2）地域格差の拡大、3）集団感染、4）結核菌の潜伏感染、5）多剤耐性結核菌の出現（初回耐性：1％、獲得耐性：20％、初回＋獲得耐性：2％）、6）有効な新規ワクチン開発、7）ヒト免疫不全ウイルス（HIV）感染症／後天性免疫不全症候群（AIDS）の重複感染などがある。

表1．世界における感染症による死亡数（2000）

感染症	死亡数:万人
全感染症	1,350
急性呼吸器感染症	396
AIDS（結核の合併を含む）	267
下痢性疾患	220
結核	200
マラリア	109
麻疹	89
参考：年間総死亡	5,400

世界保健機関、2000

結核菌の概要

結核菌（*M. tuberculosis*）の生物学的特徴として、1）細胞内寄生性、2）脂質成分に富む細胞壁、3）好気性、4）遅発育性、5）空気（飛沫核）感染、6）慢性炎症および7）遺伝子の解読などがある（表2）[1]。結核菌など抗酸菌は基本的に外毒素や内毒素非産生性であるが、例外的に*M. ulcerans*（西アフリカ諸国で猛威を奮っているBuruli潰瘍の原因菌）が外毒素（mycolactone、別名：polyketide toxin、宿主組織に壊死を惹起する）を産生する[2]。炎症病変や組織障害は結核菌に対する感染免疫応答過程で宿主から産生されるサイトカインをはじめとする生理活性物質に依存している。結核菌の細胞壁は長鎖脂肪酸（ミコール酸）に富み、グラム染色では難染色性を示す。そのため、抗酸性（Ziehl-Neelsen、Kinyoun）染色や蛍光染色が用いられる。抗酸性染色は石炭酸フクシンで加温染色後，塩酸アルコールで脱色，メチレンブルーで後染色する。抗酸菌は“赤い桿菌”として観察される。抗酸菌以外の通常細菌やヒト組織・細胞は後染色のメチレンブルーにより“青く”対比染色される。抗酸菌をオーラミンやローダミンなどの蛍光色素を用いる蛍光染色法も広く用いられている．分裂倍加時間は約12～15時間の遅発育菌であり、感染伝播は飛沫核（空気）感染による。宿主防御機構では、マクロファージ—サイトカイン—T細胞応答系、すなわち、細胞性免疫が役割を演じ、細胞内殺菌物質として、ガス状物質（反応性酸素化合物質や反応性窒素化合物質）が寄与している。その結果、結核菌感染者の約10％が一生涯において結核を発病する。病変は慢性炎症、肉芽腫、乾酪壊死、空洞形成や線維化などが特徴的である。*M. tuberculosis* H37Rvの全ゲノム塩基配列が解明された[3]。今後、遺伝子解析を基盤とした科学的戦略が推進され、分子／遺伝子標的を視点とした新規診断法、抗結核薬の開発、薬剤耐性獲得機構の解明や新規ワクチン開発が展開されるであろう[4]。

表2．結核菌の特徴

細胞内寄生性：	桿菌（0.2-0.6 x 1-10 μm）、宿主細胞、特に、マクロファ-ジ内で抗菌機構から逃れて増殖
細胞壁：	脂質成分が豊富なため、疎水性であり、化学物質にも安定、グラム染色に難染色性、抗酸性
好気性：	酸素分圧の高い臓器（肺など）で増殖し、病変を形成
遅発育性：	至適温度：37℃、倍加時間：約12-15時間、培養集落形成に4-8週間
感染形式：	飛沫核／空気感染
病原性：	慢性炎症、肉芽腫、乾酪壊死、空洞形成、線維化
遺伝子：	全ゲノム（約4.41 Mb）の解読

結核菌の病原性

結核菌の病原性（表3）として、1）宿主防御機構からの逸脱や2）遅延型過敏反応（細胞性免疫応答の負の側面）の誘導があり、その結果、結核菌感染から発病に至る長期の潜伏期間、組織破壊を伴う肉芽腫炎症が特徴的である[5]。

表3．抗酸菌の病原性と病原因子

病原性	宿主防御機構から逸脱 遅延型過敏反応の誘導	
	化学的性状	生物学的活性
病原因子	細胞壁表層ミコール糖脂質（TDM、SL）	肉芽腫誘導やP-L融合の阻害
	Lipoarabinomannan	マクロファージ活性化の阻害、TNF-α誘導による組織障害、Th1 細胞応答抑制性サイトカインの誘導
	補体活性化因子	補体受容体3や4、オプソニン化による貪食能の亢進
	65 kDa熱ショック蛋白	宿主の免疫的交叉反応性による自己免疫の誘導
	DNA結合蛋白	接着や侵入の促進
	外毒素	mycolactone（*M. ulcerans*）

結核菌の潜伏感染

肺結核患者（特に、喀痰塗抹陽性）から曝露された約30％に結核菌感染が成立し、感染者の約10％が一生涯において結核を発病する。有効な感染防御応答により、90％の感染者は結核菌を封じ込め、発病を回避している。しかし、結核菌は宿主

内で潜伏感染している。潜伏感染した結核菌は宿主免疫機構の破綻（老化、免疫抑制薬／副腎皮質ステロイド薬投与、栄養障害、HIV感染／AIDSなど）により、発育・増殖を再開し、結核を発病するに至る（内因性再燃）。人類の約1/3が結核菌に潜伏感染している事実を考慮すると、潜伏感染機序を解明することは新規抗結核薬やワクチン開発を促進し、その結果、結核制圧に寄与するであろう[6]。潜伏感染した結核菌の特性として、1）定常期に発現するσ因子（*sigF*遺伝子、対数増殖期には未発現）や2）糖代謝から脂質代謝への変換が知られている。*sigF*欠損結核菌は肉芽腫内で生存不能であり、肉芽腫内生存結核菌はglyoxylate shunt（脂肪酸から糖代謝への変換酵素系、哺乳動物の冬眠におけるエネルギー代謝変換に類似）を亢進させている[7]。

肉芽腫炎症と感染防御の統御

結核菌など抗酸菌は細胞内寄生病原体であり、宿主防御にマクローファージ—サイトカイン—CD4陽性1型ヘルパーT（Th1）細胞応答系、細胞性免疫が貢献している。細胞性免疫の起動サイトカインとして、interleukin（IL）-12、IL-18やinterferon（IFN）-γがTh1細胞分化や活性化など、重要な役割を演じている[8]。しかし、結核菌感染に対する遅延型過敏反応を含む細胞性免疫の発現は抗結核菌防御と組織傷害に貢献、すなわち、功罪の二面性（諸刃の剣）を表現する[9]。また、遺伝的因子として、ヒト第2染色体に存在する遺伝子（*NRAMP1*：natural resistance associated macrophage protein 1、別名＝*SLC11A1*）が感染防御に関与し、この機能はマクロファ-ジに表現されている。初回の結核菌曝露の場合、宿主の炎症応答は非特異的であり、普遍的な細菌感染に対する炎症応答に類似している。感染約4～6週後に乾酪壊死を伴う肉芽腫炎症が生じ、また、結核菌ツベルクリン蛋白質抗原に遅延型過敏反応、ツベルクリン皮内反応が成立する（図1）。この機序として、病変部において炎症惹起性サイトカイン（IL-1やtumor necrosis factor α：TNF-α）、Th1細胞関連サイトカイン（IL-12、IL-18、IFN-γ）や単球走化性ケモカイン（monocyte chemoattractant protein 1：MCP-1やmacrophage inflammatory protein 1αなど）が産生され、マクロファ-ジの局所的集積（肉芽腫）、加えて、細胞性免疫（遅延型過敏反応を含む）が誘導される。

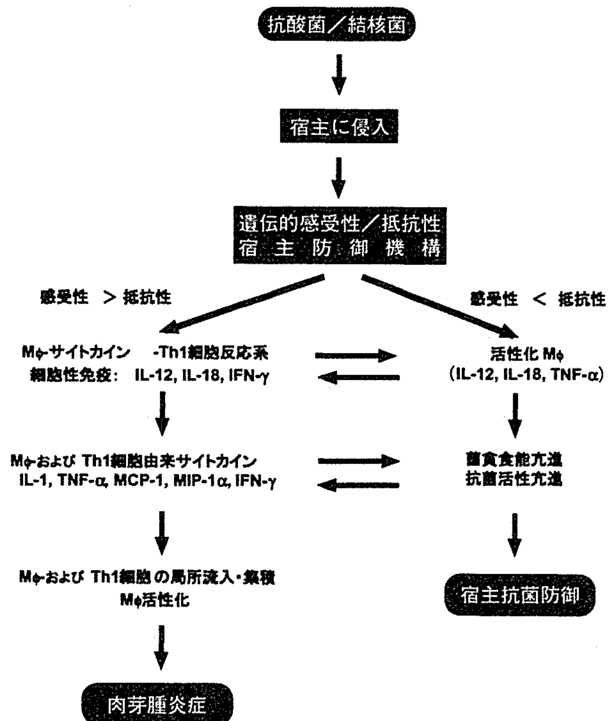

図1. 抗酸菌／結核菌感染における宿主細胞および機能分子応答機構
Mo：マクロファージ

結核菌の病原因子

結核菌病原因子として、細胞壁表層構成成分が関与していると考えられている[1]。病原因子として、1）細胞壁表層糖脂質、2）lipoarabinomannan、3）補体活性化因子、4）熱ショック蛋白質や5）抗酸菌DNA結合蛋白質などがある（表3）。本稿では、特に、細胞壁表層糖脂質や抗酸菌DNA結合蛋白質について、概説する。

細胞壁糖脂質

結核菌の脂質は乾燥菌体重量の10%以上、細胞壁の20%以上を構成し、他の一般細菌に比し、極めて多い。事実、結核菌の全ゲノムは約4.4 Mb

（大腸菌：4.6 Mb）であり、蛋白質を規定している遺伝子は約4000、脂肪酸代謝に関与している酵素は250以上、大腸菌が50であることから、結核菌の脂質代謝が極めて旺盛であることが遺伝子情報からも判明している[3]。結核菌細胞壁の脂質として、mycolyl-arabinogalactan-peptidoglycan complex、lipoarabinomannan（LAM）、lipomannan、phosphatidyl-*myo*-inositol、sulfolipid（SL）、trehalose 6,6'-dimycolate（TDM）/cord factor、phenolic glycolipidやlipooligosaccharidesなどの糖脂質が特徴的である[10]。

特に，アシル化trehalose脂質化合物であるTDM/cord factorやSL（図２）が結核菌に特徴的であり、結核菌-宿主関係、すなわち、病原性や毒性の発現に関与している。また、“抗酸性”に主として関与する菌体表層成分はミコール酸などの脂質成分であり、ミコール酸は天然で稀なα位に分枝鎖、β位に水酸基を持つ長鎖脂肪酸（結核菌では炭素数：60～90）である。TDMの構造—活性連関で側鎖炭素鎖長が病原性に関与している[11]。

宿主マクロファージは結核菌を貪食し、食胞体（phagosome）を形成するが、アシル化trehalose脂質化合物（SLやTDM）が加水分解酵素を含むリソゾーム（lysosome）との融合（P-L fusion）を阻害することにより、酸性化されず、食胞体内、すなわち、宿主細胞内での生存を可能にしている。

肉芽腫炎症は発症機序により、異物性（T細胞非依存性）および過敏性（T細胞依存性）に大別（図３）される。TDMを無胸腺ヌードマウスや未免疫マウスに投与することにより、肉芽腫を惹起できること、TDM免疫マウスではTDM誘導肉芽腫が増強されることや遅延型過敏反応／細胞性免疫の指標である足蹠腫脹反応が誘導されることから、TDMは異物性および過敏性の両機序を介して、肉芽腫を形成する[12]。事実、TDM誘導肉芽腫病変は多量の細胞性免疫起動性サイトカイン（IL-12やIFN-γ）を含み、活動性に伴い、消長する[12]。従って、結核肉芽腫の発現に異物性および過敏性の両機序が複合関与し、結核肉芽腫は混合性肉芽腫である。肉芽腫形成には病変局所への血中単球の流入や活性化が必須であり、このため、局所のcc-ケモカイン産生や血管新生が重要な役割を演じる。TDMは血管内皮細胞細胞増殖因子（vascular endothelial growth factor：VEGF）を誘導し、局所の血管新生に寄与している[13]。加えて、TDMは宿主免疫担当細胞（胸腺や脾臓）にアポトーシスを誘導し，その結果，細胞内寄生病原体の増殖や生存を困難にし，さらに胸腺内自己反応性T細胞を除去，Th1/Th2細胞の分化を制御することにより自己免疫疾患の発症を防止している可能性がある[14]。しかし、SLはP-L fusion阻害（宿主細胞内生存）を発揮したが、炎症・免疫惹起やアポトーシス誘導活性を全く示さず、TDMと対照的である[12)~14]。アシル化trehalose脂質化合物は結核菌細胞壁表層に存在し、１）結

Trehalose 6,6'-dimycolate/Cord factor (TDM)

2,3,6,6'-tetra acyl trehalose 2'-sulfate/Sulfolipid (SL)

図２．アシル化トレハロース脂質の化学構造

結核菌-TDM
マクロファージ
炎症性サイトカイン：IL-1, TNF-α
CC-ケモカイン：MCP-1, MIP-1α
T細胞非依存性
T細胞依存性
マクロファージの流入
マクロファージ活性化
異物性肉芽腫
Th1誘導性サイトカイン：IL-12
Th1細胞の分化・誘導・活性化
IFN-γ
細胞性免疫
遅延型過敏反応
過敏性肉芽腫

図３．結核性肉芽腫炎症機序と細胞壁TDM

核菌の宿主細胞内生存、2）炎症・免疫惹起物質（肉芽腫炎症、遅延型過敏反応、血管新生など）や3）アポトーシス誘導活性を有する多機能分子である（図4）。

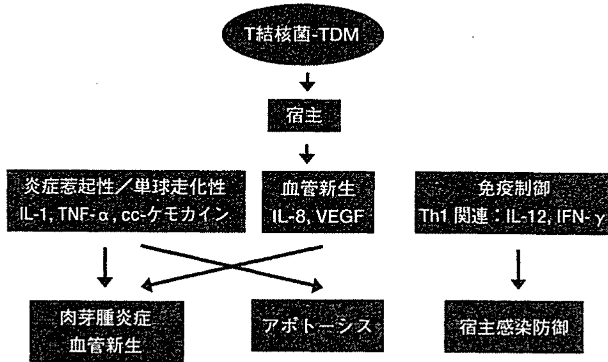

図4．結核菌細胞壁TDMと宿主応答の分子機序

抗酸菌DNA結合蛋白質
(mycobacterial DNA-binding protein 1 ：MDP1)

遅発育型抗酸菌が定常期や休眠期において最も大量に発現する蛋白質であるMDP1（分子量：28 kDa）は抗酸菌体内や表層に存在する抗酸菌特異的蛋白質である。機能的に細胞質内MDP1は核酸結合性を示し、転写および翻訳阻害活性を有し、そのため、休眠機構、さらに、潜伏感染に関与していることが示唆されている。他方、菌表層MDP1の生物学的意義は不明であった。MDP1が宿主細胞表面に存在するglycosaminoglycan、特に、ヒアルロン酸に結合し、抗酸菌の接着／侵入に関与することが判明した[15]。ヒアルロン酸は細胞外マトリックスの主要構成成分として組織の形態決定や細胞浸潤に関与、さらに、細胞表面受容体を介して細胞を活性化・増殖や分化を促す生理活性物質でもある。低分子ヒアルロン酸はCD44介在性にマクロファージを集積させ、抗酸菌殺傷活性を有する一酸化窒素産生を促す。加えて、ヒアルロン酸は抗原提示樹状細胞の活性化を誘導する。すなわち、ヒアルロン酸は宿主感染防御の成立に関与している。ヒアルロン酸は多機能分子であり、ヒアルロン酸が抗酸菌に対する宿主細胞受容体としての機能以外に、結核病態形成や防御への関与を示唆している。

病原因子の将来展望・臨床応用

細胞壁表層糖脂質や抗酸菌DNA結合蛋白質に関し、臨床医学的視点（診断、治療および予防）に立脚し、現在の状況を概説する。

抗酸菌細胞壁糖脂質を抗原とした血清診断

感染宿主は抗酸菌細胞壁糖脂質に対し抗体産生など液性免疫応答を表現するため、抗酸菌細胞壁糖脂質抗原を用いた血清診断が開発されている。結核菌細胞壁糖脂質抗原（TDM）による血清診断は感度（80%）および特異度（95%）であり、特に、喀痰塗抹陰性や培養陰性結核に有用な診断方法であることが示唆されている[16]。

M. avium complex（MAC）感染症は結核など抗酸菌感染症の約20%を占める。MACは環境菌であり、普遍的に存在し、臨床および病原体診断の確定には臨床経過を考慮するため、長期間を要する。MAC特異的細胞壁表層糖ペプチド脂質（GPL）抗原（図5）を用いた迅速・簡便血清診断法は感度および特異度ともに良好な成績（90%以上）を示し、また、血清抗GPL抗体価はMAC感染症の疾患活動性を反映した[17]。従って、GPL抗原を用いた血清診断はMAC感染症の診断や疾患活動性の評価に有用であり、今後、大規模臨床試験など、臨床応用が期待される。

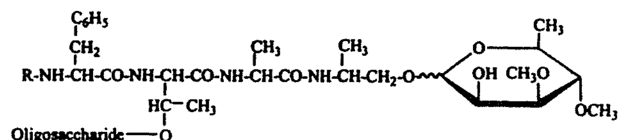

図5．MAC特異的細胞壁表層糖ペプチド脂質（GPL）の化学構造

抗酸菌の宿主細胞接着や侵入を標的とした治療・予防介入

抗酸菌の宿主細胞接着や侵入に関するMDP1-ヒアルロン酸関係をマウス気道抗酸菌感染モデルにて検証した。その結果、ヒアルロン酸をBCGと同時、或いは、感染成立後に投与することにより、

感染菌数が著しく減少した。BCG-宿主細胞結合をヒアルロン酸が阻害し、定着菌数が減少した結果であろう[15)]。現行の治療戦略に抗酸菌の接着や侵入を標的とした治療予防戦略は存在しないため、MDP1やヒアルロン酸が結核予防や治療戦略の開発に有望な候補になる可能性を示している。

おわりに

抗酸菌病原因子や宿主応答の分子機序は未解明な部分が多く、抗酸菌感染症の制圧には抗酸菌病原因子や宿主応答の分子機序の解明、さらに、この機序の解明は新規治療戦略やワクチン開発に寄与するであろう。

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Molecular Pathogenesis of Mycobacterial Diseases

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Key words : disease progression, host defense, mycobacterial DNA binding protein, serodiagnosis, surface glycolipid

Mycobacterial diseases, including tuberculosis, leprosy, and disease due to nontuberculous mycobacteria, are the major cause of death from infectious diseases around the world. About one-third of the world population is latently infected with *Mycobacterium tuberculosis*. Over 8 million new cases and nearly 2 million deaths occur each year. Tuberculosis presents a significant health threat to the world. The pathogenicity of mycobacteria is related to their ability to escape killing by ingested macrophages, latent infection, and induce delayed type hypersensitivity. This has been attributed to several components of the mycobacterial cell wall, such as surface glycolipids, lipoarabinomannan, complement activation factor, heat-shock protein, and mycobacterial DNA binding protein. From the aspect of my research interests, I have focused on mycobacterial glycolipids and mycobacterial DNA binding protein in this article. Surface molecules of mycobacteria exert pleiotropic activities in both the microbe and host, and thus participate in the pathogenesis of mycobacterial diseases. The better understanding of mycobacterial pathogenicity may open the new avenue for the development of therapeutic and prophylactic interventions.

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感染症と移植
PART 4

結　核
―世界最大の感染症―

小 林 和 夫*

Tuberculosis : Tuberculosis as a major public health threat

世界では，約20億人（人類の1/3）が結核菌（*Mycobacterium tuberculosis*）に既感染，毎年800万人が結核を発病，200万人が死亡している．日本では，年間3万3,000人（罹患率人口10万対：25.8）が結核を発病，2,300人（死亡率：1.8）が死亡し，結核は単一病原体による世界最大の感染症である．結核制圧目標は，喀痰塗抹陽性肺結核罹患率：0.1/10万人以下であるが，日本の現状は9.4であり，制圧目標には程遠い．類縁である非結核性抗酸菌（*M. avium* complex や *M. kansasii* など）感染症は結核など抗酸菌感染症の約20％を占める．非結核性抗酸菌は環境菌であり，また，*M. avium* complex は抗微生物薬に対し多剤耐性を示すため，治療に難渋している．結核対策の重点項目として，① 直接監視下短期抗結核化学療法の普及，② 潜在性結核菌感染対策，③ HIV 感染/AIDS と結核の重複感染に対する効果的な戦略，④ 多剤耐性結核に対する抗結核薬，⑤ 迅速，簡便な診断法，⑥ 有効なワクチンの開発，⑦ 集団や院内感染対策，が推進され，結核が制圧されることを期待している．

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key words : 再興感染症（re-emerging infectious disease），多剤耐性結核（multidrug-resistant tuberculosis），直接監視下短期抗結核化学療法（directly observed treatment, short course：DOTS），施設内感染（intrainstitutional infection），潜在性結核感染（latent tuberculosis infection）

結核は代表的な再興感染症であり，世界および日本においても，単一病原体感染症として，人類に甚大な健康被害を提供している．世界の感染症による年間死亡（2000年）は1,350万人（総死亡：5,400万人の約1/4）を占め，感染症死亡の内訳では，呼吸器感染症：約400万人，後天性免疫不全症候群（AIDS）（結核合併を含む）：約270万人，下痢性疾患：220万人，結核：200万人，マラリア：110万人である[1,2]（**表1**）．

呼吸器感染症や下痢性疾患の原因病原体は単一でなく，多種多様である．そのため，世界保健機関や G8 サミット[3]は，① ヒト免疫不全ウイルス（HIV）感染/AIDS，② 結核，③ マラリア（熱帯熱マラリア）による死亡が年間約500万人，患者発生が3億人であることから，これら三大疾患を最重要感染症に認定し，世界が協調して対策を構築することを宣言している．また，1993年に世界保健機関，1999年に厚生省（現厚生労働省）が“結核緊急事態宣言”を発表，結核問題を再認識し，制圧対策を推進している．

結核の発生動向

世界では，約20億人（全人口の1/3）が結核菌（*Mycobacterium tuberculosis*）に既感染（ほとんどは

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表1 世界における感染症による死亡数(2000年)

感染症	死亡数(万人)
全感染症	1,350
急性呼吸器感染症	396
AIDS(結核合併を含む)	267
下痢性疾患	220
結核	200
マラリア	110
麻疹	90
破傷風	38
百日咳	30
性感染症	18
髄膜炎	17

(世界保健機関, 2000)

表2 世界および日本の結核発生動向

	結核菌既感染者数	年間死亡数	新規登録患者数	有病者数
世界	20億人	200万人	800万人	2,200万人
日本	0.3億人	0.23万人	3.3万人	3.2万人

潜在性), 毎年800万人が結核を発病, 200万人が死亡し, 有病者は2,200万人である[4]. なお, 結核関連死亡(結核, 結核とHIV感染/AIDSの重複)は350万人である. すなわち, 毎秒, 新規結核患者が発生し, 毎15秒に1人が結核で死亡, 1人の無治療結核患者が年間10～15人の感染者を生じさせ, 世界の人口の約1%が毎年, 結核菌に感染している. なお, 結核菌感染後の生涯にわたる発病率は5～10%である. 世界保健機関は, 今後約20年間に10億人の新規感染者が発生, 1億5,000万人が結核を発病, そして, 3,600万人の結核死亡を予測している[5].

日本では, 年間3万3,000人(罹患率人口10万対: 25.8)が結核を発病, 2,300人(死亡率: 1.8)が死亡し, 有病者は3万2,000人(有病率: 25.4)であり, 結核は単一病原体による最大の感染症である[6](**表2**).

日本における結核対策の課題(**表3**)として, ① 急速な人口の高齢化に伴う高齢者結核の増加(70歳以上の占める割合: 約42%), ② 国内地域格差の拡大(最高罹患率は大阪市: 74.4, 最低は長野県: 12.5), ③ 集団や院内感染の続発および増加(1998～2001年合計: 166件, 結核集団感染の定義は同一の感染源が, 2家族以上にまたがり, 20人以上に結核を感染させた場合, なお, 発病者1人は感染者6人と換算する), ④ 多剤耐性結核菌の出現(初回耐性: 1～2%, 再治療: 10～20%, 多剤耐性結核菌の定義はイソニアジド: INHとリファンピシン: RIFに少なくとも同時耐性), ⑤ 特異的, 迅速かつ簡便な結核菌感染の検査法の開発, ⑥ 潜在性結核菌感染対策などがある. 加えて, HIV感染/AIDSが着実に増加している現状[7]を考慮した場合, 日本においても, ⑦ HIV感染/AIDSと結核の重複感染は将来的に重要な課題となることが想定される. 今後, 再興感染症として, 結核の重要性を認識し, 確実な治療や予防対策を推進することが肝要である.

表3 結核対策の課題

- 高齢者結核の増加
- 国内地域格差の拡大
- 集団や院内感染の続発および増加
- 多剤耐性結核菌の出現
- 特異的, 迅速かつ簡便な結核菌感染の検査法の開発
- 潜在性結核菌感染対策
- HIV感染/AIDSと結核の重複感染

感染症の発生動向将来予測として, 社会基盤の整備, 抗微生物化学療法やワクチンで治療・予防可能な疾患(下痢性疾患, 肺炎, 麻疹など)は減少することが考えられるが, HIV感染/AIDSや結核は, 今後も現状維持あるいは増加することが予測されている[8].

結核の増加要因

結核の増加要因は, 社会要因, 宿主要因, および病原体要因に大別される[9].

社会要因として, 国際化, 交通機関の発達による高速・大量移動, 都市化による過密, 貧困, 受診や診断の遅延, 結核対策の軽視などが寄与している. 宿主要因として, 感染抵抗力の減弱(人口の高齢化, 糖尿病, 慢性腎不全, HIV感染/AIDS, 免疫抑制薬/臓器移植や免疫疾患など)が

表4 結核の増加要因

社会要因
● 高速，海外旅行および移民の増加(年間海外旅行者：約1,600万人，海外からの旅行者：約500万人，外国人登録者：約150万人)
● 貧困や衛生状態の低下を伴う人口過密都市
● 受診や診断の遅延
● 結核対策の不備や軽視
宿主要因
● 易感染性宿主の増加(人口の高齢化，糖尿病，慢性腎不全，HIV感染/AIDS，免疫抑制薬/臓器移植や免疫疾患など)
病原体要因
● 薬剤耐性結核菌の出現
● 病原性の変化

表5 薬剤耐性結核の出現状況 (%)

	いずれの1薬剤	多剤耐性
全体	12.6	2.2
初回耐性	9.9〜10.7	1.0〜1.4
獲得(再治療)耐性	23.3〜36.0	9.3〜13.0

いずれの1薬剤：INH，RIF，エサンブトール(EMB)，ストレプトマイシン(SM)

易感染性を招来している．また，病原体要因として，薬剤耐性結核菌の出現および病原性の変化などが増加に関与している(**表4**)．

特に，憂慮すべき増加要因は，① HIV感染/AIDS，および，② 多剤耐性結核(MDR-TB)の出現である．

2003年12月現在，世界のHIV感染者/AIDS患者は4,200万人，結核菌とHIVの重複感染は約1,400万人，結核を発症した患者でHIV陽性は約8%を占めている[10]．結核菌感染に対する宿主防御は細胞性免疫(マクロファージ—サイトカイン—1型ヘルパーTリンパ球連関)の発現に依存している[11〜13]が，HIV感染/AIDSは細胞性免疫を破壊するため，結核菌感染や発病を惹起しやすくする．実際，HIV陽性者の発病の相対危険度はHIV陰性者の約10倍である[14]．また，AIDS死亡の約10%が結核を直接原因としている[5]．HIV感染/AIDS蔓延の防止は，感染経路(経血液，性的接触，母子感染)の遮断であり，性的接触，特に不特定多数との性交渉の回避，コンドーム使用による安全な性行為が重要である[10]．

薬剤耐性結核の原因は，不適切な結核医療，すなわち，抗結核化学療法薬の不適切な選択や使用，治療中断や脱落であり，医療関係者や患者の対応に起因するman-made diseaseである[15,16]．全世界で5,000万人以上が多剤耐性結核菌に既感染し，医療費は薬剤感受性結核に比し3〜100倍を要し，さらに，再発率(28%)がきわめて高く，結核制圧対策に大きな課題を提供している(**表5**)．

薬剤耐性，特に，多剤耐性結核に有効な抗結核薬や制圧戦略の開発は急務の課題である[17]．薬剤耐性結核の出現を防止する効果的な戦略は，薬剤感受性結核を確実に治療，そして，治癒させることであり，世界保健機関は直接監視下短期抗結核化学療法(directly observed treatment, short course：DOTS)を推奨している．DOTSの基本は，標準的な抗結核化学療法薬として，INH，RIF，EMBおよびピラジナミドの4薬を併用し，かつ，患者の服薬を毎日確認することである(面前服用も含む)．1薬剤当たりの耐性菌出現頻度は$1/10^{6\text{-}8}$であるため，多薬剤を併用することにより，耐性結核菌の出現頻度を低下させることが可能である[4,5]．

なお，米国疾病管理予防センター，米国感染症学会や米国胸部学会の勧告では，結核菌のSM耐性が増加しているため，SMの選択順位は低下している[18]．

集団感染や施設内(院内)感染対策

日本における集団感染は166件(1998〜2001年の合計)であり，事例は増加傾向を示している[19]．主要な発生場所は，学校(38%)，事業所(29%)，病院(20%)であるが，その他(施設，遊興場，飲食店など)にも拡大している．

この理由として，① 社会的活動の旺盛な世代における結核菌未感染者の増加──►初感染結核や発病の増加(結核の場合，外来性再感染が比較的少ないため)，② 国民や医療従事者の結核に対する関心の低下により，受診および診断の遅延──►

感染性結核患者の放置→蔓延などがある．

医療従事者の健康管理として，機関の管理者は労働安全衛生法や結核予防法[20]などに基づき，健康診断を実施し，徹底する．管轄保健所長や医療機関管理者は，医療機関において結核患者が発生した場合，協力して定期外健康診断を実施する．

結核の主要な感染源は，排菌量の多い喀痰塗抹陽性肺結核や喉頭結核患者(塗抹陰性や肺外結核の感染性は低い)であり，塗抹陽性結核患者の喀痰は10,000結核菌/mL以上を含む．結核菌の感染経路は飛沫核(空気)感染様式であり，結核菌が直径1～5 μmの落下しがたい，浮遊性飛沫核に含まれ，吸入することで感染する[21]．感染は結核菌の被曝露(吸入)者の約30%に成立する[22]．

結核のみならず感染症の蔓延防止は，感染源，感染経路および感受性宿主対策を基本としている．施設内結核感染防止対策は，① 管理対策(administrative measures)，② 環境や設備整備計画(engineering or environmental programs)，および，③ 個人防御対策(personal respiratory protection)から構成される[23](**表6**)．

表6 施設内結核感染防止対策

管理対策
- 施設内感染防止委員会や感染制御部隊(infection control team)の設置
- 院内結核発生動向調査
- 医療従事者へ結核に関する啓発
- 医療従事者のツベルクリン皮内反応
- 持続性(2週間以上)咳嗽患者の優先診療
- 喀痰塗抹陽性結核患者の早期発見や迅速な個室収容/隔離，有効な抗結核化学療法

環境や設備整備計画
- 陰圧個室の整備
- 換気(7回以上/時間)
- 紫外線照射やHEPAフィルター装備

個人防御対策
- 飛沫核を除去できるマスク(例：N95)
- 咳嗽を誘発する医療行為(例：気管支内視鏡検査や気管内挿管)における細心の注意
- 未発病感染者の化学予防(INH服用など)

未発病感染者の化学予防を実施する際，日本ではBCG(bacille Calmette-Guérin)接種が普及しているため，新規結核菌感染者をツベルクリン皮内反応(TST)で選定することは困難である．すなわち，① 現行TSTはBCGと結核菌に双方に共通な蛋白質抗原を用いているため，BCG接種と結核菌感染による反応の区別が不可能，② TST反応は経時的に減弱すること，さらに，③ 減弱した反応は再度のTSTによって，回復する(免疫学的ブースター効果)などが判定を困難にしている．

院内結核感染防止対策として，職員の新規採用に際し，39歳以下の場合，TST二段階法(two-step testing)を行い，その成績を発赤長径ミリ数，副反応の種類・有無を含めて記録しておくことが望まれる(なお，米国など諸外国はTST結果を硬結径で表現し，日本とは異なる)．第2回目の成績をTST基礎値として，その後の定期的TST，または結核患者発生時の接触者検診で実施するTSTに際して，結核菌感染の判定に有力な参考となる．実際的には，第1回目のTST強陽性者を除き，全員に第2回目のTSTを1～3週間後に行う方式が勧められる．

定期外検診(特に，塗抹陽性結核患者発生)におけるTSTは発生約2カ月後に施行し，結核菌感染判定基準として，① 発赤≧30 mmや，② TST基礎値に比し，10 mm以上の増強などが一般的である[23]．この基準に従い，未発病感染者(潜在性結核菌感染者)を選定し，化学予防を実施している．結核菌の潜伏感染者や感染した未発病医療従事者に対する発病予防戦略は，抗結核化学療法薬の6カ月間投与が一般的である．INHによる化学予防の場合，発病予防効果は50～70%，効果持続は約10年間である．ただし，感染源の結核菌がINH感受性であることを確認する．INH耐性の場合，RIFによる化学予防を考慮する．

潜在性結核菌感染の診断と対策

結核菌とBCGは結核菌群に属し，相同性がきわめて高く，BCGは乳幼児結核の発病予防ワクチンとして繁用されている．ツベルクリンは結核菌培養濾液から精製された蛋白抗原であり，

表7 内因性再燃における発病相対危険度

状況	発病相対危険度
● HIV感染者	9.4〜9.9
● 陳旧性結核	5.2
● 慢性腎不全	2.4
● 抗サイトカイン療法(IL-1拮抗薬, TNF抗体や可溶性受容体)	2.0
● 糖尿病(管理不良)	1.7
● 珪肺症	1.2〜1.7
● 低体重(基準値の10%以上のるいそう)	1.6
● 胃切除	1.4

TSTが臨床応用されている．TST陽性は結核菌感染，非結核性抗酸菌感染(*Mycobacterium avium* complexや*M. kansasii*など)やBCG接種を示唆し，TSTは結核菌感染，非結核性抗酸菌感染やBCG接種を区別することは不可能である．結核菌感染者の治療(抗結核薬の発病予防内服)に際し，結核菌感染者の特定をするうえで，TSTは支障をきたしている．

結核菌とBCGのゲノム情報から，BCGで欠失している遺伝子領域(region of deletion 1：RD1)産物(early secreted antigen target 6：ESAT-6や，culture filtrate protein 10：CFP-10)は結核菌特異的蛋白であり，TSTでは識別できなかった結核菌感染，非結核性抗酸菌感染やBCG接種によるTST陽性をESAT-6やCFP-10抗原による特異免疫反応(末梢血にRD1抗原を添加培養し，上清に産生されるinterferon γを定量)で結核菌感染のみを検出することが可能となった[24〜26]．RD1領域を利用した結核菌特異的診断法は，潜在性感染の発見，集団感染の拡がりの把握や化学予防対象者の選定に有用である．

結核の発病様式は，① 潜在性結核菌感染(約20億人，人類の1/3)を起源とした内因性再燃と，② 外来性再感染に大別される．結核菌感染後の発病率は5〜10%であるが，その多くは"内因性再燃"機序である．したがって，潜在性結核菌感染者を科学・効率的に発見し，発病高危険群(**表7**)や濃厚接触者などの潜在性感染者に治療(発病予防)介入することは結核制圧に新戦略を提供するであろう[27]．

BCGの有効性

現行の結核発病予防ワクチンであるBCGの有効性に関し，根拠に基づく医療(evidence-based medicine：EBM)の観点から，見直しが進められた．その結果，乳幼児結核(結核性髄膜炎など播種性結核)の有効性は認められたが，成人肺結核に対するBCGの有効性は実証されていないこと，さらに，BCG再接種によるTSTの陽転化が結核菌感染の診断を妨げること[28]などの事由により，結核予防法を改正し，BCGは乳幼児期(原則として，生後6カ月までにTSTを省略したBCG接種)の初回接種のみに限定し，BCG再接種およびTST(小学1年生および中学1年生時)は2003年4月から廃止された[29]．

BCGを凌駕する安全，かつ，有効な新規ワクチンの研究・開発は大きな課題である[22]．

移植医療と抗酸菌感染症

結核など抗酸菌感染に対する宿主防御は細胞性免疫に依存しており，細胞性免疫の低下した易感染性宿主(compromised hosts)における日和見感染症(opportunistic infections)でもある．臓器移植(造血幹細胞や固形臓器)の普及に伴い，被移植者における抗酸菌感染症が増加している．その理由として，① 被移植者の増加，② 移植臓器拒絶反応を回避するための強力な免疫抑制療法，③ 被移植者の寿命延長，および，④ 診断技術の向上が考えられる．

一般人口の抗酸菌感染症の罹患率と比較した場合，被移植者における結核は40〜70倍，非結核性抗酸菌感染症は50〜600倍のきわめて高い罹患率が報告されている．抗酸菌感染症は移植3カ月以降に発症することが多い．病型では，① 限局型，② 肺外型，③ 播種型があるが，②および③が60%以上を占め，非定型的であることが多い[30]．

おわりに

最近，世界を震撼させたSARS-コロナウイルスによる重症急性呼吸器症候群の集団発生事例からも明らかなように，感染症は現在でも，人類の健康に甚大な健康被害を提供し，さらに，新興・再興感染症や薬剤耐性病原体感染症は世界的に増加しており，油断できない状況にある[31]．代表的再興感染症である結核は単一病原体感染症として，人類の健康に最大の脅威であり，一般国民や医療従事者への啓発・教育，感染源，感染経路および感受性宿主対策などの結核対策は，将来も重要な課題である．結核制圧目標は喀痰塗抹陽性肺結核罹患率：0.1/10万人以下であるが，日本の現状は9.4であり，制圧目標には程遠く，中進国である．加えて，結核が呼吸器のみならず，全身性感染症であることを考慮すると，結核を念頭において日常の診療に従事することが肝要である．

今後の結核対策における重点項目として，① DOTSの普及，② 潜在性結核菌感染対策，③ HIV感染/AIDSと結核の重複感染に対する効果的な戦略，④ 多剤耐性結核に対する抗結核薬，⑤ 迅速・簡便な診断法，⑥ 有効なワクチンの開発，⑦ 集団や院内感染対策，が推進され，結核が制圧されることを期待している．

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Identification of an Immunomodulating Agent from *Mycobacterium leprae*

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A search for an immunomodulating agent from mycobacteria was carried out using *Mycobacterium leprae*. The antigenicity of each fraction of the bacterial membrane, which contains the most antigenic components of *M. leprae*, was assessed by using sera from paucibacillary leprosy. N-terminal sequencing of the serum-reactive protein and functional assessment of the membrane fractions using monocyte-derived dendritic cells (DCs) identified major membrane protein II (MMP-II) as one of the efficient T-cell-activating candidates. Purified MMP-II stimulated DCs from healthy individuals to produce interleukin-12 p70 and up-regulated the surface expression of major histocompatibility complex class I and II, CD86, and CD83 molecules. Also, there was an increase in the percentage of CD83$^+$ cells in the DC population. Furthermore, MMP-II-pulsed DCs expressed their derivatives on their surfaces. Using Toll-like receptor 2 (TLR-2)-dependent receptor constructs, we found that TLR-2 signaling was involved in DC maturation induced by MMP-II. Taken together, MMP-II can be recognized as an immunomodulating protein in terms of activation of antigen-presenting cells and innate immunity.

Mycobacterial infection is a major public health risk worldwide, and around one-third of the world's population is estimated to be latently infected. *Mycobacterium tuberculosis*, the causative agent of human tuberculosis, accounts for 8 to 10 million new active cases and 2 million deaths annually (12, 41). Nontuberculoid mycobacterial infections of immunocompromised individuals, such as human immunodeficiency virus type 1 (HIV-1)-infected patients (8, 28), evoke serious concern, and *Mycobacterium leprae* induces a chronic progressive peripheral nerve injury which leads to systemic deformity (16, 37). The sole immunomodulating agent currently available for human use against mycobacterial diseases is *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). However, its protective effect against mycobacterial infection is unconvincing, especially to elderly people, and thus, its use is limited (3). Various efforts are currently being made to develop immunomodulating agents, but convincing protection against mycobacterium-induced diseases has not been achieved to date. The development of other useful molecules as immunomodulating agents is greatly desired. In this study, we attempted to find such antigenic molecules in *M. leprae* subfractions.

M. leprae is the causative agent of human leprosy, for which a broad disease spectrum is clinically observed (34). Most individuals infected with *M. leprae* do not manifest leprosy, but a few manifest the disease, depending on their immunological status. The representative spectra are the tuberculoid, or paucibacillary (PB), leprosy and the lepromatous, or multibacillary (MB), leprosy. In the former disease spectrum, localized skin and nerve lesions are observed, and T cells act chiefly to localize bacterial spread and, thus, disease lesions (20, 31, 36). In contrast, in the latter disease spectrum, such cell-mediated immune responses are not efficiently evoked; rather, T cells show *M. leprae* antigen (Ag)-specific anergic responses. In both types of leprosy, the protective effect of antibody (Ab) is not observed. These observations indicate that the bacterial component Ags capable of modulating immune responses should be identified. Previously it has been demonstrated that monocyte-derived dendritic cells (DCs), which are the most potent antigen-presenting cells (APCs), are capable of stimulating both memory and naïve CD4$^+$ and CD8$^+$ T cells (14, 15, 21). Also, we reported that DCs played a central role in stimulating T cells (10, 18, 22); however, macrophages stimulated T cells less efficiently. Furthermore, we showed that among subcellular components of *M. leprae*, the cell membrane fraction was quite antigenic and contained molecules which stimulated DCs to produce interleukin-12 (IL-12) p70 (22). However, the molecules associated with DC activation have not been elucidated. For identification of an APC-associated immunomodulator, the following issues should be addressed: (i) the ability of the immunogen to activate APCs, including both DCs and macrophages; (ii) the ability of the immunogen to be processed and presented on the surfaces of these APCs, because mycobacteria are intracellular parasitic pathogens. In this context, we fractionated the *M. leprae*-derived cell membrane fraction, screened the fractions for such a protein by using DCs as APCs, and subsequently evaluated the newly identified molecule, major membrane protein II (MMP-II), in terms of innate immunity.

MATERIALS AND METHODS

Preparation of cells and bacteria. Peripheral blood was obtained with informed consent from healthy individuals who were positive for purified protein derivative (PPD) due to *M. bovis* BCG vaccination. We are aware that PPD-negative individuals would help to provide full information for these experi-

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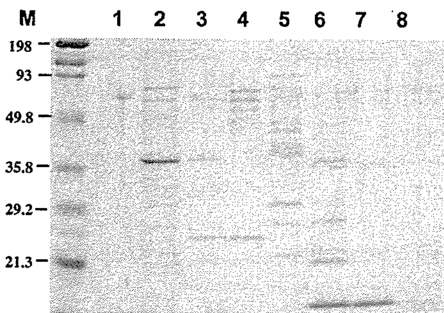

FIG. 1. *M. leprae* membrane fractions were separated into eight fractions by gel filtration as described in Materials and Methods. Then 3 μg of each fraction was run on a 12% SDS-polyacrylamide gel, and silver staining of the gel was performed.

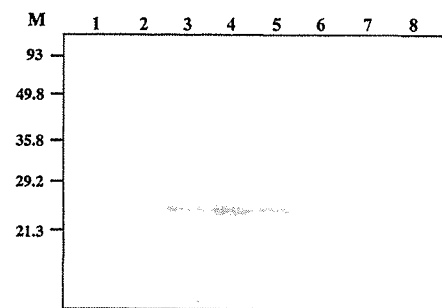

FIG. 3. Western blot of *M. leprae* membrane fractions. Five micrograms of various membrane fractions of *M. leprae* was run on a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane, which was further probed with an anti-MMP-II MAb. An alkaline phosphatase-conjugated anti-mouse IgG Ab was used as the secondary Ab, and the protein was detected with NBT/BCIP reagent.

ments, however, in Japan, such individuals are not available for study, because *M. bovis* BCG vaccination is compulsory for children (0 to 4 years old). Moreover, PPD-negative individuals in the Japanese population are those who do not respond to BCG vaccination, and therefore, it is likely that they suffer from an unknown human disease or immune insufficiency. Therefore, these individuals cannot be used as controls for our experiments. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described (23). Macrophages were differentiated by culturing plastic-adherent CD14$^+$ monocytes with RPMI 1640 medium containing 20% of fetal calf serum (FCS) and 1% penicillin G (Katayama Chemical, Osaka, Japan) in the presence of 5 ng/ml of macrophage colony-stimulating factor (R & D Systems, Abingdon, United Kingdom), as previously described (40). For preparation of the monocytes, CD3$^+$ T cells were removed from either freshly isolated heparinized blood or cryopreserved PBMCs using immunomagnetic beads coated with an anti-CD3 monoclonal antibody (MAb) (Dynabeads 450; Dynal, Oslo, Norway). The CD3$^-$ fraction of the PBMCs was plated on collagen-coated plates and cultured for 60 min at 37°C. The non-plastic-adherent cells were then removed by extensive washing, and the remaining adherent cells were used as monocytes and precursors of macrophages and DCs (23). Monocyte-derived DCs were differentiated from the plastic-adherent cells as described previously (23, 24). Briefly, the plastic-adherent cells were cultured in 3 ml of RPMI 1640 medium containing 10% FCS for 5 days in the presence of 50 ng of recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) (Pepro Tech EC Ltd., London, England) and 10 ng of rIL-4 (Pepro Tech) per ml. rGM-CSF and rIL-4 were supplied every 2 days, and 400 μl of medium was replaced as described previously (24). In some cases, DCs unpulsed or pulsed with Ags were further treated with a soluble form of CD40 ligand (CD40L) (Pepro Tech) to obtain fully matured DCs capable of efficiently activating T cells. The purity of DCs obtained was 90.5% as judged by the expression of CD1a. Since *M. leprae* cannot be cultivated or grown in vitro, *M. leprae* (Thai-53) was obtained from an armadillo liver that had been previously infected with *M. leprae*. The isolated bacteria were counted by Shepard's method (35) and were frozen at −80°C until use. The viability of *M. leprae* was assessed by using a fluorescent diacetate/ethidium bromide test (17).

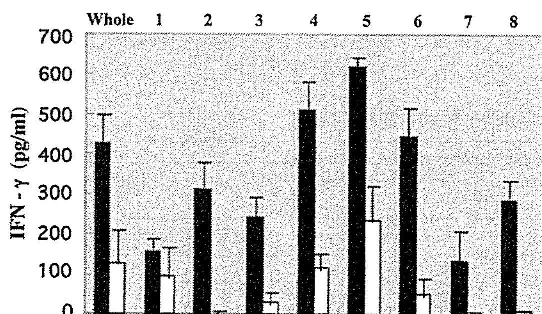

FIG. 2. IFN-γ production by T cells stimulated with membrane fraction-pulsed DCs. The responder CD4$^+$ and CD8$^+$ T cells (10^5/well) were stimulated for 4 days with autologous DCs which had been previously stimulated with 10 μg/ml of various membrane fractions of *M. leprae* obtained by gel filtration. Solid bars, IFN-γ production from CD4$^+$ T cells; open bars, IFN-γ production from CD8$^+$ T cells.

Fractionation of *M. leprae* protein and N-terminal sequencing. The fractionation of the mycobacterial proteins into cell wall, membrane, and cytosolic fractions was carried out according to previous reports (13, 18, 22). Briefly, the mycobacterial suspension was mixed with zirconium beads in the presence of protease inhibitors at a ratio of approximately 1:1 (vol/vol) and homogenized using Beads Homogenizer, model BC-20 (Central Scientific Commerce, Tokyo, Japan), at 1,500 rpm for 90 s three to four times. The beads were separated, and the suspension was centrifuged at 10,000 × *g* for 30 min. The supernatant was then further ultracentrifuged at 100,000 × *g* for 1 h. The resulting pellet was suspended in phosphate-buffered saline, washed twice, and taken as the membrane fraction. For the identification of *M. leprae* antigenic molecules, the membrane fraction was further fractionated using a fast protein liquid chromatography system (Amersham Bioscience, New Jersey). Four hundred micrograms of protein was run on a Superose 12 column (Amersham Bioscience) in 50 mM Tris-HCl, 0.5 M NaCl, and 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) at a flow rate of 0.5 ml/min. Fractions were collected, concentrated, buffer exchanged to 50 mM Tris-HCl using Microcon centrifuge filter units YM-3 (Millipore, Bedford, MA), and run on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The gel was stained with "Daiichi" silver stain (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. Western blotting was performed using PB patients' pooled sera, at a dilution of 1:25, which had been preadsorbed with the *M. leprae* cytosolic fraction. Alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG) (Biosource, Camarillo, CA) was used as the secondary Ab, and detection was performed by using the nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolylphosphate (BCIP) reagent (Calbiochem, San Diego, CA). N-terminal peptide sequencing of the protein which reacted to the sera was performed at the Center for Instrumental Analysis, Hokkaido University.

Analysis of cell surface Ags. The expression of cell surface Ags on DCs was analyzed using FACScalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma Chemical Co., St. Louis, MO), and 10^4 live cells were analyzed. For analysis of cell surface Ags, fluorescein isothiocyanate-conjugated MAbs against HLA-ABC (G46-2.6; PharMingen, San Diego, CA), HLA-DR (L243; PharMingen), CD86 (FUN-1; PharMingen), and CD83 (HB15a; Immunotech, Marseille, France) were used. A murine MAb against MMP-II was raised by immunizing a mouse with purified MMP-II. The optimal concentrations of MAbs were determined in advance.

Identification and purification of MMP-II. The MMP-II gene was PCR amplified from *M. leprae* chromosomal DNA and cloned into an *Escherichia coli* expression vector. Briefly, the MMP-II gene was inserted into the expression plasmid pET28 (Novagen Inc., Madison, WI) and transformed into *E. coli* strain ER2566 (New England Biolabs Inc., Beverly, MA). The expressed protein was eluted using Whole Gel Eluter (Bio-Rad Laboratories, Hercules, CA) and used for all experiments. The amount of lipopolysaccharide (LPS) in the purified MMP-II protein was determined by using a *Limulus* amebocyte lysate assay (Whittaker Bioproducts, Walkersville, MD) and found to be less than 70 pg per mg of MMP-II, a level that did not affect the maturation of DCs.

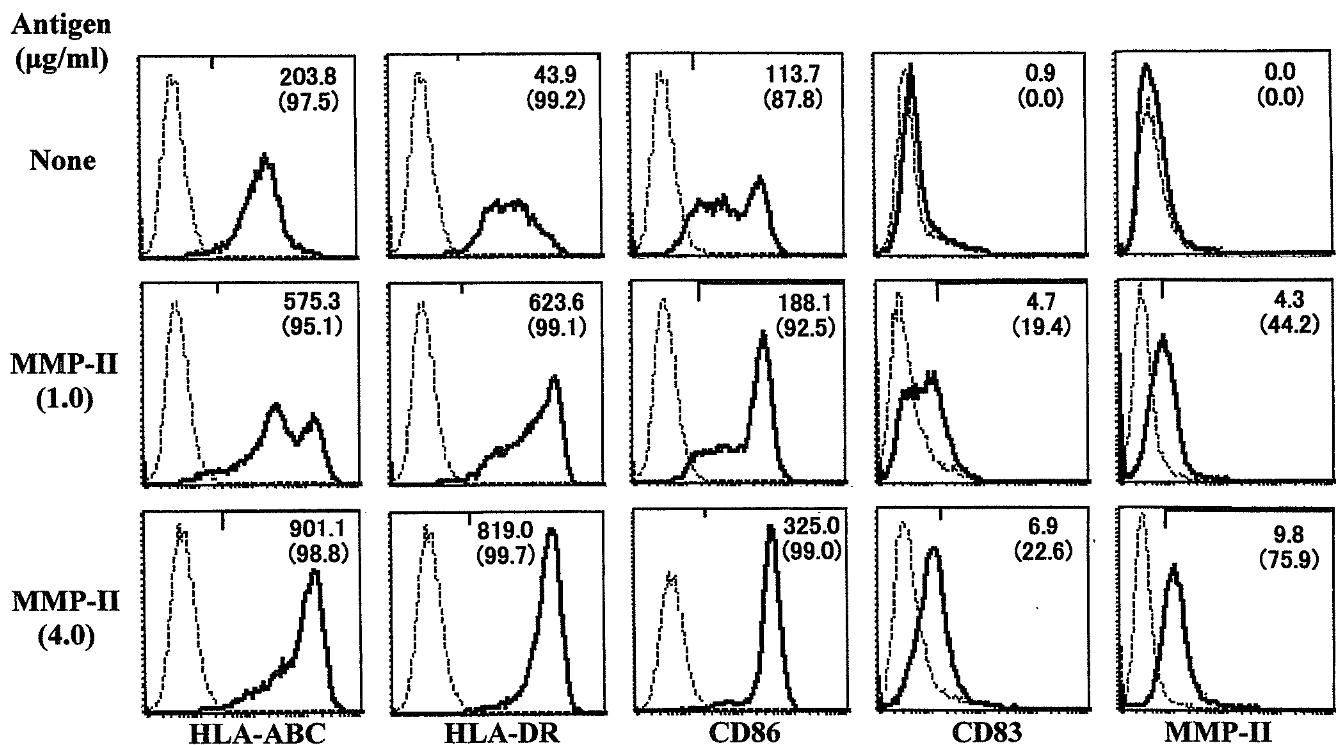

FIG. 4. Expression of various molecules on DCs pulsed with MMP-II. Monocyte-derived DCs from healthy individuals (PPD positive) were pulsed with the indicated dose of MMP-II. DCs were gated and analyzed. Solid curves, isotype-matched control IgG; broken curves, the indicated MAb. The number in the top right corner of each panel represents the difference in mean fluorescence intensity between the control IgG and the test MAb. The number in parentheses is the percentage of positive cells. Results of one experiment representative of three separate experiments are shown.

Assessment of APC function of DCs pulsed with cell membrane fractions. The ability of DCs pulsed with various fractions of the *M. leprae* cell membrane to stimulate autologous T cells was assessed using an autologous stimulator–T-cell mixed reaction as previously described (10, 24). The Ag-pulsed DCs were treated with 50 μg/ml of mitomycin C, washed extensively to remove extracellular Ags, and used as a stimulator. T cells were prepared as follows: freshly thawed PBMCs were depleted of major histocompatibility complex (MHC) class II$^+$ cells by using magnetic beads coated with a MAb to MHC class II Ag (Dynabeads 450; Dynal) and were further treated with beads coated with either a CD4 or a CD8 MAb to select T cells negatively as previously reported (10). The purity of CD4$^+$ T cells or CD8$^+$ T cells was more than 98%. The supernatant of the stimulator–T-cell mixture was collected on day 4 of coculture, and the level of gamma interferon (IFN-γ) produced was measured by an Opt EIA Human ELISA Set (BD PharMingen International).

Assessment of cytokine production. Levels of the following cytokines were measured: tumor necrosis factor alpha (TNF-α), IL-10, and IL-12 p70 produced from either macrophages or DCs by stimulation with MMP-II for 24 h in the presence or absence of a soluble form of CD40L (Pepro Tech). The murine MAb against TLR-2 (clone 2392; IgG1) with neutralizing activity was obtained from Genentech (San Francisco, CA). The optimal concentration of the anti-Toll-like receptor 2 (anti-TLR-2) Ab was determined in advance. The concentrations of IL-12 p70, IL-10, and TNF-α were quantified using the Opt EIA Human ELISA Set enzyme assay kits, available from BD PharMingen International.

Cell transfection and luciferase assay. Human embryonic kidney cells (HEK293) were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% FCS, 50 mg/ml penicillin/streptomycin, and nonessential amino acids (Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator with 5% CO_2. The cDNA of human TLR-2 was PCR amplified using a human spleen cDNA library (BD Biosciences, San Jose, CA) and inserted into pCIneo (Promega, Madison, WI). HEK293 cells (2 × 10^4) were transiently transfected with a mixture of plasmids—200 ng of pCIneo hTLR2, 25 ng of p5×NF-κB-luc

TABLE 1. Cytokine production from DCs stimulated with MMP-II[a]

DC stimulation (dose)	Concn (pg/ml) of the following cytokine:					
	IL-12 p70		TNF-α		IL-10	
	CD40L (−)	CD40L (+)	CD40L (−)	CD40L (+)	CD40L (−)	CD40L (+)
None	2.6 ± 0.2*,†	17.7 ± 0.4‡,§	2.6 ± 0.4¶,‖	15.4 ± 3.8**,††	2.0 ± 0.1	3.4 ± 0.1
MMP-II (1 μg/ml)	51.2 ± 0.5*	782.0 ± 8.7‡	345.4 ± 9.9¶	345.7 ± 19.3**	2.5 ± 0.3	1.7 ± 0.1
MMP-II (4 μg/ml)	404.0 ± 9.8†	1624.0 ± 11.0§	773.8 ± 11.1‖	747.3 ± 18.7††	2.2 ± 0.1	2.8 ± 0.3
LPS (0.3 pg/ml)	2.8 ± 0.3	18.7 ± 0.6	5.0 ± 1.3	36.0 ± 9.2	2.0 ± 0.3	3.0 ± 0.2

[a] Monocyte-derived DCs (10^5/well) were stimulated for 24 h with the indicated dose of MMP-II in the absence [CD40L (−)] or presence [CD40L (+)] of a soluble form of CD40L (1.0 μg/ml). The DCs were also stimulated for 24 h with 0.3 pg/ml of LPS, which is estimated to be present in 4 μg/ml of MMP-II. Results representative of more than three separate experiments are shown. Assays were done in triplicate, and results are expressed as means ± standard deviations. Titers with the same symbols are statistically compared by Student's *t* test, as follows: *, $P < 0.001$; ‡, $P < 0.001$; ¶, $P < 0.0005$; **, $P < 0.001$; †, $P < 0.001$; §, $P < 0.001$; ‖, $P < 0.0001$; ††, $P < 0.0005$.

TABLE 2. Cytokine production from macrophages stimulated with MMP-II[a]

Macrophage stimulation (dose)	Concn (pg/ml) of the following cytokine:		
	IL-12 p70	TNF-α	IL-10
None	2.0 ± 0.2	19.3 ± 1.8*,†	45.3 ± 8.8‡,§
MMP-II (1.0 μg/ml)	2.1 ± 0.3	122.2 ± 6.8*	149.9 ± 20.3‡
MMP-II (4.0 μg/ml)	1.9 ± 0.2	568.6 ± 12.4†	561.2 ± 31.9§
LPS (0.3 pg/ml)	1.8 ± 0.1	10.0 ± 2.0	15.6 ± 3.2

[a] Monocyte-derived macrophages (10^5/well) were stimulated for 24 h in the presence of a soluble form of CD40L (1.0 μg/ml) with the indicated dose of MMP-II. Results representative of more than three separate experiments are shown. Assays were done in triplicate, and results are expressed as means ± standard deviations. Titers with the same symbols are statistically compared by Student's t test, as follows: *, $P < 0.001$; ‡, $P < 0.005$; †, $P < 0.0005$; §, $P < 0.001$.

(Stratagene, La Jolla, CA), and 10 ng of pRL-TK-*Renilla* luciferase plasmid (Promega)—using the FuGENE 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN), as previously described (38). Thirty-six hours after transfection, cells were treated with various amounts of glutathione *S*-transferase (GST), MMP-II, or peptidoglycan (PGN) as a positive control (for TLR-2-dependent luciferase activity) for a further 6 h. The cells were lysed in 70 μl of 1× passive lysis buffer (Promega), and luciferase activity in 10 μl of the cell lysate was measured using the Promega Dual-Luciferase Reporter Assay System according to the protocol provided by the manufacturer. Data were expressed as fold induction relative to the activity of *Renilla* luciferase, which is an internal control for transfection efficiency.

Statistical analysis. Student's t test was applied to demonstrate statistically significant differences.

RESULTS

Identification of *M. leprae*-derived antigenic molecules. The cell membrane fraction from *M. leprae* was found to be the most T-cell stimulating (10, 22), although it may also contain some inhibitory molecules (10). The *M. leprae*-derived cell membrane fraction was solubilized and further fractionated using a gel filtration column to search for the antigenic molecules. Figure 1 shows the silver staining of each fraction, which revealed several proteins. Then we pulsed healthy donor-derived DCs with each of these fractions individually and examined the antigenicity of each fraction by monitoring IFN-γ production by DC-stimulated $CD4^+$ and $CD8^+$ T cells derived from PPD-positive healthy individuals (Fig. 2). Among the eight fractions, fractions 4 through 6 seemed to be efficient at stimulating cytokine production by $CD4^+$ T cells, and fractions 4 and 5 appeared to be involved in the activation of both $CD4^+$ and $CD8^+$ T cells. Thus, using these two fractions of cell membrane, we identified one of the antigenic molecules. The pooled PB leprosy sera, preadsorbed with *M. leprae* cytosol fractions, were used for Western blot analysis of the fractions (not shown). N-terminal sequencing of the serum-reactive bands common to fractions 4 and 5 identified MMP-II as one of the candidates. The presence of MMP-II in fractions 4 and 5 was further confirmed by Western blotting using a MAb against MMP-II (Fig. 3). For the purification of the protein, the MMP-II gene was amplified by PCR from the genomic DNA of *M. leprae*, and MMP-II protein was subsequently expressed in *E. coli* by using the T7 expression system (pET-28). The expressed protein was confirmed to be MMP-II by Western blot analysis (not shown), by comparison to purified MMP-II, used as a positive control (donated by P. J. Brennan, Colorado University).

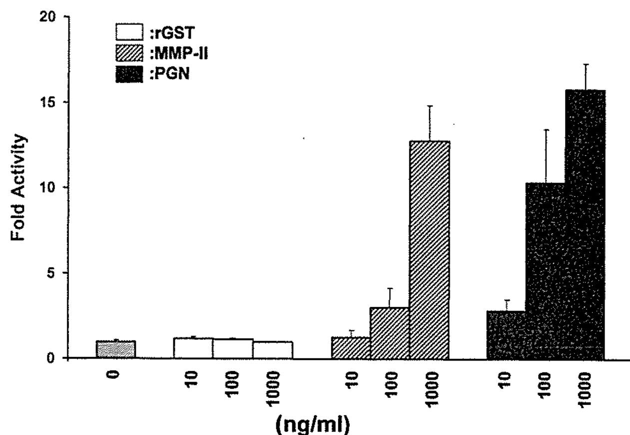

FIG. 5. NF-κB-dependent reporter gene activity of the TLR2 transfectant was measured after stimulation with or without 10, 100, or 1,000 ng/ml of rGST, MMP-II, or PGN, as described in Materials and Methods. Data are expressed as fold induction relative to the activity of *Renilla* luciferase, which is an internal control for transfection efficiency in the dual-luciferase reporter assay. Results of one experiment representative of two separate experiments are shown. Assays were done in triplicate, and the results are expressed as means ± standard deviations.

Antigenicity of *M. leprae*-derived MMP-II. The ability of MMP-II to evoke cellular immunity was assessed using DCs and macrophages as APCs. Previously we demonstrated that the cytosol fraction from *M. leprae* was less efficient at the induction of DC maturation and that the whole cell membrane fraction partially induced DC maturation (22). In contrast, when immature DCs were pulsed with MMP-II, they up-regulated the expression levels of HLA-ABC, HLA-DR, CD86, and CD83 Ags on the surfaces of DCs in an Ag dose-dependent manner, and the percentage of $CD83^+$ cells was found to increase significantly (Fig. 4). The expression of MMP-II on the surfaces of MMP-II-pulsed DCs was revealed using a MAb to MMP-II (Fig. 4). The functional aspects of MMP-II in terms of APC activation were assessed by measuring production of cytokines, such as IL-12 p70, IL-10, and TNF-α, by APCs (Tables 1 and 2). The bioactive form of IL-12 was released from DCs by pulsing MMP-II in the absence of CD40L, and the cytokine production level was enhanced by copulsing DCs with CD40L and MMP-II (Table 1). Obviously IL-12 was not produced from DCs by stimulation with the amount of LPS estimated to be present in 4 μg/ml of MMP-II. Furthermore, DCs produced TNF-α in the presence or absence of CD40L, but they did not produce any significant amount of IL-10 due to MMP-II stimulation. These results suggested that MMP-II could activate DCs and induce their maturation. Macrophages derived from monocytes did not produce IL-12 p70 by stimulation with MMP-II, but they produced TNF-α and IL-10 (Table 2), which are found predominantly in granulomatous mycobacterium-infected lesions. These results indicate that MMP-II also activated macrophages, but macrophages and DCs seem to have distinct functional roles. All cytokines were produced in an Ag dose-dependent fashion.

Involvement of TLR-2 in activation of DCs. In order to elucidate the mechanism by which MMP-II activates DCs, we examined the relationship of MMP-II and TLR-2, because

TABLE 3. Effect of the TLR-2-antagonistic Ab on IL-12 p70 production by DCs[a]

DC stimulation (dose)	IL-12 p70 production (pg/ml) in the presence of:				
	TLR-2-antagonistic Ab at the following concn (μg/ml):			Control IgG at the following concn (μg/ml):	
	0	5.0	10.0	5.0	10.0
MMP-II (1.0 μg/ml)	603.1 ± 11.0*†	491.2 ± 10.2*	178.1 ± 8.8†	658.2 ± 11.3	675.9 ± 10.7
MMP-II (4.0 μg/ml)	1,210.2 ± 20.0‡,§	949.0 ± 9.3‡	805.3 ± 7.9§	1,290.3 ± 12.4	1,403.8 ± 31.5

[a] Monocyte-derived immature DCs (10^6/ml) were treated with the indicated dose of a TLR-2-antagonistic MAb or an isotype-matched control IgG and were subsequently stimulated for 24 h with MMP-II in the presence of CD40L (1.0 μg/ml). Results representative of more than three separate experiments are shown. Assays were done in triplicate, and results are expressed as means ± standard deviations. Titers with the same symbols were statistically compared by Student's *t* test, as follows: *, $P < 0.0001$; †, $P < 0.0005$; ‡, $P < 0.0001$; §, $P < 0.0001$.

TLR-2 is reported to be highly associated with induction of innate immunity against mycobacterial infection (1, 4, 30). When HEK293 cells that had been cotransfected with pCIneo TLR-2, p5×NF-κB-luc, and pRL-Tk-*Renilla* luciferase were pulsed with MMP-II, significant levels of luciferase activity were induced in an Ag dose-dependent manner, levels comparable to those induced by PGN, a well-defined TLR-2-associated bacterial Ag (Fig. 5). Similar results were also obtained using *M. leprae*-derived MMP-II. Such changes were not induced by rGST, a negative-control protein. Furthermore, when the surface TLR-2 Ag on DCs was masked by an antagonistic Ab to TLR-2, IL-12 p70 production by DCs stimulated with MMP-II was significantly, though partially, suppressed (Table 3). The isotype-matched control IgG did not affect IL-12 p70 production by MMP-II-stimulated DCs. As expected, the TLR-2-antagonistic Ab did not suppress IL-12 p70 production by DCs stimulated with LPS (a ligand for TLR-4) (not shown). These results indicate that MMP-II might use TLR-2 as its ligand on APCs, resulting in stimulation of DCs.

DISCUSSION

Leprosy is a broad-spectrum disease (34). One representative manifestation is PB leprosy. Studies on clinical specimens of the skin lesions indicate that the infection is localized and the spread of *M. leprae* is suppressed as a consequence of activation of cellular immune responses (20, 31, 36). On the other hand, MB leprosy usually manifests widespread infection due to the lack of an efficient response to *M. leprae* components. The mechanisms leading to the broad spectrum are not fully understood yet, but these observations suggest the presence of an Ag with immunomodulating activities that modify the immune responses in vivo. So far, however, such Ags have not been identified. Previously we evaluated the APC function of professional APCs and found that DCs were superior to macrophages in activating T cells (10). When we examined the DC-mediated antigenicity of subcellular components of *M. leprae* for identification of immunomodulating molecules, we found that the cell membrane fraction was more suitable than other fractions (22). Therefore, the *M. leprae* membrane fraction was size fractionated, and each fraction was examined for its T-cell-stimulating ability by using DCs as APCs. Two of the fractions with high activity were examined by reaction to PB leprosy sera, and subsequently the N terminus of the reactive protein was sequenced. As a result, MMP-II was identified as one of the antigenic cell membrane proteins, and the result was confirmed by Western blotting of the various fractions using an anti-MMP-II antibody (Fig. 3).

MMP-II was originally identified from *M. leprae* as a major native protein in 1990 (13) and was recognized as being identical to mycobacterial bacterioferritin (32). Purification of MMP-II by reverse-phase chromatography revealed a large molecular mass of 380 kDa and a ferroxidase center residue. MMP-II contains 1,000 to 4,000 atoms of iron per molecule of protein (32). A homology search on the mycobacterial nucleotide database revealed that MMP-II is conserved among *M. leprae*, *M. tuberculosis*, and *M. avium*. The percent homology at the amino acid level is about 86% among these species. The previous studies reported that MMP-II was recognized in vivo by B and T cells. Sera from patients were reported to have higher IgG titers to MMP-II, regardless of the clinical type of leprosy, than sera from healthy individuals (7). Also, T cells from leprosy of both the PB and the MB type were stimulated by MMP-II to proliferate and to produce both IFN-γ and IL-5 (29). However, tuberculosis patients or individuals who have had contact with leprosy patients have not been examined yet. Also, the influence of MMP-II on the innate immune response has not yet been clarified.

MMP-II stimulated DCs to produce TNF-α and a bioactive form of IL-12 (IL-12 p70) (Table 1) and induced their maturation, as observed by their phenotypic changes (Fig. 4). Further, MMP-II also stimulated macrophages to produce TNF-α and IL-10 (Table 1). These cytokines were produced by stimulation with either MMP-II derived from *M. leprae* (not shown) or MMP-II overexpressed in *E. coli* (Table 1). DCs and macrophages play distinct roles in the host defense against mycobacterial infection (9). DCs are central to the initiation of Ag-specific T-cell responses (6, 27, 36), and in our preliminary experiments, DCs pulsed with purified MMP-II stimulated both $CD4^+$ and $CD8^+$ T cells from PPD-positive healthy individuals to produce IFN-γ (not shown). The activated form of macrophage is involved in the formation of tuberculoid granulomatous lesions (5, 9). These results indicate that MMP-II might contribute to the immune regulation of host cells against mycobacteria. Then we investigated what could be the MMP-II ligand that is expressed on APCs. TLR-2 is associated mainly with innate immunity and has been shown to recognize the molecular pattern of pathogens (4, 11, 18, 26, 33). In mycobacterial infection, it has been reported that a 19-kDa lipoprotein isolated from *M. tuberculosis* ligated TLR-2 (4, 19), and the *M. leprae* 33-kDa lipoprotein could be another candidate participating in the TLR-2-associated innate immune system

(19). In our study using the TLR-2 reporter assay with HEK293 cells, we found that TLR-2 is likely to be involved in the recognition of MMP-II in spite of the fact that MMP-II lacks the triacylated region. This finding surprised us, but a similar ligation of protein to TLR-2 has also been reported for neisserial porins HSP60 and HSP70, which have no posttranslational modification of acylation (2, 25, 39). IL-12 production by MMP-II-stimulated DCs was partially inhibited by a TLR-2-antagonistic Ab, which indicates that other receptors are also involved in signals leading to IL-12 production.

The data in this report, taken together, indicate that MMP-II has an immunomodulating activity and contributes to the activation of innate immunity. Further study should be pursued to evaluate its host defense-associated activity against leprosy and other mycobacterial infections that pose a worldwide threat.

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