cord factors might activate PKC in the cells and then induce carcinogenesis through signal transduction of tumor promotion in the lungs.

Cord factors are structurally varied. Trehalose 6-monomycolate (rTMM), trehalose 6,6′-dimycolate (rTDM), mannose 6-monomycolate (rMMM) and glucose 6-monomycolate (rGMM) with C_{44-46} mycolic acids were isolated from *Rhodococcus ruber*,[12] in addition to mTMM and mTDM isolated from *M. tuberculosis*. They were subjected to assay of PKC activation to test their structure-function relationships.

We previously reported that tumor necrosis factor-α (TNF-α), which is induced by activated macrophages, stimulated transformation of BALB/3T3 cells initiated with 3-methylcholanthrene (MCA), and induced clonal expansion of Bhas 42 cells, which are BALB/3T3 cells containing viral-H-*ras* gene.[13] The results indicated that TNF-α is an endogenous tumor promoter, that is, it induces clonal growth of initiated cells. Administration of cord factor isolated from *M. tuberculosis* induced high levels of TNF-α in plasma of mice,[14] and i.v. administration of cord factors induced granuloma formation in the lungs of mice, associated with accumulation of macrophages.[15] Thus, we think activation of PKC by cord factors might be linked to release of TNF-α in the lung. Here we present evidence that a single i.p. administration of mTMM to mice induced TNF-α release in serum and

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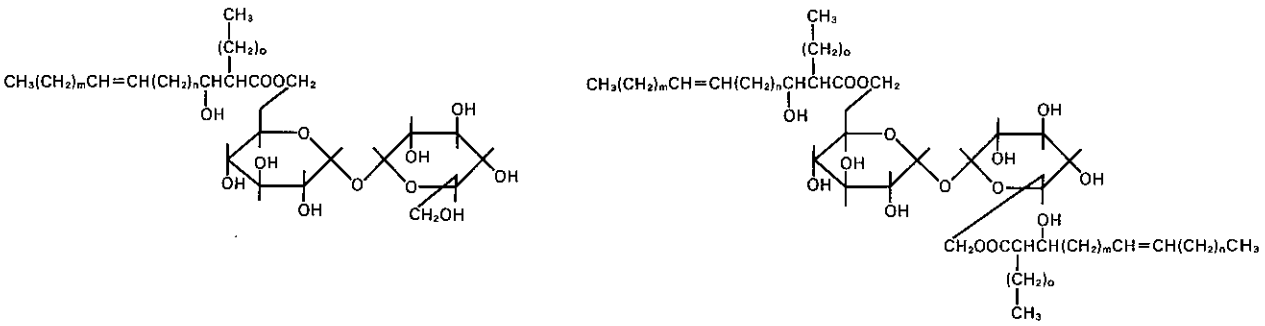

Fig. 1. Structures of trehalose 6-monomycolate (mTMM) and trehalose 6,6'-dimycolate (mTDM). These cord factors, isolated from *M. tuberculosis*, are glycolipids containing mycolic acid. Mycolic acid is a 3-hydroxy fatty acid with a C_{80-86} long-chain alkyl branch at the 2-position.

in the lungs, suggesting that TNF-α synthesis is induced by activation of PKC in lung macrophages treated with cord factors. We discuss the possibility that mTMM, a new enhancer of PKC activation, promotes tumor development in the lungs of patients with a history of pulmonary tuberculosis through induction of TNF-α, an endogenous tumor promoter.

MATERIALS AND METHODS

Cord factors mTMM and mTDM with C_{80-86} mycolic acids were isolated from *M. tuberculosis*,[4] and rTMM, rTDM, rGMM and rMMM with C_{44-46} mycolic acids were isolated from *R. ruber*,[12] as reported previously.

Chemicals Suppliers of chemicals used were as follows: calf thymus H1 histone from Boehringer Mannheim GmbH; [γ-^{32}P]ATP from Amersham; phospholipids, diolein and arachidonic acid from Serdary Research Laboratories; 12 - O - tetradecanoylphorbol - 13 - acetate (TPA) from Sigma; monoclonal anti-rabbit PKCα, β and γ antibodies from Seikagaku Co.; ELISA kit from Genzyme Co. Other chemicals were purchased from commercial sources.

Animals Male BALB/c mice, 6 weeks old, were obtained from Charles River Japan Inc., Kanagawa.

Isolation of PKC A cytosolic fraction of mouse brain was subjected to DEAE-cellulose column chromatography, as reported previously,[16] affording a PKC fraction. The PKC mixture was further purified by hydroxyapatite column chromatography, according to the method of Sekiguchi *et al.*,[17] to separate PKCα, β and γ subspecies, which were identified by Western blotting using rabbit anti-PKCα, β and γ antibodies.[17] Each subspecies used was almost pure.

PKC assay The enzyme activity was assayed routinely with histone H1 as a phosphate acceptor. The reaction mixture (0.25 ml) contained 20 m*M* Tris-HCl (pH 7.5), 10 m*M* Mg $(CH_3COO)_2$, 10 μM [γ-^{32}P]ATP (1.0×10^5 cpm), 50 μg of Hl histone, and other chemicals including $CaCl_2$ (1 μM), PtdSer (8 μg/ml), diolein (1.3 μM), and cord factors. Cord factors were dissolved in chloroform/methanol (2/1), and arachidonic acid was dissolved in hexane. These solutions were evaporated under nitrogen and the residues were sonicated in a buffer solution for 5 min for 37°C under nitrogen. PtdSer and diolein were first mixed, dried under nitrogen and then sonicated in buffer solution for 5 min at 30°C, as reported previously.[11] The reaction mixture was incubated for 3 min at 30°C immediately after addition of PKC. The amount of PKC used for each assay was approximately 0.05 U under the standard conditions. The acid-precipitable materials were collected on a BA85 nitrocellulose membrane filter (Schleicher & Schuell Inc.) and the radioactivity was determined with a scintillation counter.

TNF-α release mTMM dissolved in mineral oil (10 μg/0.1 ml) was sonicated, and then administered i.p. to mice. Mice were killed at various days after the administration. Their sera were stored at −80°C, and the lung tissues were excised and immediately frozen under liquid nitrogen. TNF-α levels in sera were determined by use of an ELISA kit, which contained a hamster monoclonal antibody specific for mouse TNF-α and a goat polyclonal anti-mouse TNF-α antibody. Supernatants of the lung tissue homogenized in a buffer containing 50 m*M* Tris-HCl (pH 7.5), 0.1 *M* NaCl, 1% Triton X-100, 5 m*M* EDTA, 0.02% NaN_3, 0.1 m*M* PMSF, and 1 μM leupeptin were obtained by centrifugation at 12,000 rpm for 15 min. Aliquots (1 mg protein/0.1 ml) of the supernatant fluid were used for determination of mouse TNF-α with the ELISA kit.[13] Protein concentration was determined by the Bradford method using bovine serum albumin as a standard.[18]

RESULTS

Activation of PKC mixture by arachidonic acid, mTMM and mTDM Since the effects of arachidonic acid on PKC activation had previously been reported,[11] we first confirmed that arachidonic acid at various concentrations with 1 μM Ca^{2+} activated PKC mixture, in the presence of PtdSer and diolein. The concentration of arachidonic acid giving rise to the maximum effect was slightly higher, in the range of 40–50 μM, than that reported previously (Fig. 2A).[11] Arachidonic acid with diolein, even without PtdSer, significantly activated PKC mixture in a manner similar to that seen with PtdSer and diolein. However, arachidonic acid alone did not activate the enzyme mixture. mTMM dose-dependently activated PKC mixture in the presence of PtdSer. The activation by mTMM was more strongly enhanced by addition of diolein, and maximum activation was obtained with 40–50 μM mTMM. mTMM alone showed a marginal stimulatory effect, which was not enhanced by diolein. A big difference was found between the effects of arachidonic acid and mTMM; namely, arachidonic acid showed a synergistic action with diolein, whereas mTMM was synergistic with PtdSer, but not with diolein (Fig. 2B). Activation of PKC mixture by mTDM was slightly different from that by mTMM (Fig. 2C). Although mTDM activated the enzyme in the presence of PtdSer, the enhancing activity of mTDM was slightly weaker than that of mTMM. The results indicated that mTDM showed synergistic action with PtdSer, as did mTMM. However, in the presence of both PtdSer and diolein, mTDM did not enhance PKC activation at all, and the activation was entirely different from that of mTMM. This suggests that mTMM and mTDM interact differently with the enzyme in the presence of both PtdSer and diolein (Fig. 2C).

Activation of PKC subspecies by mTMM Next, PKC activation by mTMM was determined with each PKC subspecies, α, β and γ, which were separated by hydroxyapatite column chromatography. Although the identification of PKCα, β and γ is not described here in detail, the fraction of PKCγ was eluted with 60 mM potassium phosphate; that of PKCβ, with 80 mM, and that of PKCα, with 120 mM (data not shown). Fig. 3 shows that mTMM activated each PKC subspecies, α, β and γ, in the presence of PtdSer and diolein, or of PtdSer alone. In particular, mTMM more strongly activated PKCα than PKCβ or γ in the presence of both PtdSer and diolein.

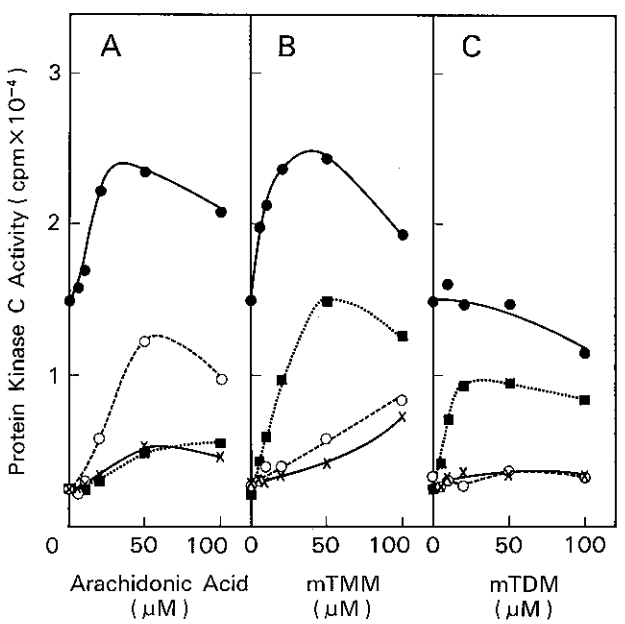

Fig. 2. Activation of PKC mixture by arachidonic acid, mTMM and mTDM. The enzyme activity was assayed as described under "Materials and Methods." Activation of PKC mixture by (A) arachidonic acid, (B) mTMM and (C) mTDM at various concentrations with 1 μM Ca^{2+} in the absence of PtdSer and diolein (×) or in the presence of diolein (○), PtdSer (■), or both (●). The concentrations of PtdSer and diolein were 8 μg/ml and 0.8 μg/ml, respectively.

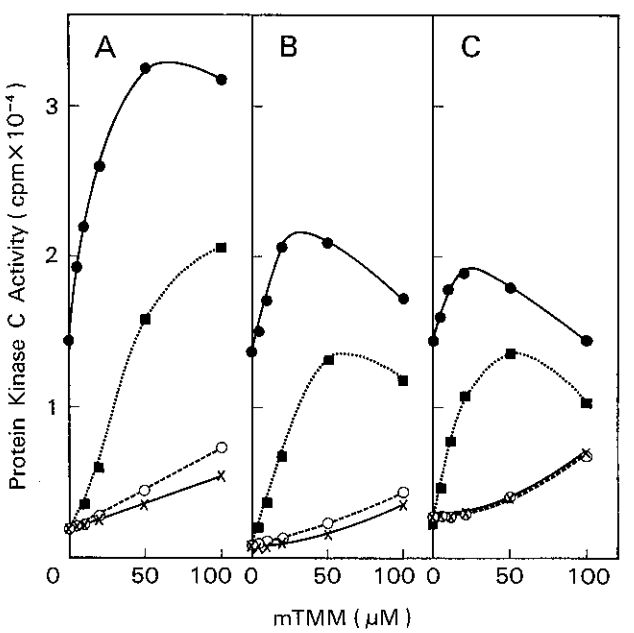

Fig. 3. Activation of PKC subspecies by mTMM. PKC subspecies were assayed in the presence of 1 μM Ca^{2+} at various concentrations of mTMM as described under "Materials and Methods" in the absence of PtdSer and diolein (×) or in the presence of diolein (○), PtdSer (■) or both (●). The concentrations of PtdSer and diolein were 8 μg/ml and 0.8 μg/ml, respectively. (A) The α subspecies, (B) the β subspecies and (C) the γ subspecies.

Effects of diolein on PKC activation by mTMM Activation of PKC subspecies α, β and γ was studied with diolein at concentrations up to 4 μM in the presence of mTMM and PtdSer, or either separately (Fig. 4). mTMM activated PKCα more strongly than PKCβ or γ. All the results suggest that mTMM enhanced the reaction velocity in the presence of both PtdSer and diolein. The results also showed that mTMM and diolein acted differently on the enzyme.

Effect of Ca^{2+} concentration on PKCα activation by mTMM Activation of PKCα was studied with various concentrations of Ca^{2+} from 10^{-7} to 10^{-3} M in the presence of mTMM, diolein and TPA (Fig. 5). mTMM alone or mTMM with PtdSer activated PKCα at unusually high concentrations of Ca^{2+}, around 0.1 to 1 mM, in the absence of diolein. Diolein and PtdSer showed similar PKCα activation to either mTMM and PtdSer or TPA and PtdSer, being Ca^{2+} concentration-dependent. This confirms that diolein acts similarly to TPA, but not in the same way as mTMM. Moreover, in the presence of mTMM and PtdSer, diolein activated PKCα differently than TPA did, depending on Ca^{2+} concentration. It is worth noting that the synergistic action of mTMM and PtdSer with diolein or mTMM and PtdSer with TPA was predominant at lower Ca^{2+} concentrations. Thus, mTMM seems to activate PKCα in the presence of PtdSer and diolein or PtdSer and TPA, independently of Ca^{2+} concentration at the basal level. The activation pattern of mTMM depending on Ca^{2+} concentration is similar to that of arachidonic acid, as reported previously.[11]

Phospholipid specificity The synergistic action of mTMM and diolein on activation of PKC mixture was observed specifically in the presence of PtdSer (Fig. 6). Phosphatidylinositol, phosphatidylethanolamine, phosphatidylcholine and phosphatidylglycerol were practically inactive.

Activation of PKC mixture by various cord factors Two cord factors with C_{80-86} mycolic acids isolated from *M. tuberculosis*, and four cord factors with C_{44-46} mycolic acids isolated from *R. ruber*, were examined for the ability to activate PKC mixture in the presence of

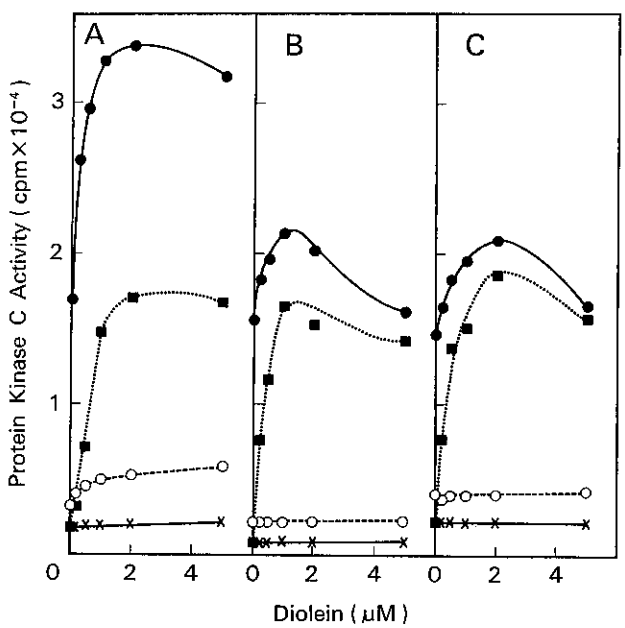

Fig. 4. Effects of diolein on PKC activation by mTMM. PKC subspecies were assayed in the presence of 1 μM Ca^{2+} at various concentrations of diolein in the absence of mTMM and PtdSer (×) or in the presence of mTMM (○), PtdSer (■), or both (●). The concentrations of mTMM and PtdSer were 50 μM and 8 μg/ml, respectively. (A) The α subspecies, (B) the β subspecies and (C) the γ subspecies.

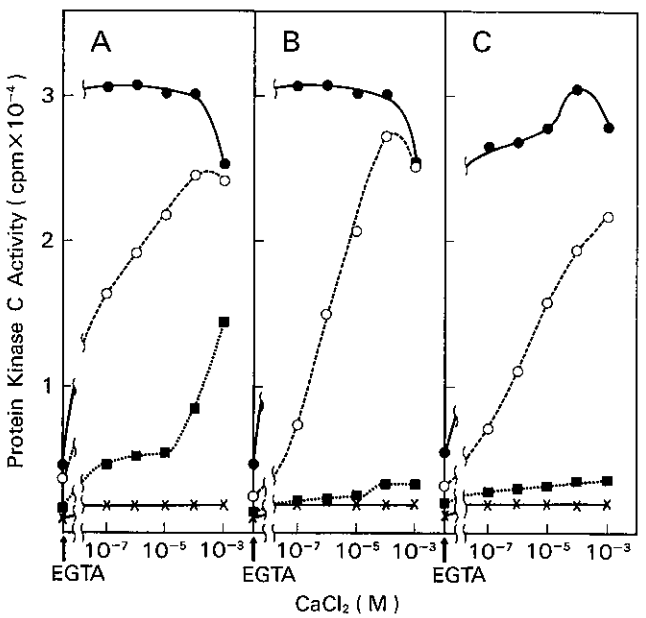

Fig. 5. Effects of Ca^{2+} concentrations on PKC activation by mTMM, diolein and TPA. (A) Activation of PKCα subspecies by 50 μM mTMM with various concentrations of Ca^{2+} in the absence of PtdSer and diolein (■) or in the presence of PtdSer (○), or PtdSer and diolein (●). (B) Activation of PKCα subspecies by 0.8 μg/ml diolein with various concentrations of Ca^{2+} in the absence of PtdSer and mTMM (■) or in the presence of PtdSer (○), or PtdSer and mTMM (●). (C) Activation of PKCα subspecies by 30 nM TPA with various concentrations of Ca^{2+} in the absence of PtdSer and mTMM (■) or in the presence of PtdSer (○), or PtdSer and mTMM (●). PKC activity in the absence of mTMM, diolein, TPA and PtdSer is shown (×). EGTA (5 mM) instead of $CaCl_2$ was added to the reaction mixtures indicated by arrows.

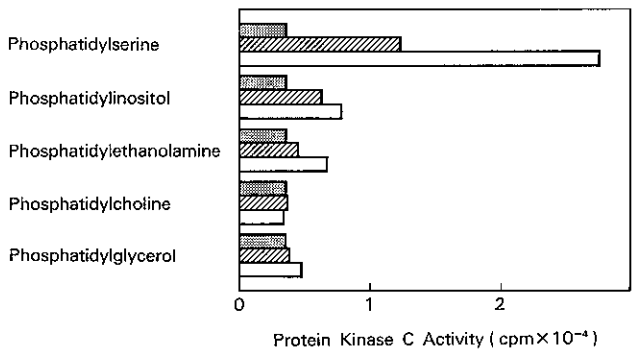

Fig. 6. Activation of PKC mixture by various phospholipids in the presence of 1 μM Ca^{2+}. PKC activity was measured in the presence of phospholipid alone (gray bars), phospholipid plus mTMM (cross-hatched bars), or phospholipid and mTMM plus diolein (white bars). The concentrations of phospholipid, mTMM and diolein were 8 μg/ml, 20 μM and 0.8 μg/ml, respectively.

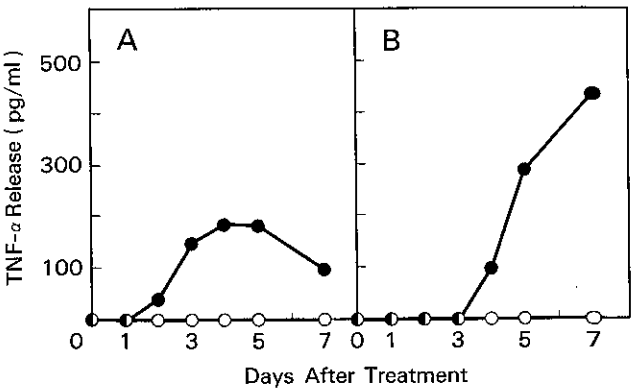

Fig. 7. TNF-α release in mouse serum and that in the lung of mouse following i.p. administration of mTMM. mTMM was i.p. administered to mice, and their sera were aspirated and the lung tissues excised at various days after the administration. Induction of TNF-α release in mouse serum (A) and that in the lung of mouse (B) was measured by use of an ELISA kit, as described under "Materials and Methods." TNF-α release by 10 μg mTMM i.p. administration (●), or by i.p. administration of 0.1 ml of mineral oil alone (○).

Table I. Activation of PKC Mixture by Various Cord Factors

Cord factor[a)]	Enhancement of PKC activation	
	cpm	(fold)
None	2,200	
M. tuberculosis		
mTMM	14,800	(6.7)
mTDM	9,300	(4.2)
R. ruber		
rTMM	5,600	(2.5)
rTDM	3,400	(1.5)
rMMM	3,000	(1.4)
rGMM	2,500	(1.1)

a) Each cord factor (50 μM) was added to the standard assay mixture in the presence of PtdSer.

PtdSer. mTMM was able to enhance PKC activation more strongly than mTDM and cord factors of *R. ruber*; the latter were not significantly active (Table I).

TNF-α release by mTMM Cord factors are thought to induce biological effects on tissues through PKC activation, and one type of target cell is assumed to be macrophages.[15] Mouse TNF-α release was found in serum 3 days after i.p. administration and the maximum level, a concentration of 180 pg TNF-α/ml, was reached around 4 and 5 days after treatment (Fig. 7). No TNF-α release was observed by treatment with mineral oil alone as a control. Macrophages are thought to be activated by mTMM. Next, TNF-α was found in supernatants of lung homogenates 4 days after treatment. TNF-α levels continuously increased up to 7 days, suggesting that mTMM induced migration of macrophages into the lungs, and macrophages containing high levels of TNF-α accumulated in the lungs. The evidence indicates that mTMM and probably other cord factors are able to induce TNF-α, an endogenous tumor promoter in the lungs.

DISCUSSION

TPA, teleocidin and aplysiatoxin, which are potent activators of PKC, are important tools in studies of signal transduction.[19,20] However, it is not yet known whether these compounds are directly involved in development of human cancers. Although diacylglycerol has a similar function to TPA, about 1000 times larger amount of diacylglycerol than TPA is required for *in vitro* PKC activation.[21] How does diacylglycerol physiologically function in the cell? Shinomura *et al.* reported the synergistic action of arachidonic acid with diacylglycerol for each PKC subspecies.[11] The ratio of arachidonic acid and diacylglycerol was 50 μM to 1.2 μM in *in vitro* PKC activation. Since arachidonic acid is produced by phospholipase A_2 during the inflammatory process,[19] the synergistic action of arachidonic acid with diacylglycerol for PKC activation could be associated with the process of tumor promotion.

We demonstrated that cord factors enhanced PKC activation and in particular, PKCα activation in the presence of PtdSer and diacylglycerol, and that mTMM mimics the action of arachidonic acid. Like arachidonic

acid, mTMM together with diacylglycerol and PtdSer increased the apparent affinity of PKC for Ca^{2+} and finally activated the enzyme at basal Ca^{2+} concentrations. However, there are some differences between the effects of mTMM and arachidonic acid; namely, mTMM was synergistic with PtdSer, whereas arachidonic acid was synergistic with diacylglycerol. Thus, mTMM and arachidonic acid interact differently with the enzyme. This conclusion was also supported by evidence that mTMM specifically activates PKCα in the presence of PtdSer and diacylglycerol, rather than PKCβ and γ, whereas arachidonic acid activates the three PKC subspecies to the same extent.[11] In addition, the mode of activation by mTMM is distinct from that by TPA, and lipid A, an endotoxic lipopolysaccharide, which acts similarly to PtdSer.[22]

We have tested six cord factors in order to look at the structure-function relationship. mTMM with monomycolic acid showed stronger PKC activation than mTDM with two mycolic acids. Four cord factors isolated from *R. ruber*, which is a non-pathogenic bacterium,[12] did not show strong activation compared with those isolated from *M. tuberculosis*. Cord factors from *M. tuberculosis* have longer chain lengths than those from *R. ruber*, suggesting that the chain length of cord factors is related to pathogenicity, and there may be an optimum length for interaction between cord factor and PKC. Mannose mycolate, fructose mycolate and arabinose mycolate (all with shorter C_{36-48} chain lengths) isolated from *Nocardia rubra*, were reported to be inactive.[10]

In this experiment, PKCα isolated from mouse brain was predominantly activated. Kosaka *et al.* reported that the brain of rat has PKCα similar to that of the lung.[23] The lung is a unique target tissue of cord factors, based on the development of interstitial pneumonitis and granuloma formation associated with infiltration of macrophages and lymphocytes.[15] Thus, macrophages are assumed to be essential for production of pulmonary lesions induced by cord factors, indicating that the induction of macrophage-derived cytokines and factors, such as TNF-α, IL-1, GM-CSF and ICAM-1 could be related to the production of pulmonary lesions.[24] It is possible that PKCα activation by mTMM develops pulmonary lesions in humans through the induction of these cytokines.

The relationship between TNF-α and tumor promoter should be briefly discussed. We recently demonstrated that human TNF-α stimulated transformation of BALB/3T3 cells initiated with MCA, and induced clonal growth of Bhas 42 cells, BALB/3T3 cells containing viral H-*ras* gene, that is, so-called initiated cells; TNF-α did not induce clonal growth of normal BALB/3T3 cells.[13] This is an essential process of tumor promotion. The results led us to conclude that TNF-α is an endogenous tumor promoter and a central mediator of tumor development.[25] Based on this concept, we studied the induction of TNF-α by mTMM in the lung. Continuous increase of TNF-α release was found in the lung, suggesting that the initiated cells are exposed to TNF-α, an endogenous tumor promoter that is released from activated macrophages in the lung tissues.

The correlation between lung cancer and history of pulmonary tuberculosis is based on cord factors, which were found to induce endogenous tumor promoter in the lung. Although it is not yet known how initiation can be caused during the process of lung tuberculosis, initiation is thought to occur often in humans. As for production of tumor promoter in lung tuberculosis, high concentrations of cytokines, such as TNF-α, are observed in miliary tuberculosis and advanced pulmonary tuberculosis.[24] Since tumor promotion is a long process, cancer development in patients with pulmonary tuberculosis may be preventable by measures to inhibit PKC activation in macrophages and TNF-α release in the lung. Our results indicate that mTMM is a new TNF-α inducer and mediates the release of this tumor promoter in the lung through PKC activation.

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REFERENCES

1) Steinitz, R. Pulmonary tuberculosis and carcinoma of the lung. *Am. Rev. Respir. Dis.*, **92**, 758–766 (1965).
2) Hinds, M. W., Cohen, H. I. and Kolonel, L. N. Tuberculosis and lung cancer risk in nonsmoking women. *Am. Rev. Respir. Dis.*, **125**, 776–778 (1982).
3) Aoki, K. Excess incidence of lung cancer among pulmonary tuberculosis patients. *Jpn. J. Clin. Oncol.*, **23**, 205–220 (1993).
4) Yano, I., Oka, S., Nakatsuhara, Y., Kato, Y., Tomiyasu, I. and Kaneda, K. Molecular structure and immunopharmacological activities of the new glycolipids containing mycolic acids in actinomycetales. *In* "Biology of Actino-

mycetes '88," ed. Y. Okami, T. Beppu and H. Ogawara, pp. 469–477 (1988). Japan Scientific Societies Press, Tokyo.
5) Natsuhara, Y., Yoshinaga, J., Shogaki, T., Sumi-Nishikawa, Y., Kurano, S., Kato, Y., Kaneda, K., Oka, S. and Yano, I. Granuloma-forming activity and antitumor activity of newly isolated mycoloyl glycolipid from *Rhodococcus terrae* 70012 (Rt. GM-2). *Microbiol. Immunol.*, **34**, 45–53 (1990).
6) Oldham, R. K. Biological response modifiers. *J. Natl. Cancer Inst.*, **70**, 789–797 (1983).
7) Bekierkunst, A., Levij, I. S., Yarkoni, E., Vilkas, E., Adam, A. and Lederer, E. Granuloma formation induced in mice by chemically defined mycobacterial fractions. *J. Bacteriol.*, **100**, 95–102 (1969).
8) Ribi, E., Takayama, K., Milner, K., Gray, G. R., Goren, M., Parker, R., McLaughlin, Ch. and Kelly, M. Regression of tumors by an endotoxin combined with trehalose mycolates of differing structure. *Cancer Immunol. Immunother.*, **1**, 265–270 (1976).
9) Matsunaga, I., Oka, S., Inoue, T. and Yano, I. Mycolyl glycolipids stimulate macrophage to release a chemotactic factor. *FEMS Microbiol. Lett.*, **67**, 49–54 (1990).
10) Natsuhara, Y., Oka, S., Kaneda, K., Kato, K. and Yano, I. Parallel antitumor, granuloma-forming and tumor-necrosis-factor-priming activities of mycoloyl glycolipids from *Nocardia rubra* that differ in carbohydrate moiety: structure-activity relationship. *Cancer Immunol. Immunother.*, **31**, 99–106 (1990).
11) Shinomura, T., Asaoka, Y., Oka, M., Yoshida, K. and Nishizuka, Y. Synergistic action of diacylglycerol and unsaturated fatty acid for protein kinase C activation: its possible implications. *Proc. Natl. Acad. Sci. USA*, **88**, 5149–5153 (1991).
12) Kaneda, K., Sumi, Y., Kurano, F., Kato, Y. and Yano, I. Granuloma formation and hemopoiesis induced by C38-48-mycolic acid-containing glycolipids from *Nocardia rubra*. *Infect. Immun.*, **54**, 869–875 (1986).
13) Komori, A., Yatsunami, J., Suganuma, M., Okabe, S., Abe, S., Sakai, A., Sasaki, K. and Fujiki, H. Tumor necrosis factor acts as a tumor promoter in BALB/3T3 cell transformation. *Cancer Res.*, **53**, 1982–1985 (1993).
14) Silva, C. L. and Faccioli, L. H. Tumor necrosis factor (cachectin) mediates induction of chachexia by cord factor from mycobacteria. *Infect. Immun.*, **56**, 3067–3071 (1988).
15) Sakamoto, Y., Goren, M. B. and Kirkpatrick, C. H. Phenotypes of infiltrating cell in trahalose dimycolate-induced interstitial pneumonitis. *Infect. Immun.*, **57**, 2098–2106 (1989).
16) Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. *J. Biol. Chem.*, **254**, 3692–3695 (1979).
17) Sekiguchi, K., Tsukuda, M., Ase, K., Kikkawa, U. and Nishizuka, Y. Mode of activation and kinetic properties of three distinct forms of protein kinase C from rat brain. *J. Biochem.*, **103**, 759–765 (1988).
18) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254 (1976).
19) Nishizuka, Y. The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature*, **308**, 693–698 (1984).
20) Fujiki, H. and Sugimura, T. New classes of tumor promoters: teleocidin, aplysiatoxin, and palytoxin. *Adv. Cancer Res.*, **49**, 223–264 (1987).
21) Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.*, **257**, 7847–7851 (1982).
22) Romano, M. and Hawiger, J. Interaction of endotoxic lipid A and lipid X with purified human platelet protein kinase C. *J. Biol. Chem.*, **265**, 1765–1770 (1990).
23) Kosaka, Y., Ogita, K., Ase, K., Nomura, H., Kikkawa, U. and Nishizuka, Y. The heterogeneity of protein kinase C in various tissues. *Biochem. Biophys. Res. Commun.*, **151**, 973–981 (1988).
24) Shijubo, N., Imai, K., Nakanishi, F., Yachi, A. and Abe, S. Elevated concentrations of circulating ICAM-1 in far advanced and miliary tuberculosis. *Am. Rev. Respir. Dis.*, **148**, 1298–1301 (1993).
25) Fujiki, H. and Suganuma, M. Tumor necrosis factor-α, a new tumor promoter, engendered by biochemical studies of okadaic acid. *J. Biochem.*, **115**, 1–5 (1994).