厚生労働科学研究費補助金（新型インフルエンザ等新興・再興感染症研究事業）
分担研究報告書

ワクチンの検定と結核を増悪させる病態の解析

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研究要旨.
ヒト糖尿病患者が、どのくらい結核に感染し、発病するかを、中国上海市で調べた。糖尿病患者は、結核にかかりやすいといえる。糖尿病患者における多剤耐性結核の割合は、有意に高い。日本での報告とは違う。治療期間も有意に長いし、再発率も高い。本研究は、多数の症例（2,141 例）を扱っているので糖尿病と結核の実態を反映している。

A. 研究目的

過去 2 年間、糖尿病ラットモデルを用いて、糖尿病ラットが、結核菌に感染・発病しやすいことを証明した。ヒト糖尿病と結核では有意な差があるかを検証するために、中国上海市で実地調査を行った。

B. 研究方法

2008 年 4 月から 2009 年 3 月までに上海市肺科病院を受診した 2,141 人の結核患者を対象とした。その中で、糖尿病患者が何人いて、治療歴、糖尿病の型別（糖尿病 1 型か 2 型か）、リファンピシンとイソニアジドに耐性な多剤耐性患者の数、退院後の結核再発率を調べた。

糖尿病患者血清を 100 倍に薄めた後、結核菌を加え、グルコースが結核菌増殖促進作用があるか否かを調べた。なお糖尿病の判定は、空腹時血糖値 126mg/dl 以上を陽性とした。インスリンの測定値は、immunoreactive insulin のことである。

倫理面への配慮　本研究の遂行に当たっては、個人情報が漏れないように留意するとともに、病院の倫理委員会の承認を得た。

C. 研究結果

2,141 の肺結核患者を対象とした。1,464 例がスメア培養とも陽性で、20.2%がスメア陰性培養陽性であった。男女比は、3.2 対 1 であった。職業は、農民、サラリーマン、無職であり、平均年齢は、42.7 歳であった。

結核患者中 203 例が糖尿病患者であった。すでに糖尿病を発病しており、結核菌感染後糖尿病にかかった例はなかった。1 型が 7 名で、2 型は 196 名であった。治療は、レギュラーインスリンまたは SU 剤で治療された。114 名が、スメア培養とも陽性で、47 名がスメア陰性培養陽性であった。糖尿病の血糖レベルは、200-700mg/dl であった。糖尿病の治療で血糖をコントロールしたのち、isoniazid, rifampicin, pyrazinamide, ethanbutol または streptomycin で治療された。表 1 に示すように、糖尿病結核患者の 36 名が多剤耐性結核患者であった。これは、17.7%にあたり、非糖尿病結核患者の 9.3%に比べて、有意に高かった。多剤耐性結核は、糖尿病コントロール不良群で多かった（32 対 4）。さらに詳細に調べてみると、標準化学療法を受けた 167 名の糖尿病結核患者のうち、退院後 2 年以内に 20 名が再発した。

グルコースが結核菌増殖促進作用があるか否かを調べるために、患者血清を 100 倍に希釈し、それに結核菌 H37Rv を加えて、1 週間培養した後、1％小川培地で 4 週間培養し、出てきたコロニー数を比べた。ある場合には、グルコースを加えて、グルコース添加の影響を調べた。1 型、2 型糖尿病患者とも、結核菌数は、正常血清に比べて、有意に増加した。その程度は、1 型糖尿病

で顕著だった。さらに、0.1%グルコースを加えて培養したところ、結核菌が増加した。

D. 考察

同様な研究が、1つ、日本で、2000年に行われており、644人の結核患者の内、116人の糖尿病患者を対象にした。症例数が少ないが、まとまった研究である。それによると、糖尿病結核患者のうち6.0%が多剤耐性結核であった。退院後30ヶ月後の再発率は、１０．３％出会ったと報告している。この研究は、例数が少ないが、それなりにまとまった報告である。我々の報告は、対象症例数が多いが、糖尿病結核患者は9.5%であり、この報告の18.0%より少ない。1型、2型の比率は同じで、圧倒的に2型が多い。多剤耐性結核の頻度は、１７．７％で、この報告の６．０％より有意に高い。再発した例も、再発期間が違うので、明確に比べられないが、我々の方が、有意に高い。

今回、日本では、多数の結核患者中にどのくらい糖尿病患者がいるかを、症例数が少ないので単一施設で行えないので、中国上海市肺科病院で行った。やはり、動物実験で得られた所見が得られた。今後、大規模な実態調査を行い、糖尿病結核患者に対する新しい治療法を策定することが望ましい。

今後、やるべき課題を挙げたい。NKT細胞もインターフェロンγを産生し、抗結核免疫に関係するので、糖尿病結核患者でどのようになっているかを調べることが必要である。第2に、CD25 T細胞も抗結核免疫に関係することが、我々の研究で明らかになっているので、糖尿病結核患者で、どのような挙動を示すのかを調べるのは意義深い。

最後に、非結核性抗酸菌症患者は糖尿病にかかりやすいのか調べる必要がある。東京病院倉島先生は、はっきりした相関関係がないと主張するが、詳しく調べる必要があろう。

E. 結論

中国上海市における結核患者に占める糖尿病患者の実態を調べた。糖尿病患者は、結核にかかりやすいと言えるが、入院後糖尿病を発症した例はなかった。糖尿病を、よくコントロールした症例では、入院期間が短かったので、糖尿病の治療は大事である。糖尿病における多剤耐性結核患者の割合は、有意に高かった。結核治療期間も有意に長いし、再発率も高い。糖尿病患者血清中で、結核菌が有意に増殖する。本研究は、症例数が多いことから、糖尿病と結核の実態を反映していると考えられる。

G. 研究発表

1. 論文発表

1) Sugawara, I., T. Udagawa, T. Aoki, and S. Mizuno. 2009. Establishment of a guinea pig model of latent tuberculosis with GFP-introduced *M. tuberculosis*. Tohoku J. Exp. Med., 219: 257-262.
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4) Sugawara, I., J. Zhang, and C. Li. 2009. Cross-resistance of *M. tuberculosis* isolates among streptomycin, kanamycin and amikacin. Ind. J. Exp. Biol., 47: 520-522.

2. 学会発表

なし

H. 知的財産権の出願・登録状況

1. 特許取得　なし
2. 実用新案登録　なし
3. その他　なし

表 1. **Drug sensitivity of tubercle bacilli cultured from TB patients with DM.**

	Drug-susceptible	MDR*	Total
Diabetics (n=203)+	82.3%	36 (17.7%)	100%
FBS≧200 mg/dl		32(15.7%)	
126≦FBS□ 199		4(2.0%)	
Non-diabetics (n=1,938)	90.7%	9.3%	100%

*p<0.01 +The detailed profiles of 203 diabetics and 1,938 non-diabetics are as follows: 67 (FBS≧200 mg/dl)(51-78 years old, man：woman=5.5：1), 136 (126≦FBS□ 199)(30-82 years old, man:woman=5.5:1), and 1,938 (FBS□ 125)(2-96 years old, man:woman=3:1).

MDR ; multidrug-resistant, FBS ; fasting blood sugar (mg/dl).

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（新型インフルエンザ等新興・再興感染症研究事業）

結核菌を含む抗酸菌の細胞壁の生合成に不可欠な遺伝子の検索と解析

分担研究報告書

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厚生労働科学研究費補助金（新型インフルエンザ等新興・再興研究事業研究事業）
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結核菌を含む抗酸菌の細胞壁の生合成に不可欠な遺伝子の検索と解析

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研究要旨.
結核菌を含む抗酸菌のゲノム解析は進んでいるものの、機能が未知の遺伝子やタンパク質が数多く残されている。これらの機能未知遺伝子・タンパク質の中には、抗酸菌の新規診断法や新規抗結核薬の開発に有用なものが存在していると考えられる。そこで本研究課題では、抗酸菌に特徴的な細胞壁の生合成に関連する遺伝子を含む新規遺伝子・タンパク質の解析を行うことにより、抗酸菌に関する新たな知見を得るとともに、新規診断法や新規抗結核薬の開発に結びつけることを目的としている。本年度は、昨年度から引き続き、本研究課題で確立した抗酸菌の新規鑑別法の有用性について ATCC 株や臨床分離株等を用いて検討した。その結果、本鑑別法は既存の遺伝子検査と比較してより多くの病原性抗酸菌が簡便に鑑別可能であることが示された。さらに、本鑑別法の主要な病原性抗酸菌種に対する特異度・感度はともに 96%以上であった。また、結核菌のゲノム上に存在する機能未知遺伝子 *Rv2613c* が特異的な一次構造と活性を持つ新規 diadenosine polyphosphate 加リン酸分解酵素をコードしていることを明らかにし、本酵素が新規抗結核薬の開発における標的候補の１つであることを示した。さらに X 線結晶構造解析により、本酵素の立体構造を決定し、構造既知の diadenosine polyphosphate 関連酵素と比較した。その結果、構造骨格部分では相同性を示すが、活性中心部位ではアミノ酸残基の種類が異なっていることを明らかにした。

A. 研究目的

結核菌を含む抗酸菌のゲノム解析は進んでいるものの、機能が未知の遺伝子やタンパク質が多数存在している。これらの機能未知遺伝子・タンパク質の中には、抗酸菌の新規診断法や新規抗結核薬の開発に有用なものが存在していると考えられる。特に結核菌を含む抗酸菌の細胞壁構造は、抗酸菌に特異的であり病原性とも深く関わっていることから、新規診断法や新規抗結核薬の有望な標的部位として予想される。そこで本研究では、抗酸菌の細胞壁構造に関連する遺伝子を含む新規遺伝子・タンパク質の解析や機能の同定を行うことにより、抗酸菌に関する新たな知見を得るとともに、新規診断法や新規抗結核薬の開発に結びつけることを目的としている。

抗酸菌症の治療では、原因となる抗酸菌の種類によって隔離の有無や治療薬の選択等が異なるため、臨床現場では迅速な起炎菌の同定が求められる。抗酸菌の簡便な鑑別として、近年では遺伝子検査が行われているが、同定可能な菌種が限られており、より多種の抗酸菌が鑑別可能な方法が求められている。そこで本研究では、抗酸菌に特徴的な細胞壁構造の合成に関与する遺伝子の情報に基づいてプライマーを設計し、PCR 反応を行うことによって抗酸菌種を鑑

別する新たな方法を開発した。具体的には、抗酸菌の細胞壁構造の合成に関与している2つの遺伝子（結核菌では*Rv3783*遺伝子と*Rv3789*遺伝子に相当）に着目した。この2つの遺伝子の塩基配列は、ゲノムが既に解読されている抗酸菌種において、高度に保存されているが、この2つの遺伝子の間に存在するORFの数や長さは菌種により大きく異なっている。従って、この2つの遺伝子上にプライマーを設計してPCR反応を行い、得られたバンドのサイズを比較することにより、主要な病原性抗酸菌を鑑別することが可能であると予想された。さらに、結核菌のみを特異的に鑑別するために、結核菌の遺伝子*Rv1330c*のみを増幅するプライマーセットも設計した。これまでに本研究課題では、設計した2つのプライマーセットを用いてPCR反応を行った後に、増幅されたバンドのパターンを比較することによって、抗酸菌を簡便に鑑別することが可能な新しい方法を確立した。本年度は、本鑑別法の有用性について、ATCC株や臨床分離株をテンプレートとして用いてさらに検討を行った。

新規抗結核薬は、既存の抗結核薬の作用機序とは異なる標的部位を持ち、結核菌に特異的に作用するものが求められる。そこで本研究課題では、新規抗結核薬の標的部位になり得ると考えられる新規遺伝子・タンパク質の詳細な機能と構造の解析を行って、新規抗結核薬の開発に結びつけることを目的とした。具体的には、結核菌のゲノム上に存在する機能未知遺伝子*Rv2613c*に着目した。本遺伝子の破壊株では野生株と比較して生育能が低下することが既に報告されていること、並びに本遺伝子が細胞壁合成酵素をコードしている遺伝子群と同一のオペロン上に存在していることから、本遺伝子は結核菌の生体内において重要な役割を果たしていることが予想された。さらに、本遺伝子と高い相同性を示す配列がヒトを含めた真核生物のゲノム上には存在しないことから、本遺伝子は結核菌に特異的に作用するような新規抗結核薬の標的部位になり得ると考えられた。そこで本遺伝子がコードするタンパク質について、詳細な酵素学的諸性質を明らかにするとともに、X線結晶構造解析による立体構造の決定を試みた。

B. 研究方法

1) 抗酸菌の新規鑑別法

*Rv3783*と*Rv3789*遺伝子に対応するプライマーセット1と、*Rv1330c*遺伝子に対応するプライマーセット2を用いたマルチPCR反応を行った後に、電気泳動でバンドを確認することによって、抗酸菌の鑑別を試みた。PCR反応条件は昨年度までに最適化した条件で行った。ATCC株と臨床分離株を含めた52菌種158株の抗酸菌（*M. tuberculosis* 5株、*M. bovis* 6株、*M. bovis* BCG 1株、*M. kansasii* 9株、*M. marinum* 5株、*M. asiaticum* 1株、*M. scrofulaceum* 5株、*M. xenopi* 1株、*M. ulcerans* 1株、*M. gordonae* 6株、*M. szulgai* 6株、*M. avium* 15株、*M. intracellulare* 8株、*M. malmoense* 9株、*M. nonchromogenicum* 5株、*M. shimoidei* 1株、*M. triviale* 1株、*M. abscessus* 1株、*M. chelonae* 1株、*M. fortuitum* 1株、*M. thermoresistibile* 11株、*M. smegmatis* 9株、*M. mucogenicum* 1株、*M. neoaurum* 7株、*M. porcinum* 1株、*M. aichiense* 1株、*M. austroafricanum* 1株、*M. chitae* 2株、*M. chubuense* 1株、*M. diernhoferi* 1株、*M. duvalii* 1株、*M. flavescns* 1株、*M. gadium* 5株、*M. gilvum* 1株、*M. komossense* 1株、*M. moriokaense* 1株、*M. parafortuitum* 1株、*M. phlei* 1株、*M. pulveris* 2株、*M. rhodesiae* 5株、*M. sphagni* 1株、*M. vaccae* 1株、*M. obuense* 1株、*M. novun* 1株、*M. gallinarum* 1株、*M. acepulcemsis* 1株、*M. paraffricum* 1株、*M. butyricum* 1株、*M. goodfellow* 1株、*M. shinshuense* 1株、*M. tokaiense* 1株、及び*M. lactae* 1株）のコロニーをテンプレートとしたPCR反応と電気泳動を行い、新規鑑別法の有用性について評価した。

2) 結核菌由来機能未知遺伝子*Rv2613c*がコードするタンパク質の機能と構造の解析

結核菌由来Rv2613c遺伝子がコードする

タンパク質を大腸菌内で大量発現させた後、2 段階のカラムクロマトグラフィーによりSDS-PAGE 上で単一バンドになるまで精製を行った。HPLC を用いて反応産物のピークを測定することにより、精製タンパク質の機能を同定した。X 線結晶構造解析では、スクリーニングキットを用いて本タンパク質の結晶化条件を決定した後、回折データの収集を放射光科学研究施設で行った。初期位相はセレノメチオニンを用いた単波長異常散乱法により求め、coot プログラムとccp4 プログラムを利用してモデルの構築と精密化を行うことにより、本タンパク質の立体構造を決定した。

倫理面への配慮　本研究は、バイオセーフティーレベルに応じた該当実験室（P2 レベルまたは P3 レベル）で行った。実験を行う際には研究所内の安全講習を受講するとともに、実験計画について安全委員会の承認を受けている。また、大臣確認実験を必要とする実験（組換え DNA 実験）については、必要書類を文部科学省に提出し認可されている。実際の実験では、関連法令を遵守した上で、安全性等に十分に配慮して行った。

C. 研究結果

1) 抗酸菌の新規鑑別法

昨年度までに設計を行った 2 種類のプライマーセットを同時に利用して PCR 反応を行った後、バンドのパターンを調べることにより、抗酸菌の鑑別を行った。PCR 反応条件は昨年度までに確立した方法に準拠した。52 菌種 158 株のコロニーをテンプレートとして本鑑別法の有用性について検討を行った結果、本鑑別法ではバンドパターンが、*M. tuberculosis*、*M. bovis*、*M. bovis* BCG、*M. kansasii*、*M. asiaticum*、*M. parafortuitum*、*M. avium*、*M. intracellulare*、*M. scrofulaceum*、*M. smegmatis*、*M. rhodesiae*、及び *M. malmoense* の 12 種類の菌種を、結核菌群のグループ（*M. tuberculosis*、*M. bovis*、*M. bovis* BCG）、*M. kansasii* のグループ（*M. kansasii*、*M. asiaticum*、*M. parafortuitum*）、MAC のグループ（*M. avium* & *M. intracellulare*）、*M. scrofulaceum* のグループ（*M. scrofulaceum* & *M. smegmatis*）、*M. rhodesiae* のグループ（*M. rhodesiae*）、及び *M. malmoense* のグループ（*M. malmoense*）の 6 種類のグループに明確に分かれることが示された。また、結核菌、MAC、及び *M. kansasii* といった主要な病原性抗酸菌に対して、本鑑別法の特異度・感度は全て 96%以上であった（Table 1）。

2) 結核菌由来機能未知遺伝子 *Rv2613c* がコードするタンパク質の機能と構造の解析

結核菌のゲノム上に存在する機能未知遺伝子 *Rv2613c* がコードするタンパク質について、大量発現・精製・機能解析を行った。その結果、この酵素は diadenosine polyphosphate 等のヌクレオチドに対して加リン酸分解活性を示すことが明らかとなった。従って、このタンパクは結核菌由来の新規 diadenosine polyphosphate 加リン酸分解酵素であることが分かった。この酵素は種々の dinucleoside polyphosphate を基質として利用出来、最適なリン酸の鎖長は 4 または 5 であった。速度論的解析の結果、diadenosine tetraphosphate に対する K_m 値は 0.10 mM であり、リン酸に対する K_m 値は 0.94 mM であった。本酵素の活性発現には 2 価の金属イオンが必須であり、特にマンガン存在下で著しい活性を示した。至適 pH は 8.0、至適温度は 30℃であり、65℃10 分の処理によってその活性は完全に失われた。これまでに報告されている酵母由来の diadenosine polyphosphate 加リン酸分解酵素と比較すると、本酵素は至適 pH や至適温度については類似していたものの、アミノ酸残基数が少なく、モチーフ構造が異なる等、一次構造上では特異な特徴を有していた。さらに、本酵素は加リン酸分解反応の逆反応を触媒しない点で既知の diadenosine polyphosphate 加リン酸分解酵素とは大きく異なっていた。

本酵素の立体構造を決定するため、結晶化条件のスクリーニングを行い、結晶構造解析に適した結晶が得られた（Fig. 1）。次

に、作製したセレノメチオニン誘導体を用いた単波長異常分散法により初期位相を求め、モデルの構築と精密化を繰り返すことによって、本酵素の立体構造を決定した（Fig. 2）。決定した本酵素の立体構造を構造既知の diadenosine polyphosphate 関連酵素と比較した結果、構造骨格部分では相同性が示されたが、活性中心部位ではアミノ酸残基の種類が異なっていることが明らかになった。

D. 考察

本研究課題で確立した抗酸菌の新規鑑別法は、既存の遺伝子検査と比較して、より多くの病原性抗酸菌を簡便に鑑別可能な特長を持っていた。また、主要な病原性抗酸菌の鑑別において、既存の遺伝子検査以上の感度と特異度を示した。臨床現場では迅速な菌種の同定が求められていることから、本鑑別法は今後、そのような要望に応えることができる新規診断法の開発につながることが期待される。

結核菌由来 *Rv2613c* 遺伝子が、新規 diadenosine polyphosphate 加リン酸分解酵素をコードしていることを明らかにした。本酵素は他の diadenosine polyphosphate と比較して、特異な一次構造と活性を有していた。さらに、*Rv2613c* 遺伝子を欠損させせることにより、結核菌の生育能が著しく低下することが報告されていることから、Rv2613c は結核菌に特異的に作用するような新規抗結核薬の標的になり得ることが示された。Rv2613c を標的とする新規抗結核薬の開発に向けて、本タンパク質の立体構造を明らかにした。構造既知の diadenosine polyphosphate 関連酵素と比較すると、構造骨格部分では相同性が見られたが、活性中心部位ではアミノ酸残基の種類が異なっていることが明らかになった。今後は、Rv2613c の機能を損なわせるような化合物をデザインすることによって、新規抗結核薬の開発に結びつくことが期待される。

E. 結論

1) 多くの病原性抗酸菌を簡便に鑑別可能な新規鑑別法を確立した。
2) 結核菌由来 Rv2613c の詳細な機能と構造を明らかにした。

G. 研究発表

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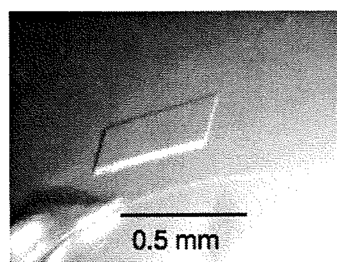
H. 知的財産権の出願・登録状況

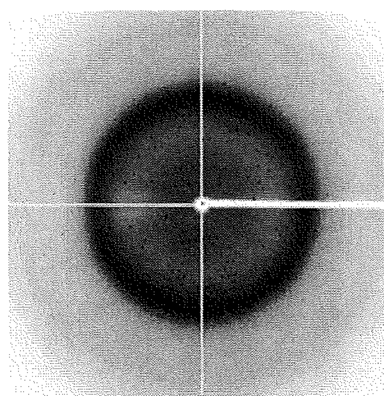
1. 特許取得　なし
2. 実用新案登録　なし
3. その他　なし

Table 1 ATCC株と臨床分離株に対する新規鑑別法の有用性

菌種	感度（%）	特異度（%）
結核菌群	100	100
MAC	96	99
M. kansasii	100	100
M. scroofulaceum & *M. smegmatis*	100	99
合計	98	98



Rv2613c（Native）の結晶



X線回折像

Fig. 1 Rv2613cの結晶とX線回折像

Cドメイン：α / β / α構造

Fig. 2 Rv2613cのリボンモデル

研究成果の刊行に関する一覧表

別紙 5-1

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の 編集者名	書　籍　名	出版社名	出版地	出版年	ページ

別紙 5-1

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Inhibition of the Multiplication of *Mycobacterium leprae* by Vaccination with a Recombinant *M. bovis* BCG Strain That Secretes Major Membrane Protein II in Mice[▽]

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The ability of a recombinant *Mycobacterium bovis* BCG strain that secretes major membrane protein II (MMP-II) of *Mycobacterium leprae* (BCG-SM) to confer protection against leprosy was evaluated by use of a mouse footpad model. C57BL/6J mice intradermally inoculated with BCG-SM produced splenic T cells which secreted significant amounts of gamma interferon (IFN-γ) in response to either the recombinant MMP-II, the *M. leprae*-derived membrane fraction, or the BCG-derived cytosolic fraction in vitro more efficiently than those from the mice infected with the vector control BCG strain (BCG-pMV, a BCG strain containing pMV-261). A higher percentage of CD8$^+$ T cells obtained from BCG-SM-inoculated mice than those obtained from BCG-pMV-inoculated mice produced intracellular IFN-γ on restimulation with the *M. leprae* antigens. BCG-SM inhibited the multiplication of *M. leprae* in the footpads of C57BL/6J mice more efficiently than BCG-pMV. These results indicate that a BCG strain that secretes MMP-II could be a better vaccine candidate for leprosy.

Leprosy, which is caused by *Mycobacterium leprae*, is an infectious disease that still affects thousands of people worldwide. According to WHO's weekly epidemiological report, 254,525 new cases were detected in 2007 (25). One reason why leprosy is still prevalent may be due to the inherent characteristics of *M. leprae*, i.e., slow growth and weak pathogenicity. It takes 12 to 14 days for *M. leprae* to replicate, so it is predicted that 2 to 5 years are necessary for the clinical manifestations to appear after an infection (1, 18). Likewise, it takes 6 to 8 months for the recognizable swelling of the footpad to appear in nude mice (22).

Leprosy is clinically divided into two major categories: multibacillary (MB) leprosy and paucibacillary (PB) leprosy. In the lesions of patients with PB leprosy, dendritic cells (DCs) and activated T cells are involved with confining *M. leprae* to a localized area. These pathological observations indicate that cell-mediated reactions are triggered and that the activation of both CD4$^+$ and CD8$^+$ T cells is closely associated with inhibition of the spread of the bacilli. In contrast, abundant foamy macrophages loaded with bacilli but not DCs appear in the lesions of MB patients (11). It can be speculated that antigen (Ag)-presenting cells such as DCs recognize the immunodominant Ags of *M. leprae* and express those derivatives on their surfaces, thereby activating T cells. Previously, using T cells from patients with PB leprosy, we have identified major membrane protein II (MMP-II), also known as bacterioferritin (ML2038), as one of the immunodominant Ags (8). We found that MMP-II activates DCs through Toll-like receptor 2, leading to higher levels of expression of major histocompatibility complex class I and class II, CD86, and CD83 Ags and increased levels of production of interleukin-12 p70. Furthermore, MMP-II-pulsed DCs derived from patients with PB leprosy activated both autologous CD4$^+$ T cells and CD8$^+$ T cells to produce gamma interferon (IFN-γ) in amounts larger than the amounts produced by T cells from patients with MB leprosy and *M. bovis* BCG-vaccinated healthy individuals, indicating that T cells from patients with PB leprosy may be primed with MMP-II in vivo.

The BCG vaccine has been used for the prevention of tuberculosis, although its role in the prevention of leprosy is still being debated. The protective efficacy of BCG against leprosy has been tested in several trials, including studies in the Karonga District of northern Malawi, in which 50% protection was observed (17). Through combined systematic analyses of experimental studies, Setia et al. found that the BCG vaccine had an overall level of protective efficacy of 26% against human leprosy (19). Their observational studies overestimated the protective effect at 61%. In another review of 29 studies, Zodpey reported that 44.8% of the reports indicated that the BCG vaccine had a level of efficacy of 50% or more (26). These observations indicate that improvements to the BCG vaccine are necessary to increase its protective effect. Recently, we produced a recombinant BCG strain that secretes MMP-II (strain BCG-SM, where SM indicates secreting MMP-II). Since MMP-II has the ability to ligate Toll-like receptor 2, we expected BCG-SM to highly activate human T cells. In fact, BCG-SM activated not only naïve CD4$^+$ T cells but also naïve CD8$^+$ T cells through DCs (9). The fact that BCG-SM was more efficient than the parental BCG strain at the activation of both subsets of naïve T cells led us to seek further insights into the protective activity of BCG-SM. In the present study, we investigated the effect of vaccination of BCG-SM on the multiplication of *M. leprae* in mice.

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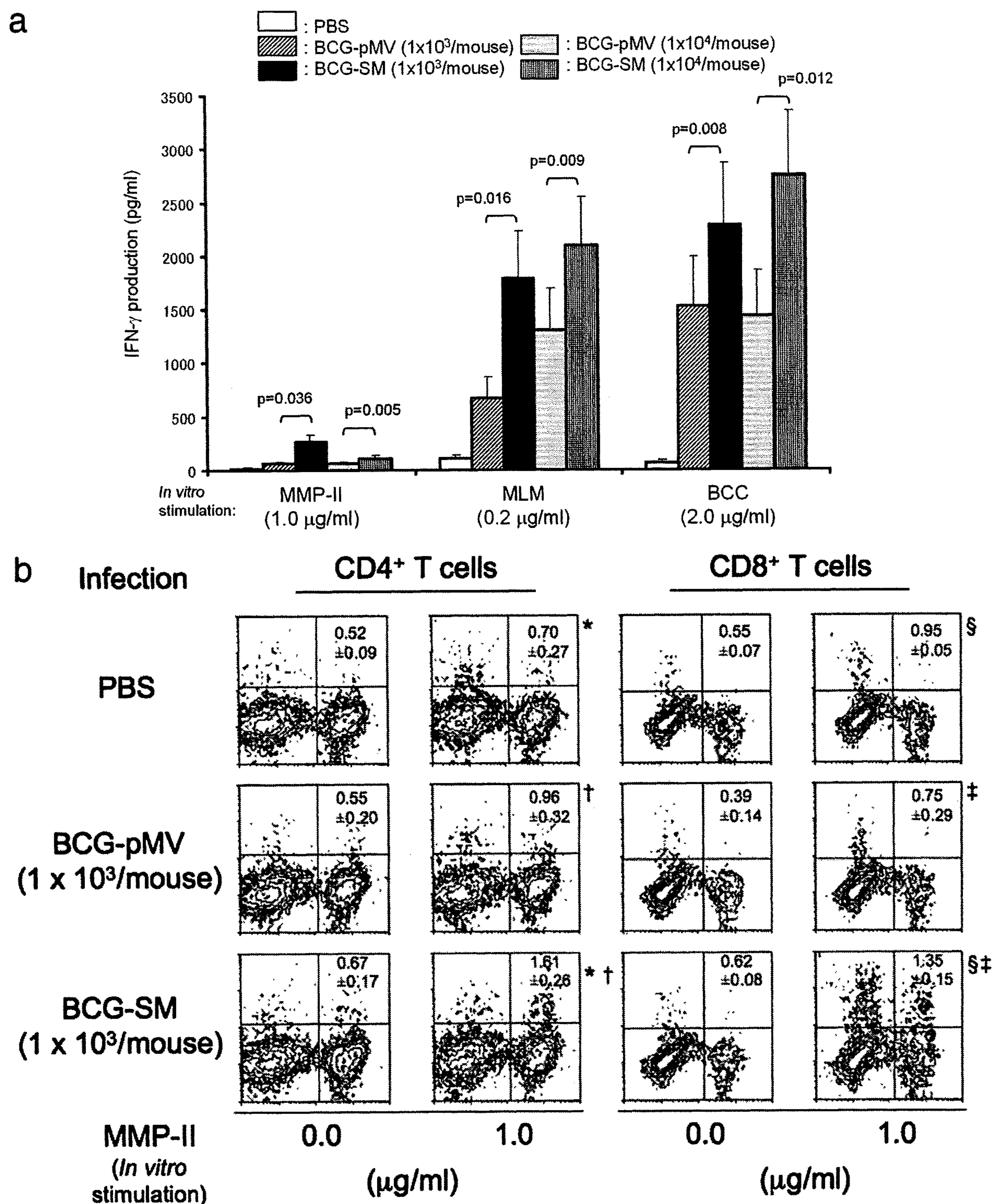


FIG. 1. (a) Production of T cells responsive to *M. leprae*-derived Ags by inoculation with recombinant BCG in mice. Five-week-old C57BL/6J mice were intradermally inoculated with the indicated dose of either BCG-pMV (the vector control BCG strain) or BCG-SM (an MMP-II-secreting BCG strain). Four weeks after the inoculation, splenocytes were restimulated in vitro with the indicated doses of various Ags for 4 days in vitro, and the level of IFN-γ production in the cell supernatant was measured by ELISA. The assays were performed in triplicate for each mouse, and the results for three mice per group are shown as the means ± standard deviations. The representative results of one of three separate experiments are shown. The titers were compared statistically by Student's *t* test. (b) Intracellular IFN-γ production by $CD4^+$ and $CD8^+$ T cells in mice intradermally inoculated with BCG by secondary stimulation. Five-week-old C57BL/6J mice were intradermally infected with 1×10^3 CFU of either BCG-pMV or BCG-SM per mouse. Four weeks after the inoculation, splenocytes (2×10^5/well) were stimulated with 1.0 μg/ml of recombinant MMP-II for 3 days in vitro. The $CD4^+$ T cells and $CD8^+$ T cells were gated separately and analyzed for the intracellular production of IFN-γ. The number at the top right-hand corner of each panel represents the mean percentage of IFN-γ-producing cells ± standard deviation (for three mice) among the gated T-cell population. A representative plot of one of three separate experiments is shown. The titers were compared statistically by Student's *t* test. *, $P < 0.0001$; †, $P < 0.005$; §, $P < 0.05$; ‡, $P < 0.05$.

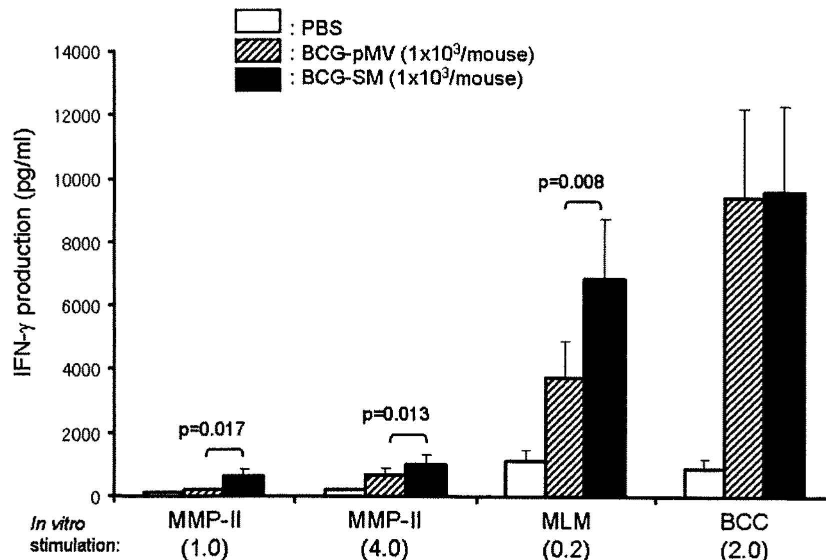


FIG. 2. Long-term effects of vaccination with recombinant BCG on the production of T cells responsive to *M. leprae*-derived Ags. C57BL/6J mice were intradermally infected with 1×10^3 CFU of either BCG-pMV or BCG-SM per mouse. Thirty-four weeks after the inoculation, splenocytes were stimulated with the indicated dose of various Ags for 4 days in vitro, and the amount of IFN-γ produced in the cell supernatant was measured. Assays were carried out in triplicate for each mouse, and the results for three mice per group are shown as the means ± standard deviations. The titers were compared statistically by Student's *t* test.

MATERIALS AND METHODS

Preparation of *M. leprae*, the recombinant BCG strain, and Ags. *M. leprae* (strain Thai-53) was maintained by serial passage in athymic BALB/c *nu/nu* mice (Clea Japan, Inc., Tokyo, Japan) by inoculation of the bacilli into both hind footpads. At 8 months postinoculation, the footpads were processed to recover *M. leprae* bacilli by a previously described method (12, 22). The isolated bacteria were counted by a previously described method (10, 21). Nonfrozen, freshly prepared bacteria were used for inoculation of the mice.

A recombinant BCG strain that secretes *M. leprae*-derived MMP-II was constructed as described previously (9). In brief, a shuttle vector, pMV-261, was used to construct pMV-SM with the MMP-II cDNA fragment. BCG substrain Pasteur was cultured in vitro in Middlebrook 7H9 broth (BD Biosciences–Pharmingen, San Jose, CA) supplemented with 0.05% Tween 80 and 10% albumin-dextrose-catalase (BD Biosciences). Expression vectors were introduced into the BCG strain by electroporation. Transformants were selected on Middlebrook 7H10 agar (BD Biosciences) plates supplemented with 10% oleic acid-albumin-dextrose-catalase (BD Biosciences) and 25 μg/ml kanamycin. The mycobacteria were subsequently grown in Middlebrook 7H9 broth containing 25 μg/ml of kanamycin. The BCG strain containing pMV-SM as an extrachromosomal plasmid is referred to as BCG-SM, while the BCG strain containing pMV-261 is referred to as BCG-pMV. In terms of in vitro growth and infectivity, there was no difference between the two strains. The recombinant MMP-II, the *M. leprae*-derived membrane fraction (MLM), and the cytosolic fraction of BCG (BCC) were obtained as described previously (8, 13).

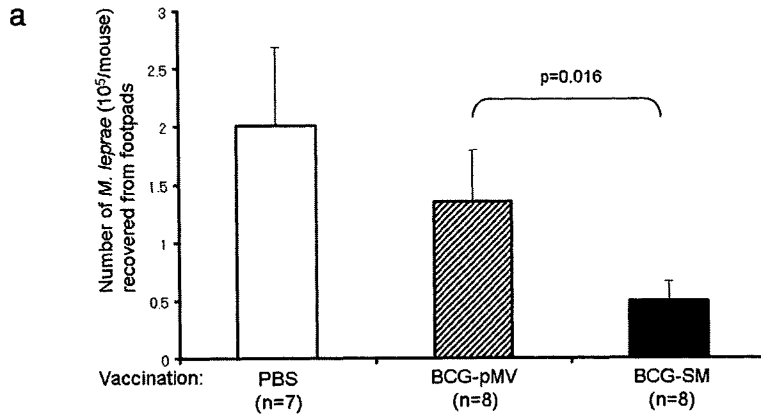
Animal studies. For inoculation into mice, recombinant BCG strains were cultured in Middlebrook 7H9 medium to the log phase of growth and were stored at 10^8 CFU/ml at −80°C. Before the aliquots were used for inoculation, the concentration of viable bacilli was determined by plating the bacilli on Middlebrook 7H11 agar. The indicated numbers of 5-week-old C57BL/6J mice (Clea Japan, Inc.) per group were inoculated intradermally with 0.1 ml of phosphate-buffered saline (PBS) or PBS containing 1×10^3 or 1×10^4 CFU of recombinant BCG per mouse. The animals were kept under specific-pathogen-free conditions and were supplied with sterilized food and water. Four or 34 weeks after inoculation, the spleens were removed and the splenocytes were suspended at a concentration of 2×10^6 cells per ml in culture medium. The splenocytes were stimulated with the indicated concentration of recombinant MMP-II, MLM, or BCC in triplicate in 96-well round-bottom microplates (8). The individual culture supernatants were collected 3 to 4 days after stimulation, and the level of IFN-γ was measured with an Opt EIA mouse enzyme-linked immunosorbent assay (ELISA) set (BD Biosciences). For the recovery of BCG in the spleen 3 weeks after inoculation, the cells were lysed with 0.2% saponin and plated on Middlebrook 7H10 agar for colony counting.

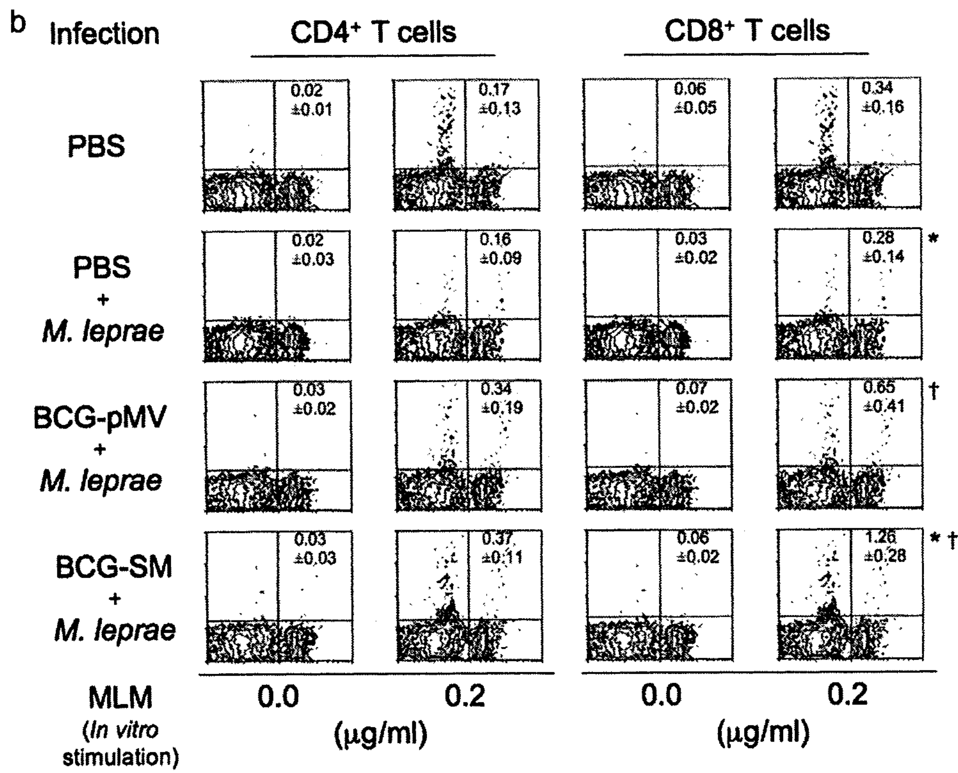
The splenocytes obtained from C57BL/6J mice infected with the recombinant BCG strains were also subjected to the identification of the T-cell subsets responsible for IFN-γ production. The level of intracellular production of IFN-γ by $CD4^+$ T cells and $CD8^+$ T cells, which were restimulated for 3 days in vitro with recombinant MMP-II or MLM, was assessed as follows: cells were treated with Golgi Stop (BD Biosciences), and Golgi transport was inhibited for 4 h. The cells were then surface stained with an allophycocyanin-labeled monoclonal antibody (MAb) to CD4 (MAb RM4-5; BD Biosciences) and a phycoerythrin-labeled MAb to CD8 (MAb H35.17-2; BD Biosciences) in the presence of 7-amino actinomycin D, after which they were washed with PBS containing 1% fetal calf serum and fixed in 1.6% formaldehyde. Subsequently, they were permeabilized with 0.1% saponin and stained with a fluorescein isothiocyanate-conjugated MAb to IFN-γ (MAb XMG1.2; BD Biosciences) or isotype control immunoglobulin G. In another set of experiments, C57BL/6J mice vaccinated intradermally with the indicated dose of BCG-pMV or BCG-SM for 4 weeks were challenged in the footpad with 5×10^3 of *M. leprae* per mouse. Thirty or 31 weeks later, the footpads and spleens were processed for further analyses. The number of *M. leprae* bacilli that grew in the footpads was enumerated by the method of Shepard and McRae (21), and the splenocytes were used to assess the level of IFN-γ production by the ELISA method and for intracellular staining for IFN-γ by flow cytometry (FACSCalibur flow cytometer; BD Biosciences). The animal experiments were reviewed and approved by the Animal Research Committee of Experimental Animals of the National Institute of Infectious Diseases and were conducted according to established guidelines.

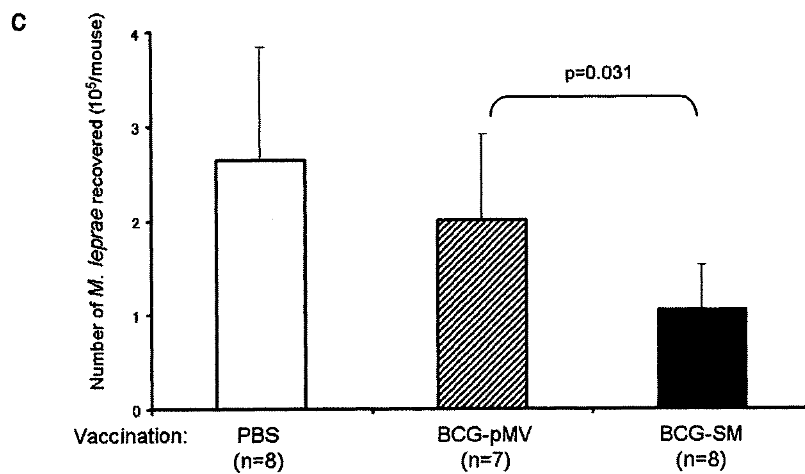
Statistical analysis. Student's *t* test and the Mann-Whitney-Wilcoxon test were used to determine statistical differences.

RESULTS

Production of *M. leprae*-derived Ag-responsive T cells in C57BL/6J mice vaccinated with BCG-SM. The purpose of vaccination is to produce T cells which can respond to *M. leprae* or *M. leprae*-derived Ags. C57BL/6J mice were intradermally infected with either BCG-pMV or BCG-SM; and their splenocytes were restimulated in vitro with the recombinant MMP-II, the MLM protein, or the BCC protein (Fig. 1a). While the


a
Number of *M. leprae* (10^5/mouse) recovered from footpads
p=0.016
Vaccination:
PBS (n=7)
BCG-pMV (n=8)
BCG-SM (n=8)


b
Infection
CD4+ T cells
CD8+ T cells
PBS
PBS + *M. leprae*
BCG-pMV + *M. leprae*
BCG-SM + *M. leprae*
MLM (*In vitro* stimulation)
0.0
0.2
(μg/ml)


c
Number of *M. leprae* recovered (10^5/mouse)
p=0.031
Vaccination:
PBS (n=8)
BCG-pMV (n=7)
BCG-SM (n=8)

splenocytes obtained from C57BL/6J mice inoculated with PBS minimally responded to these Ags, mice infected with either BCG-pMV or BCG-SM significantly responded to the Ags. However, splenocytes from the BCG-SM-vaccinated mice responded to all Ags more strongly and produced levels of IFN-γ higher than those from BCG-pMV-vaccinated mice. In order to define the T cells responsible for IFN-γ production, the T cells producing the intracellular cytokines were determined (Fig. 1b). Both $CD4^+$ T cells and $CD8^+$ T cells obtained from mice inoculated with PBS, BCG-pMV, or BCG-SM produced IFN-γ on stimulation with MMP-II. However, the two subsets of T cells from BCG-SM-infected mice responded to the stimulation more strongly than T cells from BCG-pMV-infected mice, and more than 1.0% of both $CD4^+$ T cells and $CD8^+$ T cells produced IFN-γ.

Human leprosy usually manifests long after the infection with *M. leprae*. Therefore, we evaluated the long-term effect of vaccination with BCG-SM (Fig. 2). C57BL/6J mice were vaccinated intradermally with the recombinant BCG strain for 34 weeks, and their splenocytes were examined for a secondary response to *M. leprae*-derived Ags. While the response to BCC did not differ between BCG-pMV- and BCG-SM-infected mice, significantly higher levels of IFN-γ were produced in splenocytes from BCG-SM-vaccinated mice than in those from BCG-pMV-vaccinated mice on in vitro restimulation with both the recombinant MMP-II and MLM. These results indicate that the effect of the BCG-SM vaccination persisted for a long time.

Effect of BCG-SM vaccination on multiplication of *M. leprae* in vivo. C57BL/6J mice that had been vaccinated 4 weeks earlier with either BCG-pMV or BCG-SM (1×10^4/mouse) intradermally were challenged with 5×10^3 *M. leprae* bacilli in the footpad. Thirty or 31 weeks later, the footpads were removed and the *M. leprae* bacilli recovered were enumerated (Fig. 3a). A total of 2×10^5 *M. leprae* were recovered from the mice inoculated with PBS and challenged with *M. leprae*, and BCG-pMV partially inhibited the multiplication of *M. leprae*. However, only 5×10^4 *M. leprae* bacilli were recovered from the BCG-SM-vaccinated mice, showing that BCG-SM is more effective than BCG-pMV at inhibiting the growth of *M. leprae*. In order to clarify the T-cell population responsible for the inhibition of *M. leprae* growth, $CD4^+$ T cells and $CD8^+$ T cells from BCG-vaccinated and *M. leprae*-challenged mice were restimulated with MMP-II (data not shown) or MLM (Fig. 3b) in vitro. There was no significant difference in the percentage of IFN-γ-producing $CD4^+$ T cells among uninfected *M. leprae*-challenged, BCG-pMV-vaccinated *M. leprae*-challenged, and BCG-SM-vaccinated *M. leprae*-challenged mice. However, significantly higher numbers of $CD8^+$ T cells from BCG-SM-vaccinated *M. leprae*-challenged mice than T cells from the other groups of mice produced intracellular IFN-γ in response to MMP-II. We then examined the effect of a lower dose of recombinant BCG on the multiplication of *M. leprae* in the footpads of mice. Again, a 40-fold increase in the number of *M. leprae* bacilli was observed in *M. leprae*-challenged (5×10^3/mouse) nonvaccinated mice. Also, vaccination with BCG-SM was more effective in inhibiting the growth of *M. leprae* than vaccination with BCG-pMV (Fig. 3c).

DISCUSSION

In 1991, the World Health Assembly proposed the elimination of leprosy as a public health problem by the year 2000, since the multidrug therapy was drastically effective in reducing the number of registered leprosy cases. However, at present, more than 200,000 newly manifested leprosy cases are still reported annually (25). Therefore, in order to eliminate the disease, an effective and safe vaccine is needed. The vaccine should also be widely available at a low cost. An assessment of the cost-effectiveness of BCG vaccination on childhood tuberculosis was conducted and was found to be a highly cost-effective intervention (23). However, human immunodeficiency virus (HIV)-infected infants who were vaccinated with BCG at birth were at high risk of developing disseminated BCG disease (5, 6). Therefore, care should be taken to prevent the vaccination of HIV-exposed infants with BCG. For the prevention of leprosy, a number of field trials as well as animal experiments have been conducted to test the efficacies of heat-killed *Mycobacterium leprae*, *Mycobacterium* sp. strain w, the combination of *M. leprae* and *Mycobacterium* sp. strain w, and recombinant *M. bovis* BCG as candidate vaccines in regions of endemicity (1–4, 7, 15, 20, 24). Although *M. bovis* BCG offered a certain level of protection against leprosy, its effect needs to be bolstered (19, 26). To improve BCG, its immunostimulatory activity needs to be enhanced. It is generally believed that in the host defense against mycobacteria, including *M. leprae*, both $CD4^+$ T cells and $CD8^+$ T cells play a central role. In the initial stage of a mycobacterial infection, the cells that mainly participate are the IFN-γ-producing $CD4^+$ T cells. The IFN-γ produced from $CD4^+$ T cells may activate macrophages infected with the mycobacteria, and the activated macrophages may induce the intracellular killing of the mycobacteria. In

FIG. 3. (a) Effect of vaccination with recombinant BCG on *M. leprae* multiplication. Five-week-old C57BL/6J mice were intradermally inoculated with 1×10^4 CFU of BCG-SM or BCG-pMV per mouse 4 weeks prior to challenge in the footpad with 5×10^3 of *M. leprae*. Thirty to 31 weeks later, the number of *M. leprae* bacilli recovered from the footpad was enumerated by the method of Shepard (22). The indicated number of the mice per group was used, and the numbers of bacilli recovered were compared statistically by the Mann-Whitney-Wilcoxon test. (b) Intracellular production of IFN-γ by $CD4^+$ T cells and $CD8^+$ T cells in mice vaccinated with BCG and challenged with *M. leprae*. C57BL/6J mice were vaccinated with 1×10^4 CFU of either BCG-SM or BCG-pMV per mouse for 4 weeks and challenged with 5×10^3 bacilli of *M. leprae* for 30 to 31 weeks. Splenocytes (2×10^5/well) were obtained from these mice and were restimulated with 0.2 μg/ml of MLM for 3 days in vitro. The $CD4^+$ T cells and $CD8^+$ T cells were gated separately and were analyzed for the intracellular production of IFN-γ. The number in the top right-hand corner of each panel represents the mean percentage of IFN-γ-producing cells ± standard deviation (for three mice) among the gated T-cell population. The titers were compared statistically by Student's *t* test. *, $P < 0.01$; †, $P < 0.05$. (c) Effect of vaccination with a low dose of BCG on the multiplication of *M. leprae*. Again, as described for panel a, C57BL/6J mice were inoculated but they were inoculated with a lower dose of recombinant BCG (1×10^3 CFU per mouse), and the effect on the multiplication of *M. leprae* was observed. The numbers of bacilli recovered were compared statistically by the Mann-Whitney-Wilcoxon test.

contrast, in the chronic stage, cytotoxic T lymphocytes differentiated from the activated type 1 $CD8^+$ T cells mainly act to inhibit the growth of the intracellular mycobacteria (11, 16). Thus, the activation of both $CD4^+$ T cells and $CD8^+$ T cells is essential for inhibiting the multiplication of mycobacteria.

We previously screened for *M. leprae* antigens with immunostimulatory properties and observed that a membrane protein, namely, MMP-II, stimulated human monocyte-derived DCs to produce the active form of interleukin-12 and tumor necrosis factor alpha (8). DCs pulsed with MMP-II stimulated both $CD4^+$ and $CD8^+$ T cells to produce IFN-γ. Therefore, we produced a recombinant BCG strain that secretes *M. leprae*-derived MMP-II (strain BCG-SM). The DCs infected with BCG-SM activated both human naïve $CD4^+$ T cells and naïve $CD8^+$ T cells more efficiently than the vector control BCG (9). T cells of both subsets which can respond to MLM as well as recombinant MMP-II were more efficiently produced from unprimed mice by inoculation with BCG-SM (Fig. 1). At 3 weeks postinoculation, no BCG could be recovered from the spleen. Moreover, it was found that BCG-SM effectively inhibited the multiplication of *M. leprae* in the footpads of C57BL/6J mice, possibly due to the efficient production of T cells responsive to *M. leprae*-derived Ags. It may be difficult to determine the T-cell subset responsible for the inhibition; however, $CD8^+$ T cells from mice vaccinated with BCG-SM and challenged with *M. leprae* for 30 weeks still had the ability to produce IFN-γ after stimulation with *M. leprae*-derived Ag. Furthermore, *M. leprae*-responsive $CD4^+$ and $CD8^+$ T cells persisted for 34 weeks after infection with BCG-SM. Therefore, it is possible that $CD8^+$ T cells at least partially contribute to inhibiting the growth of *M. leprae* in vivo.

Earlier efforts to produce a vaccine against leprosy have not been particularly successful. Some reports indicated that a mixture of refined components of *M. leprae* was protective, while others emphasized DNA-based vaccines (13, 14). To date, BCG-based vaccines seem to be more promising in terms of their applicability in the field due to the safety and history of global usage of BCG. Taken together, the present study indicates that a recombinant BCG strain that secretes MMP-II could be a useful candidate as a vaccine against leprosy.

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Induction of Cross-Priming of Naive CD8$^+$ T Lymphocytes by Recombinant Bacillus Calmette-Guérin That Secretes Heat Shock Protein 70-Major Membrane Protein-II Fusion Protein[1]

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Because *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) unconvincingly activates human naive CD8$^+$ T cells, a rBCG (BCG-70M) that secretes a fusion protein comprising BCG-derived heat shock protein (HSP)70 and *Mycobacterium leprae*-derived major membrane protein (MMP)-II, one of the immunodominant Ags of *M. leprae*, was newly constructed to potentiate the ability of activating naive CD8$^+$ T cells through dendritic cells (DC). BCG-70M secreted HSP70-MMP-II fusion protein in vitro, which stimulated DC to produce IL-12p70 through TLR2. BCG-70M-infected DC activated not only memory and naive CD8$^+$ T cells, but also CD4$^+$ T cells of both types to produce IFN-γ. The activation of these naive T cells by BCG-70M was dependent on the MHC and CD86 molecules on BCG-70M-infected DC, and was significantly inhibited by pretreatment of DC with chloroquine. Both brefeldin A and lactacystin significantly inhibited the activation of naive CD8$^+$ T cells by BCG-70M through DC. Thus, the CD8$^+$ T cell activation may be induced by cross-presentation of Ags through a TAP- and proteosome-dependent cytosolic pathway. When naive CD8$^+$ T cells were stimulated by BCG-70M-infected DC in the presence of naive CD4$^+$ T cells, CD62L^{low}CD8$^+$ T cells and perforin-producing CD8$^+$ T cells were efficiently produced. MMP-II-reactive CD4$^+$ and CD8$^+$ memory T cells were efficiently produced in C57BL/6 mice by infection with BCG-70M. These results indicate that BCG-70M activated DC, CD4$^+$ T cells, and CD8$^+$ T cells, and the combination of HSP70 and MMP-II may be useful for inducing better T cell activation. *The Journal of Immunology,* **2009, 183: 6561–6568.**

Leprosy is a chronic infectious disease induced by an intracellular infection with *Mycobacterium leprae* (1, 2). Host defense against *M. leprae* is chiefly conducted by adaptive immunity in which both IFN-γ-producing type 1 CD4$^+$ T cells and CD8$^+$ T cells play an important role, and the activation of these T cells inhibits the spread of *M. leprae* (3–5). The activation is induced by bacilli-loaded dendritic cells (DC),[3] which display one or more antigenic determinants of *M. leprae*. Previously, we identified major membrane protein (MMP)-II (gene name, bfrA or ML2038) as one of the immunodominant Ag of *M. leprae* (6). MMP-II activates dendritic cells (DC) by activating the NF-κB pathway as a consequence of TLR2's ligation, and DC pulsed with a rMMP-II protein activate both naive and memory-type CD4$^+$ and CD8$^+$ T cells to produce IFN-γ in an Ag-specific manner (6, 7). In the lesions of patients with paucibacillary leprosy, representative of clinical leprosy on one pole, the involvement of CD1a$^+$ DC and presence of substantially activated T cells have been observed (8, 9). Furthermore, MMP-II is thought to be recognized by both T cell subsets in *M. leprae*-infected individuals, including patients with paucibacillary leprosy (7). Therefore, MMP-II is considered to play essential roles in the induction of host defense activity against *M. leprae*. Also, we reported that T cells from lepromatous leprosy, representative of clinical leprosy on another pole, can be activated to produce IFN-γ when stimulated with MMP-II-pulsed autologous DC (7), although it is known that the T cells of lepromatous leprosy patients are usually unresponsive to *M. leprae*-derived Ags (2).

Mycobacterium bovis bacillus Calmette-Guérin (BCG) is the sole available vaccine against leprosy, and several reports have evaluated its efficacy. In some countries and endemic areas, BCG has effectively inhibited the development of leprosy, whereas in others, its efficacy is reported to be quite limited (10–12). These observations indicate that questions remain regarding the reliability of BCG as a vaccine, and, in fact, Setia et al. (13) elucidated the overall efficacy of BCG to be only 26% through meta-analyses of several studies and observations. Based on these findings, we previously produced a rBCG that secretes MMP-II intracytosolically (BCG-SM) (14). As expected, BCG-SM activated both naive CD4$^+$ and CD8$^+$ T cells (14) and inhibited *M. leprae* from multiplying to some extent, but not completely, in the footpads of C57BL/6 mice (Y. M., T. T., M. Mat., and M. Mak.; unpublished observations). It is known that the parental BCG activates chiefly CD4$^+$ T cells, and less efficiently activates naive CD8$^+$ T cells (15). That BCG-SM activated naive T cells of both subsets and, consequently, partially inhibited the multiplication of *M. leprae*,

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[3] Abbreviations used in this paper: DC, dendritic cell; BCC, *Mycobacterium bovis* bacillus Calmette-Guérin-derived cytosolic protein; BCG, *Mycobacterium bovis* bacillus Calmette-Guérin; BCG-SM, rBCG that secretes major membrane protein-II; HSP, heat shock protein; MMP, major membrane protein; MOI, multiplicity of infection.

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