ツベルクリン反応は，生体内に結核菌に反応するメモリーT細胞が存在するかを検索する方法である。ツベルクリンは，液体培地で数週間培養した結核菌を加熱・濾過処理したものである。ツベルクリン反応陽性とは，結核と同じ病原性をもつものが過去に生体内に入り，T細胞によって認識された経歴があることを示している。従って，「陽性」イコール「結核菌の感染あり」ということはできない。弱毒化牛型結核菌であるBCG接種者も陽性を示すことが多い。

(5) 結核の治療と予防

「不治の病」といわれた結核も，早期発見・早期治療の原則が守られれば6カ月で治る病気となっている。厚生労働省で認可されている薬剤は10種類以上を数えている。

基本的な治療法は，初期2カ月の強化療法とこれに続く4カ月の継続期治療の6カ月間治療である。強化療法とは，4剤（106頁参照）の併用療法であり，菌量を減少させることを目的としている。4カ月間の継続療法は，2～3剤（INH，RFPとEB）の併用療法である。日本では，1996（平成8）年厚生省の「結核治療基準」によって適用された。菌量の早期減少を図るとともに，耐性菌の出現を防ぐことを念頭において確定された治療法であるが，近年では耐性菌の数が次第に増加しており大きな問題となっている。複数の薬剤を長期服用するため，副作用の出現にも十分留意する必要がある。

予防法は，未だに確立されていない。ワクチンとしてBCGが使われてきたが，小児または青年成人の発症を防ぐうえでは有効であるが，高齢者など免疫機能が低下した人々に対しては無効である。BCGに代わる新しいワクチンの開発が切望されている。

排菌者と接触し，ツベルクリン反応検査から感染が強く疑われる場合には，「化学予防法」としてINH単剤を朝1回6カ月間の服用が有効である。

2) ハンセン病

(1) 概　要

ハンセン病は，皮膚および末梢神経が主に侵される慢性感染症である。らい菌感染により発症するが，らい菌は末梢神経障害をもたらす唯一の抗酸菌である。病変部位に存在するらい菌の数により少菌型と多菌型に分類される。らい菌に対する生体防御反応（細胞性免疫が中心）の強弱によりその病型が決定され，細胞性免疫が働かない多菌型では，全身・左右対称性に病変が出現する。多菌型では，らい菌に特異的である抗PGL-I（Phenolicglycolipid-I）抗体の検出が補助診断法として有効である。

WHOによる多剤併用療法が有効であり，近年では新規発症患者数も徐々に低下していて，日本国内での発症例は毎年20以下である。ハンセン病に対する有効なワクチンは存在せず，多剤耐性を示すらい菌も徐々に増加している。今後の課題が多い疾患である。

（2）らい菌と感染経路

らい菌は，長さ2ミクロン，幅0.3ミクロンの桿菌であり，結核菌と同じ抗酸菌に分類される。抗酸菌を増殖のスピードから分類すると発育の早い菌と遅い遅発育菌に分類されるが，らい菌はすべての細菌のなかでもっとも増殖が遅く，1回の分裂に約12日を要する（ちなみに大腸菌は20分)。らい菌を試験管のなかで人工的に増やすことはできず，アルマジロやヌードマウスを用いなければならない。らい菌の細胞壁は，結核菌と同様きわめて厚く脂質に富んだ構造をしている。このなかにらい菌に特異的な抗原であるPGL-Iが存在し，抗PGL-I抗体はハンセン病の血清診断に利用される。しかし，少菌型患者では，抗PGL-I抗体陰性の例が多い。

感染は鼻粘膜を介した飛沫感染である。従来損傷部位を介した経皮感染も考えられたが，感染には多数の菌が必要なため現在では否定的である。ヒトからヒトへの感染は，濃厚流行国の排菌患者周辺のみで起こる。

（3）疫　学

ハンセン病の濃厚流行国は，東南アジア・南米・アフリカである。1982-50（昭和57）年，WHOはハンセン病の制圧を目指し，3薬剤を同時に服用する多剤併用療法を開始した。本療法はきわめて有効であり，現在では登録患者数は44万人に，新規患者数は年間50万人にまで激減した。WHOはハンセン病の制圧を人口10万人対1以下と定義したが，現在このレベルに到達していないのは，ブラジル・インドなど数カ国のみである。しかし，濃厚流行国では，国全体としては制圧はされているものの，スポットとよばれる地域で未だに新規患者が多数発生している。

らい菌に対する生体防御反応は，結核と同じ細胞性免疫が主体であるが，結核とは異なりHIV-1感染者・高齢者など免疫状態が低下した患者に感染しても急性増悪することはない。HIV-1とらい菌の重複感染も臨床上問題とはならない。

（4）臨　床

らい菌は，マクロファージ・末梢神経のシュワン細胞および血管内皮細胞に主に感染する。そのためハンセン病は皮膚および末梢神経が主な病変部位となる。少菌型では，1個ないし少数の皮疹が左右非対称性に出現する。一方，多菌型では自覚症状は少なく，神経症状も徐々に出現する。多彩な皮疹が多数左右対称性に出現する。

ハンセン病の診断は，皮疹・らい菌の検出・末梢神経の肥厚・末梢神経の機能障害・病理組織学的検査・抗PGL-I抗体を用いた血清診断法が有効である。皮膚の病変部位をメスの刃で小さく切開し，組織液を取り，塗抹標本を作製する。Ziehl-Neelsen染色法で菌を同定する。菌が少数のみ存在し病理学的に同定しにくい場合は，病変部位を生検して組織を採取し，その組織よりDNAを抽出し，PCR法で診断する。この方法は感度のうえで優れている。病理学的検査では，少菌型は類上皮細胞性肉芽腫が観察されるが，多菌型では肉芽腫は形成されにくく，

泡沫状のマクロファージとらい菌が観察される。

末梢神経症状としては，皮疹に一致した知覚障害，とくに表在性感覚の低下が認められる。また，発汗障害・血管運動障害など自律神経系の異常を伴いやすい。これら末梢神経障害および皮疹の出現をみた場合，ハンセン病を疑って菌の検索を勧めたい。

治療は，抗菌活性の強い3剤（ダプソン[DDS]，クロファジミン[CLF：B663]，リファンピシン[RFP]）を同時に使用する多剤併用療法が基本である。ハンセン病に対する有効なワクチンは存在しない。

ハンセン病の治療中に突然“らい反応”とよばれる急性増悪がしばしば観察される。タイプ1とタイプ2の2型に分類され，タイプ1反応はらい菌抗原に対する強いアレルギー反応であり，タイプ2反応は免疫複合体が関与する。いずれも免疫抑制剤が有効である。

3）結核・ハンセン病の撲滅

結核およびハンセン病は古くから知られる慢性感染症である。ともに細胞内に寄生性に感染する病原体により発症するため，その撲滅はきわめて難しい。耐性菌が出現し，その数が増加する傾向にある現在，有効なワクチンの開発が，両疾患に共通する最大の課題である。

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Streptomycin-Dependent Exhibition of Cytokine-Inducing Activity in Streptomycin-Dependent *Mycobacterium tuberculosis* Strain 18b

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Peritoneal exudate cells of mice were stimulated with a streptomycin-dependent *Mycobacterium tuberculosis* strain, 18b. Gamma interferon production by natural killer cells depending on interleukin-12 and interleukin-18 was induced only in the presence of a high dose of streptomycin. This study suggested the requirement of active bacterial metabolism for this host response.

Mycobacterium tuberculosis is a causative agent of tuberculosis in human being. One-third of the world's population is currently infected with tubercle bacilli, and the global incidence of active tuberculosis per year is around 8 million cases (2). It is of great importance to understand the basic mechanism for the induction of the immune response of the host against *M. tuberculosis*. The generation of protective immunity against tuberculosis is dependent on the induction of $CD4^+$ Th1 cells capable of producing gamma interferon (IFN-γ) upon stimulation with specific antigen. IFN-γ contributes to the development of acquired resistance via the activation of macrophages (3). The importance of IFN-γ in the resistance of mice to *M. tuberculosis* has been confirmed by utilizing IFN-γ knockout mice and IFN-γ receptor knockout mice (1, 4, 11). IFN-γ is crucial also for the development of protective T cells. In our previous study, the treatment of mice with anti-IFN-γ antibody during primary immunization with viable cells of *M. bovis* bacillus Calmette-Guérin reduced the number of antigen-specific IFN-γ-producing cells and abolished the generation of protective immunity (26). Thus, IFN-γ is indispensable for both induction and expression of protective immunity against tuberculosis.

It has been shown that $CD4^+$ protective T cells are generated after infection with a sublethal dose of *M. tuberculosis* or *M. bovis* bacillus Calmette-Guérin, whereas such effector T cells are hardly induced by immunization with killed bacteria (14). We have found that the failure of killed bacteria to induce effective protective immunity in mice is due to the absence of an IFN-γ-inducing ability that is observed exclusively in viable bacilli (25, 26). Killed *M. tuberculosis* has been prepared generally by treatment with heating, germicides, or irradiation (7, 16, 20, 24), but such treatment may affect several bacterial components physically or chemically. In order to address whether the significant difference in the IFN-γ-inducing abilities of viable and killed *M. tuberculosis* is due to some undesirable changes introduced during the killing process or actually due to the viability itself, we have employed a streptomycin (SM)-dependent *M. tuberculosis* strain, 18b, in this study.

This particular strain, originally isolated in 1955 (6), has been maintained as a stock for a long time at the National Institute of Infectious Diseases in Japan, so we first confirmed whether this strain maintained the original SM dependency. On a Middlebrook 7H10 agar plate, strain 18b never grew during 5 weeks of observation in the absence of SM (Fig. 1). However, the addition of SM at concentrations of 50 μg/ml and above supported the growth of bacteria, resulting in the formation of countable colonies. It was confirmed that cells of strain 18b kept under even an SM-free condition never die for many weeks, as has been reported in the past (12, 19). According to the molecular characterization reported in 1995, *M. tuberculosis* strain 18b was shown to carry a novel mutation in

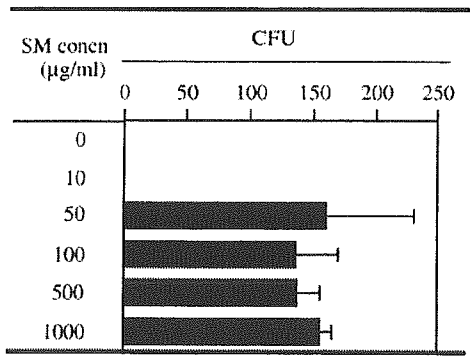

FIG. 1. SM-dependent growth of *M. tuberculosis* strain 18b. One hundred microliters of an appropriately diluted suspension of *M. tuberculosis* strain 18b was inoculated on Middlebrook 7H10 agar plates containing the indicated concentrations (concn) of SM. After 5 weeks of cultivation at 37°C, the number of visible colonies was counted. Data are shown as the means for three cultures ± standard deviations (SD). On the plates containing 0 and 10 μg/ml of SM, no colony formation was observed at the end of 5 weeks of cultivation. Two independent experiments showed similar results.

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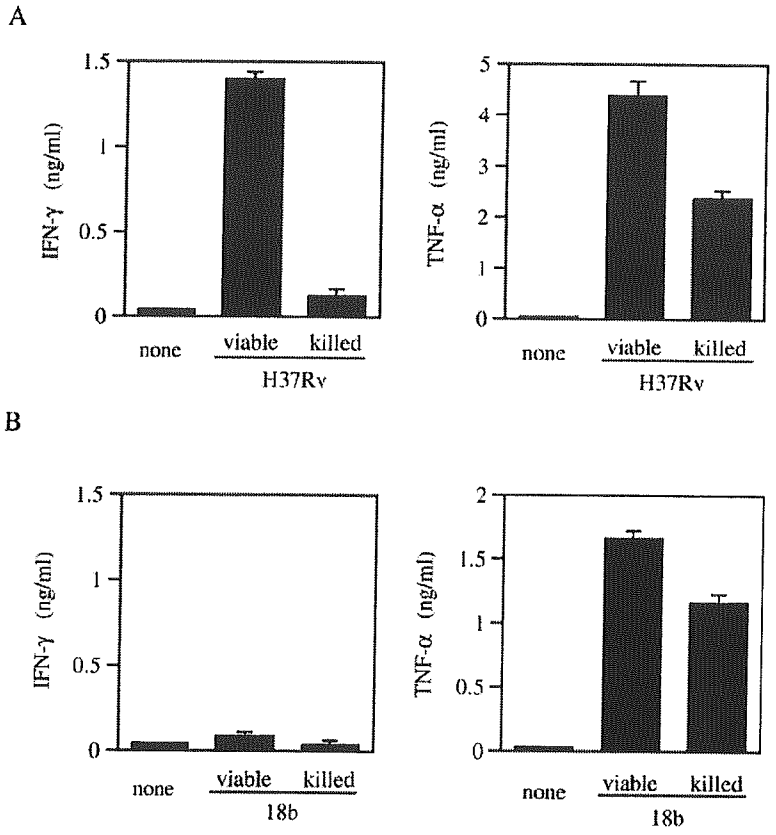

FIG. 2. Production of IFN-γ and TNF-α after stimulation of PECs with H37Rv or 18b in the absence of SM. PECs were stimulated with viable or heat-killed cells of H37Rv (A) or 18b (B) for 18 h at MOIs of 2 in the absence of SM. Killed bacteria were prepared by heating at 70°C for 2 h. The culture supernatants were collected, and the concentrations of IFN-γ and TNF-α were measured by enzyme immunoassay. Data are shown as the means for triplicate wells ± SD. The data shown are from one of two independent experiments.

the *rrs* gene, coding for 16S rRNA (8). By PCR amplification and sequence analysis of the corresponding region of genomic DNA, we were able to detect just one insertion of an additional cytosine residue (underlined) between positions 512 and 513 in the 530 loop of 16S rRNA (AGCCAGCCGCGGTAATACG TAG), as reported previously (8).

We compared the IFN-γ-inducing activities of *M. tuberculosis* H37Rv and strain 18b. Peritoneal exudate cells (PECs) were induced in C3H/HeN mice by the intraperitoneal injection of 3% thioglycolate medium, and the cells were stimulated with *M. tuberculosis* at a multiplicity of infection (MOI) of 2 for 18 h in the absence of SM to measure the cytokine produced in the supernatant. The concentration of IFN-γ was measured by a sandwich enzyme-linked immunosorbent assay constructed in our laboratory (10), and a mouse tumor necrosis factor alpha (TNF-α) enzyme-linked immunosorbent assay set purchased from BD Biosciences (San Jose, CA) was used for measuring TNF-α. Interestingly, the IFN-γ-inducing activity of viable strain 18b was considerably weaker than that of viable H37Rv (Fig. 2).

In order to consider whether the activation of the growth cycle results in the change in IFN-γ-inducing activity, we next examined the IFN-γ production induced by stimulation with viable cells of strain 18b in the presence of graded concentrations of SM in a PEC culture. The high-level production of both IFN-γ and TNF-α induced by stimulation with viable cells of strain H37Rv showed slight decreases corresponding to the increase of SM concentration in the cell culture (Fig. 3A). On the other hand, the IFN-γ production induced by stimulation with viable cells of strain 18b, which was very low in the absence of SM, showed a significant increase that depended on the concentration of SM supplemented in the culture (Fig. 3B). TNF-α production was also increased, depending on the concentration of SM. This result indicated that support of bacterial growth is essential for the maximum expression of the IFN-γ-inducing activity of *M. tuberculosis* and suggested that the initiation of active metabolism may account for the changes observed here.

In contrast to the SM concentration supporting the growth of strain 18b on Middlebrook 7H10 medium, an extremely higher concentration was required in PEC culture for the acquisition of a significant level of cytokine-inducing activity. As SM is one of the antibiotics that are believed to have difficulty in penetrating cell membranes of PECs (15), it was plausible that this high concentration of SM was necessary to provide an intracellular SM concentration required by the bacteria inside

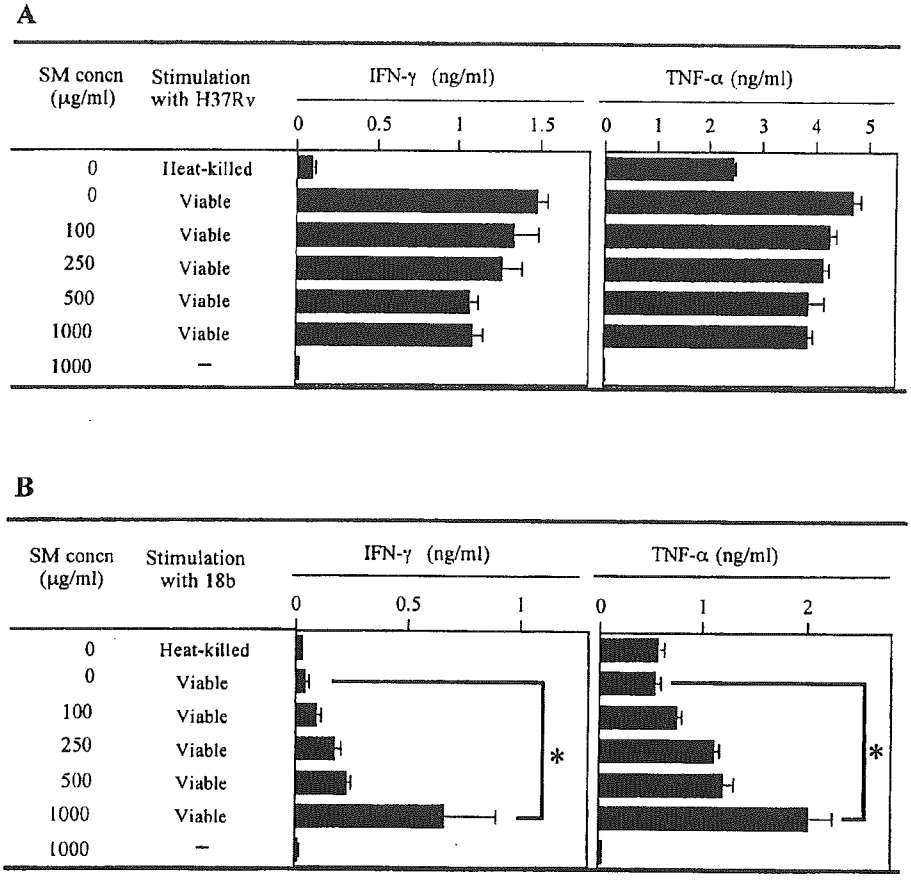

FIG. 3. Production of IFN-γ and TNF-α after stimulation of PECs with H37Rv or 18b in the presence of various concentrations (concn) of SM. The indicated concentrations of SM were added to the PEC culture. Two hours later, PECs were stimulated with viable cells of H37Rv (A) or 18b (B) for 18 h at MOIs of 2. The culture supernatants were collected and the concentrations of IFN-γ and TNF-α were measured. The data shown are the means for triplicate wells ± SD. The data shown represent three independent experiments that showed the same tendency. *, $P < 0.01$ according to Student's t test.

macrophages. To address this possibility, PECs were cultured in the presence of various concentrations of SM for 20 h, a period equivalent to that used in the cytokine assay. After extensive washings, cells were lysed, and the SM was titrated by using a RIDASCREEN streptomycin assay (AZmax Co. Chiba, Japan). Then, the intracellular concentrations of SM were calculated from the packed-cell volume. The results indicated that the intracellular concentration of SM was highly dependent on the concentration in the cell culture medium. As shown in Fig. 4, the intracellular concentration of SM was estimated to be above 100 μg/ml when added to the cell culture at 1,000 μg/ml. This finding supported an idea that the IFN-γ-inducing activity of *M. tuberculosis* is highly dependent on the presence of active metabolism. As one of the parameters for bacterial metabolism, the concentration of ammonium ion produced by *M. tuberculosis* (5) was measured by using a PACK TEST (PACK TEST Ammonium; Kyoritsu Chemical-Check Laboratory Corp., Tokyo, Japan). There was an increase in the concentration of ammonium ion in the supernatant of 18b cultured in RPMI 1640 medium for 20 h in the presence of 100

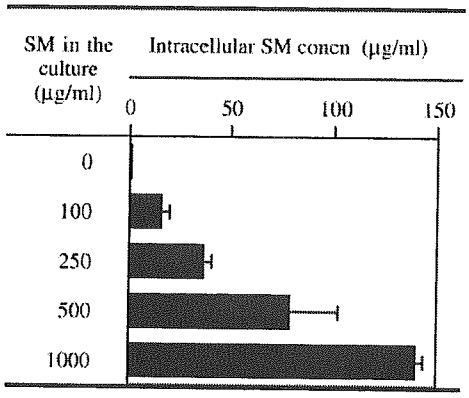

FIG. 4. Determination of intracellular concentration (concn) of SM in the PECs incubated at different concentrations of SM in media for 20 h. After three washes with PBS, the intracellular concentrations of SM were calculated. Data are shown as the means for triplicate wells ± SD. The data shown are for one of two independent experiments.

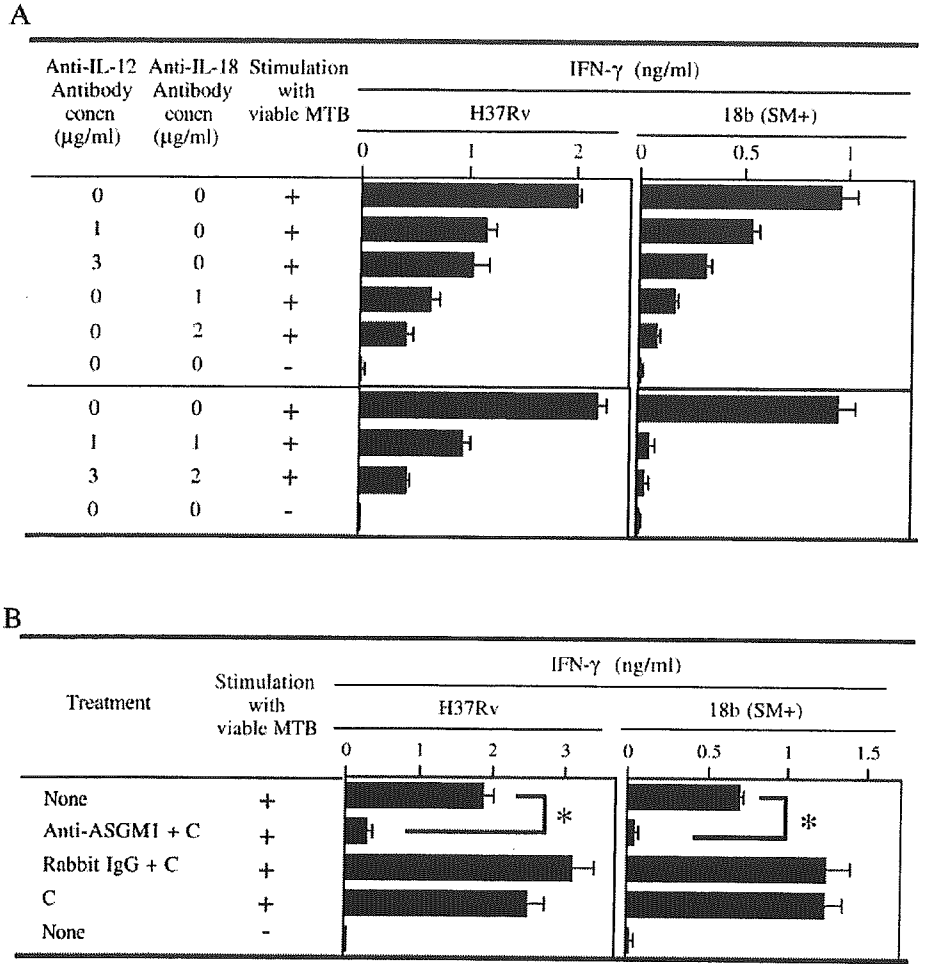

FIG. 5. Cytokines and the cells responsible for the IFN-γ production in the culture of whole PECs stimulated with *M. tuberculosis* (MTB). (A) Neutralizing antibodies to IL-12 or IL-18 were added to the culture and the change in the level of IFN-γ production was examined. (B) PECs were treated either with complement (C) alone, with 100 μg/ml of control rabbit immunoglobulin G (IgG), or with 100 μg/ml of anti-asialo GM1 (ASGM1) antibody followed by complement. Then, the cells were washed and stimulated with H37Rv or with 18b in the presence of SM. Data are shown as the means for four wells ± SD. The data shown are from one of two independent experiments. * $P < 0.01$ according to Student's *t* test. concn, concentration.

μg/ml of SM compared to that in the absence of SM (data not shown), suggesting that the active metabolism was induced by SM during this period of culturing.

Based on these findings, it was likely that there are some mycobacterial factors existing in viable bacteria, but not in killed bacteria, which contribute to the induction of IFN-γ production. Changes of the transcriptional or translational levels in various *M. tuberculosis* genes inside macrophages have been reported previously (17, 22). This may be an important strategy of *M. tuberculosis* for intracellular survival, and it is probable that some components synthesized inside the phagosome may play a role in triggering IFN-γ production. The present finding appeared to be consistent with an old observation that acquired immunity was not induced in mice by immunization with strain 18b cultivated in an SM-starved condition (13).

In order to know whether the IFN-γ production observed in this study was induced in similar manners in the stimulations by both H37Rv and 18b plus SM, the cytokines and effector cells involved in the final IFN-γ production were examined with special reference to interleukin-12 (IL-12) and IL-18, which are known to induce IFN-γ production by natural killer (NK) cells in PECs (27). The addition of neutralizing antibody to IL-12 or IL-18 resulted in appreciable levels of inhibition of IFN-γ production after stimulation with H37Rv or 18b plus SM, and a marked inhibition was observed when IL-12 and IL-18 were neutralized simultaneously (Fig. 5A). Next, the whole PECs were depleted of NK cells by using anti-asialo GM1 antibody (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and complement. The reason for the increase in the IFN-γ production after treatment with the complement alone or with control immunoglobulin is not clear, but the treatment

of PECs with anti-asialo GM1 antibody followed by treatment with the complement almost completely abolished the IFN-γ response after stimulation with H37Rv and 18b plus SM (Fig. 5B). These findings suggested that, first, Il-12 and Il-18 were produced from adherent macrophages infected with *M. tuberculosis* H37Rv or with 18b, and then these IFN-γ-inducing cytokines activated NK cells, just as we previously observed in the cells and cytokines involved in the IFN-γ response to *Listeria monocytogenes* (18).

A recent study has shown that IFN-γ induction does occur after infection with *M. tuberculosis* in mice knocked out for MyD88, a cytoplasmic adaptor molecule for the Toll-like receptor signaling pathway (23). Several lines of evidence indicated that there are intracellular pattern recognition receptors, including nucleotide-binding oligomerization domain proteins, that respond to bacterial products (9, 21). It is probable that viable cells of *M. tuberculosis* may stimulate a type of such intracellular pathways, resulting in the induction of Th1 cytokine production.

The present study employing an SM-dependent *M. tuberculosis* strain, 18b, presented a piece of the mechanism accounting for the highly potent IFN-γ-inducing activity that was virtually unobserved in killed *M. tuberculosis* but was observed in the viable form of this organism. The use of strain 18b may provide more insights into the essential role of metabolic products for the host response in addition to clarifying the precise molecular mechanism of SM dependency of this unique mutant strain in the future.

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4. 抗酸菌の病原性に関する分子遺伝学的研究

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はじめに

結核菌の増殖に関する因子およびその遺伝子に関しては既に多くの報告がある。また菌の休眠状態に関する増殖制御系の遺伝子群の報告も近年相次いでいる。

われわれは放線菌 *Streptomyces kasugaensis* の *orf3*, *orf5* 遺伝子が増殖の制御に関する遺伝子で，結核菌の *mIHF*, Rv1390とアミノ酸レベルで高いホモロジーを有するという情報を得た (from K. Akagawa. personal communication)。そこで結核菌の integration host factor (*mIHF*) とその近傍遺伝子を *Mycobacterium smegmatis* J15CS株に導入し，これらの遺伝子がJ774細胞内での *M. smegmatis* の増殖を制御するという現象を見出したので報告する。

材料と方法

（1）形質転換体作成

大腸菌・抗酸菌シャトルベクターとして，pYT 923[1] に hygromycin耐性を組み込んだ改良型の pYT 923hyg α を，宿主には *M. smegmatis* J15CS株を用いた。ヒト型結核菌 *M. tuberculosis* H37Rv の *mIHF*, *mIHF-gmk*-Rv1390 遺伝子を含む断片をベクター pYT923hyg α に挿入し，これら組換え体を *M. smegmatis* J15CS株に形質転換して，J15CS/pYT 923hyg α，J15CS/*mIHF*，J15CS/*mIHF-gmk*-Rv1390を得た。

（2）*In vitro* 実験

普通寒天培地での培養と Ziehl-Neelsen染色を行った。Tween 80加 L-broth，37℃で培養し，増殖曲線を作成した。普通寒天培地上，7日培養のコロニーについては，走査型，透過型電子顕微鏡観察を行った。

（3）*Ex vivo* 実験

マウスマクロファージ系 J774細胞を Lab-Tek chamber slide あるいは24 well plate に monolayer に調整した。普通寒天培地で7日間培養した後，5 μmのフィルターでろ過し，$10^{6\sim7}$ CFU/well感染させた。3時間食菌後，2回洗浄し，amikacin 200 μg/ml加 RPMI 1640 medium でさらに2時間培養し，細胞外の菌を殺菌した。medium交換後，5%CO_2 incubator で37℃3日間培養した。Ziehl-Neelsen染色と細胞内生菌数を計測した。また *M. smegmatis* J15CS/pYT 923hyg α，J15CS/*mIHF-gmk*-Rv1390 の感染2日目の細胞について透過型電子顕微鏡で観察した。

結　果

M. smegmatis J15CS，J15CS/pYT 923hyg α，J15CS/*mIHF*, J15CS/*mIHF-gmk*-Rv1390の普通寒天培地上でのコロニーの大きさ，Ziehl-Neelsen染色による菌の大きさ，抗酸性，液体培地での増殖曲線に差はなかった (Fig. 1)。走査型電子顕微鏡像に違いは認められなかったが，透過型電子顕微鏡で菌体内微細構造を観察した結果，J15CS/*mIHF-gmk*-Rv1390の細胞壁の内側が肥厚していた。

J774細胞感染直後の取り込みに差はなかった。しかし1日培養後には J15CS/*mIHF-gmk*-Rv1390以外の形質転

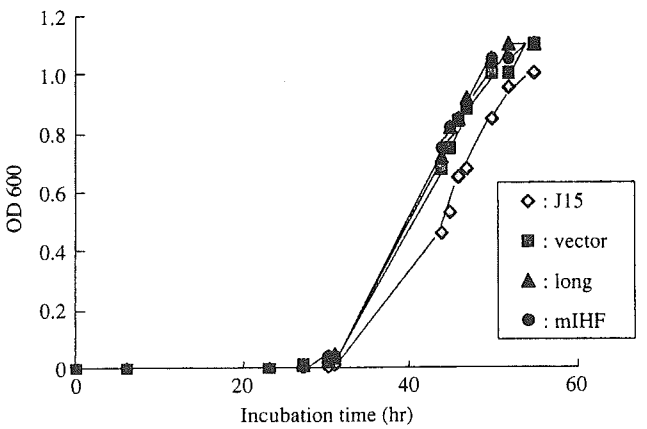

Fig. 1 Proliferation curve (Tween 80 containing L-broth)
J15: *M.smegmatis* J15CS, vector: J15CS/pYT923hyg α,
long: J15CS/*mIHF-gmk*-Rv1390, mIHF: J15CS/*mIHF*

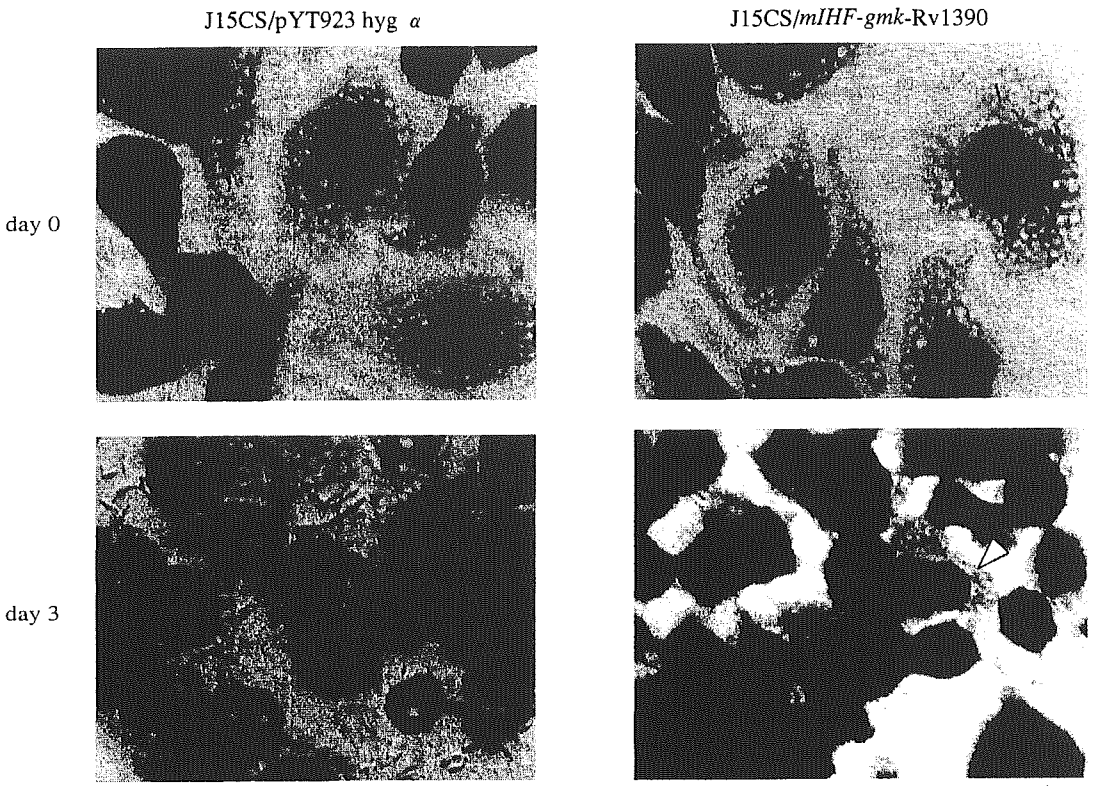

Fig. 2 Proliferation of bacteria in J774 cells (Ziehl-Neelsen staining)

換体は菌の伸長が認められ，細胞内で生残し，明瞭な抗酸性を示していたが，J15CS/*mIHF-gmk*-Rv1390は菌の伸長が認められず，抗酸性も低下していた。3日目にはこの差は顕著であった。また宿主のJ774細胞も変化していた (Fig. 2)。J15CS/pYT 923hyg α，J15CS/*mIHF-gmk*-Rv1390を透過型電子顕微鏡で観察した結果，菌がJ774細胞の食胞内に取り込まれている像が認められた。J15CS/pYT 923hyg αは小さな食胞内に正常な菌が観察された。しかしJ15CS/*mIHF-gmk*-Rv1390は大きな食胞内に変化した菌が認められた。菌体内に膜様構造の形成が認められ，電子密度の低い内容構造が見られた。しかし，これらの細胞内生菌数を計測した結果，差は認められず，J15CS/*mIHF-gmk*-Rv1390はJ774細胞内で生残していることが確認された。

考　察

M. smegmatis J15CSはJ774細胞のみでなく，ヒト肺胞上皮細胞であるA549細胞でも取り込まれ，生残した。しかし，小川培地で培養した菌は容易に殺菌され，生残するためには感染させる時の菌の状態が重要であった。遺伝学的背景が同じ結核菌であっても，感染性が異なることが知られており，ヒト側の感受性遺伝子の解析等が行われているが，菌側の要因として，この現象の解明は貴重な情報を提供すると考えられる。

結核菌の*mIHF-gmk*-Rv1390の3つの遺伝子を破壊することなく*M. smegmatis* J15CSに挿入したものは，*in vitro*では差がなかったが，電子顕微鏡では細胞壁の内側が肥厚している像が観察された。この部位は結核菌の細胞壁のペプチドグリカン層の位置であった。この物質については現在解析中である。

J774細胞，A549細胞内増殖に関しては，3日目には*mIHF-gmk*-Rv1390を有する菌は空洞内で休眠状態になった結核菌の像と酷似していた。透過型顕微鏡による微細構造では，J15CS/pYT 923hyg αは狭い食胞内に正常な菌体が認められ，細胞内寄生性細菌であるレジオネラが細胞内で分裂・増殖している時の像に似ていた。しかしJ15CS/*mIHF-gmk*-Rv1390の場合は，食胞が広く，その中にいる菌は菌体内構造が変化し，膜様構造が形成されているものがあった。

S. kasugaensis のORF5はRNA polymeraseのω subunitであると報告されている[2]。ω subunitはRNA polymeraseの立体構造維持に重要な役割をしており，σ factorとの相互作用にも影響を与えるものである。ストレス下ではω subunitに変化が生じ，σ factorも定常状態とは異なるものが選ばれ，合成されるmRNAも異なってくると思われる。結核菌のRv1390タンパクは*S. kasugaensis*のORF5と高い相同性を示し，結核菌のω subunitではないかと考えられる。一方，mIHFタンパクはstationary phase

で合成が始まり，菌の生存に必須の遺伝子であることが *M. smegmatis* を用いた研究で明らかになっている[3]。*S. kasugaensis* の ORF 3 タンパクは結核菌の mIHF タンパクと高い相同性を示し，増殖様式の変換に，ORF5 とともに重要であることが報告されている。結核菌の Rv1390 単独か，または *mIHF-gmk*-Rv1390 の 3 つの遺伝子が関連して *M. smegmatis* の増殖を休眠状態に誘導していることが示唆され，さらに解析が必要である。

結核菌は増殖が遅く，凝集性が強く，バイオハザードの面からも実験上の制約が大きい。さらにカルタヘナ議定書の批准に伴い，組換え DNA 実験は 2004 年より法規制となった。結核菌を用いた組換え DNA 実験は大臣承認となり，さらに実験上の厳しい規制が生じた。ここで紹介した *M. smegmatis* J15CS と pYT 923hyg α の宿主ベクター系を用いた結核菌の *mIHF-gmk*-Rv1390 遺伝子の研究は，結核菌の細胞内増殖性，休眠状態への移行を分子遺伝学的に研究するうえできわめて有用な系であると考える。

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DNA Augments Antigenicity of Mycobacterial DNA-Binding Protein 1 and Confers Protection against *Mycobacterium tuberculosis* Infection in Mice[1]

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Mycobacterium **consists up to 7% of mycobacterial DNA-binding protein 1 (MDP1) in total cellular proteins. Host immune responses to MDP1 were studied in mice to explore the antigenic properties of this protein. Anti-MDP1 IgG was produced after infection with either bacillus Calmette-Guérin or *Mycobacterium tuberculosis* in C3H/HeJ mice. However, the level of Ab was remarkably low when purified MDP1 was injected. MDP1 is considered to be associated with DNA in nucleoid, which contains immunostimulatory CpG motif. Therefore, we examined coadministration of MDP1 and DNA derived from *M. tuberculosis*. Consequently, this procedure significantly enhanced the production of MDP1-specific IgG. Five nanograms of DNA was enough to enhance MDP1-specific IgG production in the administration of 5 μg of MDP1 into mice. Strong immune stimulation by such a small amount of DNA is noteworthy, because >1,000- to 100,000-fold doses of CpG DNAs are used for immune activation. A synthetic peptide-based study showed that B cell epitopes were different between mice administered MDP1 alone and those given a mixture of MDP1 and DNA, suggesting that DNA alters the three-dimensional structure of MDP1. Coadministration of DNA also enhanced MDP1-specific IFN-γ production and reduced the bacterial burden of a following challenge of *M. tuberculosis*, showing that MDP1 is a novel vaccine target. Finally, we found that MDP1 remarkably enhanced TLR9-dependent immune stimulation by unmethylated CpG oligo DNA in vitro. To our knowledge, MDP1 is the first protein discovered that remarkably augments the CpG-mediated immune response and is a potential adjuvant for CpG DNA-based immune therapies.** ***The Journal of Immunology,*** **2005, 175: 441–449.**

Tuberculosis is a disease caused by infection with *Mycobacterium tuberculosis* and remains a serious threat to health in the world. Annually, 8 million people contract tuberculosis, and nearly 2 million people die from the disease. Worldwide, 32% of the population is persistently infected with *M. tuberculosis*, and some of these bacteria are thought to be in a nonreplicating dormant state (1). The majority of the disease arises from reactivation of persisting, previously implanted bacteria (2–5).

Bacillus Calmette-Guérin (BCG)[3] is an attenuated live vaccine against tuberculosis and has been given to >2 billion individuals to date. BCG is safe, inexpensive, and effective against both meningitis and miliary tuberculosis in infants, but frequently fails to protect from the most prevalent form of the disease, adult pulmonary tuberculosis (6–9). In addition, there is the possibility of causing opportunistic disease in immunocompromised hosts, such as AIDS patients, because BCG is a live vaccine and can survive in the hosts. Accordingly, there is an urgent need to develop a more effective and safer vaccine than BCG. Extensive studies to date have evaluated possible vaccine candidate proteins, such as a 6-kDa early secretory antigenic target (10); Ag 85 complexes A, B, and C (11); MTB39 and MTB48 (12); and heat shock protein 60 (13).

Mycobacterial DNA-binding protein 1 (MDP1) is produced by the genus *Mycobacterium* and is a major cellular protein, consisting of up to 7% of the total cellular protein (14). MDP1 has nucleic acid-binding activity mediated through interaction with guanine and cytosine residues in DNA (14, 15). Thus, MDP1 is presumed to be a component of the mycobacterial nucleoid and has been shown to localize to the 50S ribosomal subunit and on the bacterial surface (14, 16). The cellular content of MDP1 is increased in the stationary growth phase of mycobacteria relative to the exponential growth phase (14). Dick et al. (17) found that histone-like protein (HLP), the homologue of MDP1, was substantially upregulated in the dormant state of *Mycobacterium smegmatis*. Our previous study showed that MDP1 inhibited macromolecular biosyntheses in vitro and substantially suppressed bacterial growth (18). Taken together, it is conceivable that MDP1 has fundamental

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[3] Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; Ag85B, Ag 85 complex B; HLP, histone-like protein; HLP*Mt*, histone-like protein of *Mycobacterium tuberculosis*; HrpA, heat stress-induced ribosome-binding protein A; KO, knockout; LBP-21, laminin-binding protein of 21 kDa; Me-oligo B, synthetic oligo DNA containing methylated CpG sequence; MDP1, mycobacterial DNA-binding protein 1; ODN, synthetic oligodeoxynucleotide; PPD, purified protein derivative; RIB, RIBI adjuvant system; rMDP1, recombinant histidine-tagged MDP1.

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roles in the suppression of growth from both stationary and dormant phases of mycobacteria.

Of interest, MDP1 localizes on the bacterial surface as well as intracellularly (14, 16, 19, 20). During host-bacterium interaction, MDP1 may play a role as an adhesin. Shimoji et al. (20) found that a 21-kDa protein could bind to laminin-2, which is thought to be an *Mycobacterium leprae* receptor involved in attachment to Schwann cells (21). They designated this protein as laminin-binding protein of 21 kDa (LBP-21) and showed it to be a homologue of MDP1 in *M. leprae*, although it was deficient for DNA-binding activity (20). Thus, LBP-21 may have a role in the invasion of *M. leprae* into peripheral nerves, presumably cooperating with another adhesion molecule, phenolic glycolipid-1 (22). In addition to laminin, we recently found that MDP1 binds to glycosaminoglycans (16), which are a major component of the extracellular matrix. Glycosaminoglycans are important in the attachment of mycobacteria, especially in the interaction with nonphagocytic cells such as fibroblasts and epithelial cells (23), which are possible reservoirs of persisting *M. tuberculosis* in healthy humans (24).

Prasad et al. (25) used T cell blot assay to identify an immunodominant protein in healthy contacts with tuberculosis patients. They designated that protein as histone-like protein of *M. tuberculosis* (HLP*Mt*), which is the same molecule as MDP1. Both humoral and lymphoproliferative responses against recombinant HLP*Mt*/MDP1 were greater in healthy tuberculin reactors than in nonreactors or tuberculosis patients (25). This suggests that HLP*Mt*/MDP1 is an immunodominant Ag that may have an important role in host defense.

In this study we report a series of studies that analyze the antigenicity of MDP1 in a mouse model. We show that both humoral and cellular immune responses to MDP1 are stimulated by the presence of bacterial DNA that contains immunostimulatory CpG motifs (26, 27) that initiate immune responses through TLR9 (28). Simultaneous immunization with MDP1 and DNA, but not MDP1 alone, promotes protection against an *M. tuberculosis* challenge. An in vitro study demonstrated that a complex of MDP1 and CpG DNA markedly stimulates the production of proinflammatory cytokines in a TLR9-dependent manner. Proteins produced by pathogenic organisms are major targets of host immune responses that lead to protective immunity. Our data demonstrate that immunostimulatory cellular components that interact with these proteins have significant effects on protein recognition by the host and the subsequent development of protective immunity.

Materials and Methods

Mice

Female A/J, BALB/c, C3H/HeJ, and C57BL/6 mice were purchased from Japan SLC at 5–7 wk of age. TLR9 knockout (KO) mice (B6 129F2 background) were supplied by Dr. S. Akira (Osaka University, Osaka, Japan) (28). All mice were kept under specific pathogen-free conditions.

Bacterial strains and culture

BCG (strain Tokyo) was grown at 37°C in Middlebrook 7H9 media (Difco) supplemented with 10% albumin, dextrose, and catalase enrichment (Difco) and 0.05% Tween 80. When the OD at 630 nm was ~0.5, bacteria were collected by centrifugation and suspended in sterilized water to adjust for an OD of 1.0. Mice were infected i.p. with 5–10 $\times$ 10^6 CFU of BCG in 0.2 ml of normal saline. Two weeks later, mice were boosted with the same dose of BCG i.p. The bacterial dose was determined by counting CFUs 3 wk after plating serial 10-fold dilutions of suspension onto Middlebrook 7H11 agar containing oleic acid, dextrose, albumin, and catalase enrichment (Difco; 7H11-OADC agar).

Antigens

Recombinant histidine-tagged MDP1 (rMDP1) was purified from *Escherichia coli* transfected with pET21b$^+$-*mdp1* by methods described previously (16). Native MDP1 was purified from BCG (Tokyo strain) using the method described previously (14). Ag 85 complex B (Ag85B) purified from *M. tuberculosis* H37Rv was a gift from Dr. S. Nagai (29). Heat stress-induced ribosome-binding protein A (HrpA), purified as a recombinant protein (30), was supplied by Drs. N. Ohara and T. Tabira (Nagasaki University, Nagasaki, Japan). Bovine histone H1, histone H2A, and histone H3 were purchased from Roche. Bacterial DNA was purified from *M. tuberculosis* H37Rv by phenol-chloroform extraction (31). Briefly, 5 g of *M. tuberculosis* H37Rv (wet weight) was suspended in 5 ml of 10 mM Tris-HCl and 1 mM EDTA (pH 7.5; TE buffer), mixed with the same volume of chloroform/methanol (2/1), and incubated for 5 min to remove lipids. The suspension was centrifuged at 2,500 $\times$ *g* for 20 min, and both organic and aqueous layers were decanted to leave a packed bacterial band. Delipidated bacteria were incubated at 55°C for 20 min to remove traces of organic solvents and were resuspended in 5 ml of TE buffer and 0.5 ml of 1 M Tris-HCl (pH 9.6). Lysozyme (Sigma-Aldrich) was added to a final concentration of 100 μg/ml and incubated for 2 h. Then 0.1 vol of 10% SDS and 0.01 vol of proteinase K (Sigma-Aldrich) were added and additionally incubated overnight. To remove contaminating proteins, the same volume of phenol was added, gently mixed for 20 min, and centrifuged at 12,000 $\times$ *g* for 20 min. The aqueous layer was transferred to the fresh tube, and the protein-removing step was repeated again. Then the same volume of chloroform/isoamyl alcohol (24/1) was added and gently mixed for 10 min. The tube was centrifuged at 12,000 $\times$ *g* for 10 min, then the supernatant was transferred to new tube. DNA was precipitated by gently mixing after adding 0.1 vol of 3 M sodium acetate (pH 5.2) and 2.5 vol of ethanol. The tube was then centrifuged at 12,000 $\times$ *g* for 10 min, and the DNA pellet was rinsed with 70% ethanol. The pellet was resolved in pure water, and the concentration was determined by the absorbance at 260 nm. The endotoxin level of Ags was <50 pg/100 μM, as determined by a *Limulus* test.

Immunization of mice with protein Ags and BCG

Protein Ags were emulsified using the RIBI adjuvant system (RIB; Corixia), which consists of synthetic trehalose dicorynomycolate and monophosphoryl lipid A, or by IFA (Difco). In some cases, Ags were mixed with various amounts of DNA for 10 min at 37°C and then emulsified. Five micrograms of protein with or without DNA was injected i.p. Three weeks later, mice were boosted using the same method as the primary immunization. The same protocol was used for BCG immunization. Five to 10 $\times$ 10^6 CFU of BCG was i.p. injected per mouse. Three weeks after the boost, peripheral blood was obtained from the retro-orbital plexus of anesthetized mice, and sera were isolated and stored at −80°C until the assays.

Western blot

One microgram of purified MDP1 was fractionated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and reacted with antisera diluted 1/200.

ELISA

Ninety-six-well ELISA plates (Sumitomo) were coated with individual protein Ags, such as MDP1, HrpA, Ag85B, histone H1, histone H2A, and histone H3, by overnight incubation in carbonate buffer (pH 9.6) at 4°C. Wells were then blocked by PBS containing 3% BSA for 2 h at room temperature. Equal volumes of sera from at least five mice were mixed in each experimental group. Sera were serially diluted in PBS containing 1% BSA, added to wells, and incubated overnight at 4°C. The wells were washed four times with PBS containing 0.05% Tween 20, and HRP-conjugated goat anti-mouse IgG (DakoCytomation), IgG1, IgG2a, IgG2b, IgG3 (Santa Cruz Biotechnology), or IgG2c (Bethyl) diluted in PBS containing 1% BSA was added and incubated for 2 h at room temperature. After washing as before, 100 μl of 80 mM citrate-phosphate buffer (pH 5.0) containing 0.4 μg/ml *o*-phenylendiamine dihydrochloride (Wako Pure Chemicals) was added to the wells, and absorbance at 492 nm was measured by an MTP-300 microplate reader (Corona Electronic).

To determine B cell epitopes, overlapping peptides covering the entire sequence of MDP1 were synthesized previously as 20-mer molecules with 10-aa overlaps with the neighboring peptides, with exception of the C-terminal (15). Each peptide was dissolved in PBS at a concentration of 10 μg/ml and immobilized onto type A ELISA plates (Sumitomo) after activation of the wells by 2% glutaraldehyde. Sera diluted 1/200 by PBS containing 0.05% Tween 20 was added and incubated at 4°C overnight. The ELISA procedure described above was performed, and B cell epitopes were defined by color development with *o*-phenylendiamine dihydrochloride.

Lymph node cell culture and stimulation

Mice were killed 3 wk after the booster injection of Ags, and mesenteric lymph node cells were prepared. Cells were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich), 25 mM HEPES, 2 mM L-glutamine, 5.5×10^{-5} M 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete RPMI medium) in the presence or the absence of 10 μg/ml, MDP1, Ag85B, or purified protein derivative (PPD; Kyowa) in a humidified incubator at 37°C under 5% CO_2. IFN-γ in the culture supernatant was measured with ELISA kits (Genzyme Techne).

Prior immunization and challenge with M. tuberculosis

C3H/HeJ or BALB/c mice were s.c. immunized with 5 μg of RIB-emulsified MDP1 with or without 5 ng of *M. tuberculosis* DNA, DNA alone (5 ng), or 5 μg of Ag85B. BCG Tokyo at a dose of 10^6 CFUs was inoculated using the same procedure without emulsification. After 3 wk, mice were boosted i.p. by the same Ags and were challenged 3 wk later i.v. with 1×10^6 CFU of *M. tuberculosis* Kurono strain (ATCC 35812; American Type Culture Collection). On days 14 and 28, lungs were removed and homogenized using an LS-50 homogenizer (Yamato). The lung homogenates were serially diluted and inoculated onto 7H11-OADC agars. Bacterial numbers were calculated and expressed as CFU.

Spleen cell culture and stimulation

Synthetic oligodeoxynucleotides (ODNs) of sequence GGGGGGAACGT TGGGGGGGGGGGGGGGG were purchased from Nisshinbo and designated oligo B. As a control, cytosine-methylated oligo B was synthesized (Me-oligo B). The endotoxin level was <50 pg/100 μM, as determined by a *Limulus* test. Spleens obtained from C57BL/6 and TLR9 KO mice were cut into small pieces and homogenized. These cell suspensions were depleted of erythrocytes using a Ficoll gradient (Lympholyte-M; Cedarlane Laboratories) and centrifuged for 20 min at 1000 × *g* at room temperature. Spleen cells (1×10^6 cells/well) were cultured in the presence or the absence of MDP1 and ODNs at final concentrations of 0.5 and 1 μM, respectively. After 10-min incubation at 37°C, the MDP1-ODN mixture was added to the cell cultures and incubated for 24 h. As a control, cells were also stimulated with LPS derived from *E. coli* 0111 134 (Difco) at concentration of 100 ng/ml. The amounts of TNF-α and IL-6 in the culture supernatants were measured with ELISA kits (Genzyme Techne).

Statistical analyses

Statistical analysis was conducted with a Power Macintosh G4 using StatView 5.0 (SAS Institute). ANOVA was used to determine the significance of differences in means between multiple experimental groups. The significance level of the test was <5%.

Results

Anti-MDP1 IgG production in mice

To explore the antigenicity of MDP1, we first analyzed the humoral immune response to MDP1 in mice. BCG was inoculated into three strains of mice, including A/J, BALB/c, and C3H/He. Western blot analysis showed that MDP1 elicited a humoral immune response in all strains (Fig. 1*A*). Sera from nonimmunized mice did not react with MDP1 (data not shown). Additionally, anti-MDP1 IgG was produced in C3H/He and BALB/c mice challenged with *M. tuberculosis* H37Rv (data not shown).

We next assessed the antigenicity of purified MDP1. Five micrograms of MDP1 was emulsified in RIB and injected into C3H/He mice. In contrast to inoculation of BCG, we could not detect a significant level of anti-MDP1 IgG (Fig. 1*A*). MDP1 presumably binds to DNA, which includes immunostimulatory CpG motifs (27). Therefore, we tested the simultaneous administration of MDP1 and DNA. Five micrograms of MDP1 was incubated with 0.5 μg of DNA derived from *M. tuberculosis* H37Rv, and the mixture was injected into C3H/He mice. Western blot analysis showed that a combination of MDP1 and DNA elicited MDP1-specific IgG production, whereas MDP1 or DNA alone did not (Fig. 1*A*).

We next determined the optimal dose of DNA that could enhance anti-MDP1 IgG production. Using RIB, 5 μg of MDP1 was administered to C3H/He mice with or without 10-fold serial dilutions of DNAs ranging from 5 μg to 5 ng. Three weeks after the booster injection, the level of anti-MDP1 IgG was measured by ELISA (Fig. 1*B*). The production of IgG was dependent on the amount of DNA; interestingly, 5 ng of DNA most efficiently stimulated IgG production against MDP1. We observed enhanced anti-MDP1 IgG production by coadministration of DNA and MDP1 in the presence of IFA (Fig. 1*C*), and the result was similar to that

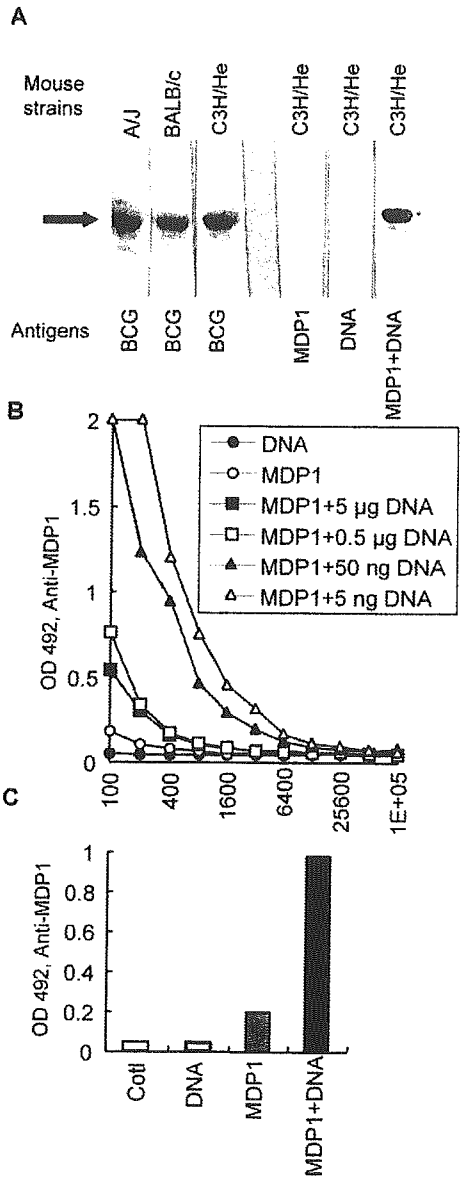

FIGURE 1. Humoral immune responses to MDP1 in mice. *A*, Western blot analysis. Purified MDP1 was blotted onto the membrane and incubated with 200/1 diluted antisera. Mouse strains are indicated along the *top* of the panel. The injected Ags, such as BCG, MDP1 alone (MDP1), *M. tuberculosis* DNA alone (DNA), and MDP1 plus DNA (MDP1+DNA) are shown along the *bottom*. *B*, DNA dose effects on anti-MDP1 IgG production. C3H/He mice were immunized with MDP1 (5 μg/mouse) with or without various amounts of DNA (5 μg to 5 ng) emulsified in RIB adjuvant, and levels of anti-MDP1 IgG were determined by ELISA. The horizontal axis shows dilution factors of antisera. *C*, Immunization with MDP1 plus DNA emulsified in IFA augmented anti-MDP1 IgG production. C3H/He mice were immunized with Ags emulsified in IFA. Immunized Ags are described *below* the horizontal axis. Cotl, IFA alone. The sera from at least five mice of each experimental group were mixed and diluted to 1/400, and the levels of anti-MDP1 IgG were determined by ELISA.

observed using RIB adjuvant, suggesting that the immunostimulatory effect of DNA on anti-MDP1 IgG production is not restricted to RIB adjuvant. The results prompted us to explore whether DNA-dependent IgG production varies between mouse strains. The same immunization procedure using RIB as an adjuvant was performed in other mouse strains, including A/J, BALB/c, and C57BL/6. The results revealed that simultaneous inoculation of MDP1 and DNA augmented the production of IgG against MDP1 in all tested strains (Fig. 2).

To determine whether DNA-mediated enhancement of anti-MDP1 IgG production is restricted to the particular IgG isotype, we analyzed the distribution of subclasses of IgG by ELISA. As shown in Fig. 3, each mouse strain possessed a specific pattern of MDP1-specific IgG isotypes, but DNA enhanced only IgG subclasses produced in mice immunized with MDP1 alone. Thus, a small dose of DNA augments the humoral response to MDP1 without altering the pattern of IgG isotypes.

MDP1-specific, DNA-dependent stimulation of IgG production

Our data showed that a small amount of DNA (5 ng) magnified anti-MDP1 IgG production (Fig. 1*B*). In contrast, 1,000- to 100,000-fold higher amounts (5–500 μg) of bacterial DNA and CpG ODNs have been applied as adjuvants in immunization with foreign Ags (32–34) or immunotherapeutic treatments (34–38). Therefore, we next examined whether 5 ng of DNA stimulated Ab production against other immunogenic mycobacterial proteins such as HrpA (39) and Ag85B (11). These Ags did not bind to DNA, as determined by gel retardation assay (data not shown). Five micrograms of each Ag was injected into BALB/c, C3H/He, and C57BL/6 mice, with or without 5 ng of DNA. We could not detect enhanced Ab production by coadministration of DNA in any of the three mouse strains (Fig. 4, *A* and *B*).

Next we examined whether DNA combined with DNA-binding proteins other than MDP1 stimulates IgG production. Bovine histone H1, histone H2A, and histone H3 were injected into three strains of mouse (BALB/c, C3H/He, and C57BL/6) with or without 5 ng of DNA. We could not detect the production of IgG against both histones H2A and H3 in any of mouse strains tested (data not shown). In contrast, anti-histone H1 Ab was detectable in all mouse strains, but DNA alone did not stimulate anti-histone H1 IgG production (Fig. 4*C*). Although we have not tested all DNA-binding proteins, these results imply that enhanced Ab production by a very small amount of bacterial DNA is a unique feature of MDP1.

DNA alters B cell epitopes of MDP1

To examine humoral immune responses against MDP1 more precisely, we defined the region(s) recognized by anti-MDP1 IgG. B

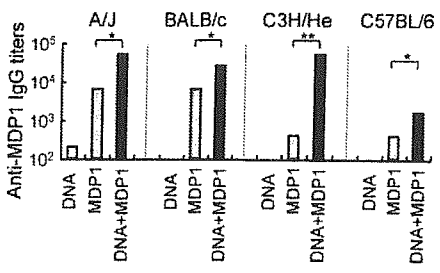

FIGURE 2. DNA stimulates the production of anti-MDP1-IgG in mice. Four strains of mice, including A/J, BALB/c, C3H/He, and C57BL/6, were immunized with DNA alone (□), MDP1 (5 μg/mouse) alone (▨), or MDP1 plus DNA (■). The titer of anti-MDP1-IgG was determined by ELISA. *, $p < 0.05$; **, $p < 0.01$ (by ANOVA).

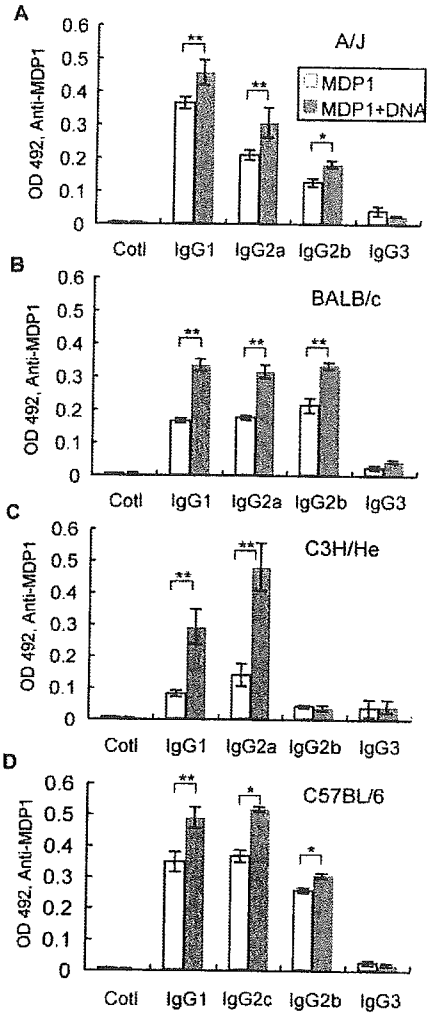

FIGURE 3. Isotypes of anti-MDP1 IgG. The levels of IgG subclasses were measured using isotype-specific Abs against IgG1, IgG2a, IgG2b, IgG2c, and IgG3. Cotl, Controls without secondary Ab. □, Immunization with MDP1 alone; ▨, coadministration of MDP1 and DNA. Antisera were diluted 1/100 (*A–C*) or 1/50 (*D*). *, $p < 0.05$; **, $p < 0.01$ (by ANOVA).

cell epitope mapping was conducted by ELISA using synthetic 20-mer peptides covering the entire MDP1 sequence. Antisera were obtained from four strains of mice, including A/J, BALB/c, C3H/He, and C57BL/6, immunized with MDP1 alone or with 5 ng of DNA and were reacted with each peptide. In A/J mice, IgG from animals immunized with MDP1 alone did not react with peptides, although it was bound to MDP1, suggesting that IgG in these mice recognized the conformational structure of MDP1 (Fig. 5*A*). In contrast, two peptides corresponding to aa 61–80 and 71–90 of MDP1 were recognized by anti-MDP1 IgG in mice immunized with MDP1 plus DNA (Fig. 5*A*). In BALB/c mice, anti-MDP1 IgG induced by injection of both MDP1 alone and MDP1 plus DNA reacted with the peptide corresponding to 51–70 of MDP1 (Fig. 5*B*). In C3H/He mice, the level of anti-MDP1 IgG was insignificant when MDP1 alone was used (Fig. 5*C*). In contrast, anti-MDP1 IgG was produced in animals immunized with MDP1 plus DNA and reacted with peptides corresponding to 141–160 and 151–170 (Fig. 5*C*). Thus, the epitope was likely to be the 151–160 region of MDP1. In C57BL/6 mice, Abs from mice immunized with MDP1 alone and MDP1 plus DNA reacted with the 61–80 and 1–20 regions, respectively (Fig. 5*D*).

Although the anti-MDP1 Ab titer was higher in BALB/c mice injected with MDP1 plus DNA than in mice immunized with

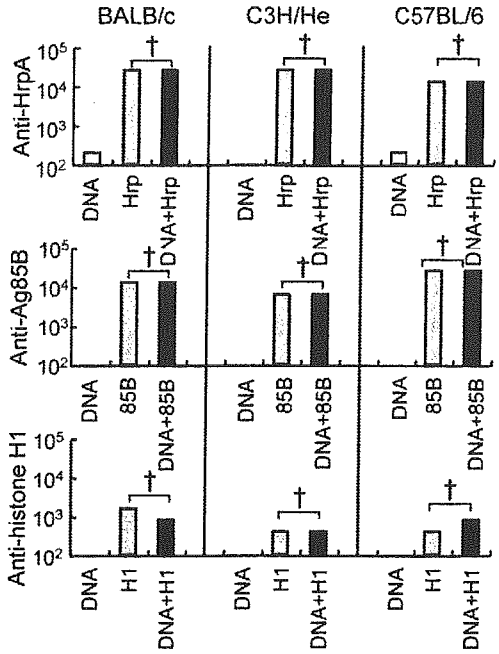

FIGURE 4. DNA fails to stimulate production of IgGs against mycobacterial Ags, HrpA and Ag85B, and a DNA-binding protein, histone H1. BALB/c, C3H/He, and C57BL/6 mice were immunized with 5 μg of HrpA (Hrp), Ag85B (85B), and histone H1 (H1) with (■) or without (▨) 5 ng of DNA. □, Immunization with DNA alone. The titer of Ag-specific IgG was measured by ELISA. †, $p > 0.1$ (by ANOVA).

MDP1 alone, the level of anti-MDP1 IgG against the defined epitope (aa 51–70) was reversed (Fig. 5*B*). This suggests that anti-MDP1 IgG recognizes mainly conformational epitopes in mice immunized with a mixture of MDP1 and DNA. To examine this possibility, inhibition assays were performed. The interaction between MDP1 and IgG from mice immunized with MDP1 alone (Fig. 6*A*), but not with MDP1-DNA (Fig. 6*B*), was inhibited by exogenously added peptide corresponding to aa 51–70 of MDP1 (Fig. 6, *A* and *B*). In contrast, the same molar amount of exogenously added MDP1 alone inhibited both reactions (Fig. 6, *A* and *B*). These data indicate that in BALB/c mice, administration of MDP1 alone produces IgG that recognizes only the 51–70 region. In contrast, administration of MDP1 plus DNA induces anti-MDP1 IgG targeting conformational epitopes on MDP1 in addition to the 51–70 region.

Similar inhibition experiments were conducted using sera from BALB/c mice injected with live BCG. The 51–70 peptide failed to abrogate the IgG-MDP1 interaction (Fig. 6*C*), although MDP1 itself did. This suggests that MDP1 is actually binding to DNA in vivo and is targeted by the host immune response.

MDP1 stimulates IFN-γ production

Protective immunity against *M. tuberculosis* infection is mediated primarily by Th1-type cell-mediated immunity (40, 41). IFN-γ triggers Th1-type cell-mediated immune responses and plays a critical role in host defense against *M. tuberculosis* infection in mice (42, 43). To investigate whether MDP1 participates in BCG-mediated protection against tuberculosis, we examined IFN-γ production induced by MDP1 stimulation. Lymph node cells from C3H/He mice immunized with BCG were cultured in the presence or the absence of MDP1, and the level of IFN-γ in culture supernatants was measured by ELISA. The results show that MDP1 stimulated IFN-γ production in a manner similar to Ag85B and PPD (Fig. 7*A*). We next examined isotypes of anti-MDP1 IgG in

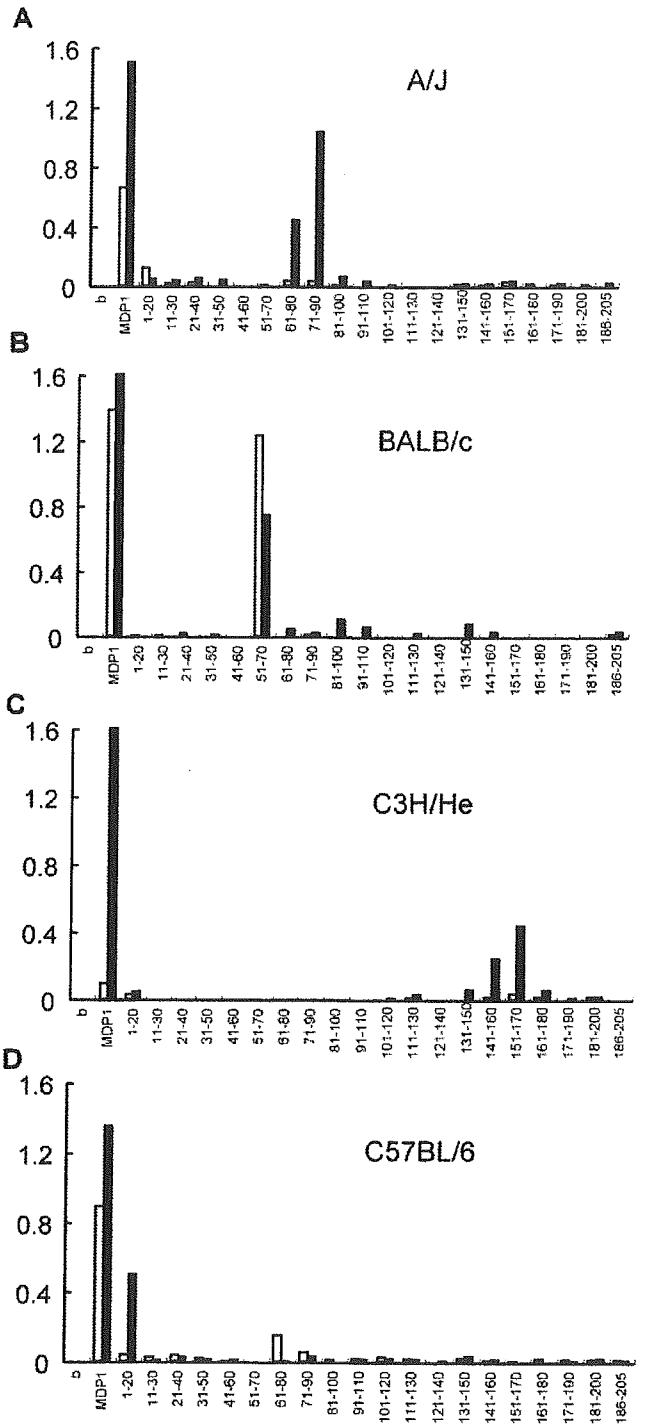

FIGURE 5. B cell epitope mapping of anti-MDP1 IgG. Epitopes of anti-MDP1 IgGs were determined by ELISA. MDP1 or 20-mer synthetic peptides covering the entire MDP1 sequence were coated on the ELISA plate as indicted at the *bottom* of each graph. b, Blank well without Ag coating. The same antisera as those described in Fig. 2 were diluted 1/200 and applied to the wells. *A–D*, Analysis of antisera derived from A/J, BALB/c, C3H/He, and C57BL/6 mice, respectively. □, Antisera from mice inoculated with MDP1 alone; ■, antisera from mice inoculated with MDP1 plus DNA. The ELISA units represent the average of duplicate samples.

BCG-immunized C3H/He mice. BCG inoculation stimulated the production of MDP1-specific IgG1 and IgG2a, but not IgG2b or IgG3 (Fig. 7*B*). Interestingly, the pattern of IgG isotypes was similar to that observed in the same mouse strain immunized with both

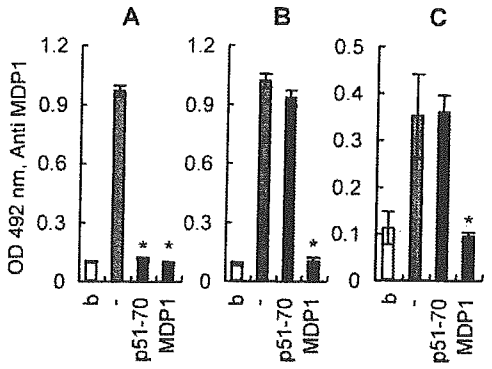

FIGURE 6. Production of anti-MDP1 IgG-targeting conformational epitopes in BALB/c mice immunized with MDP1 plus DNA or BCG, but not with MDP1 alone. Antisera from BALB/c mice immunized with MDP1 alone (*A*), MDP1 plus DNA (*B*), and BCG (*C*) were reacted with immobilized MDP1 on ELISA plates with or without exogenously added peptide corresponding to the 51–70 region of MDP1 (p51–70) or MDP1 (MDP1). b, Blank without Ag coating; −, positive controls without inhibitors. *, $p < 0.05$ (by ANOVA, vs controls without inhibitors (−)).

MDP1 and DNA (Fig. 3*C*). IFN-γ induces IgG2a production (44), whereas both Th1-related and Th2 cytokines stimulate IgG1 production (45, 46). The predominant production of IgG2a implies that the immune response to MDP1 is polarized toward the Th1 type. It is likely that MDP1 is one of the Ags that induce protective immunity after BCG immunization in C3H/He mice.

Next we examined whether the administration of purified MDP1 induces IFN-γ production. C3H/He mice were immunized with MDP1 alone or with MDP1 plus DNA. As controls, RIB and DNA alone were administered to mice as well. Lymph node cells were cultured with or without 10 μg/ml MDP1, and the production of IFN-γ was assessed. The results showed that MDP1 stimulates IFN-γ production (Fig. 7*C*). However, immunization with MDP1 mixed with DNA produced much more IFN-γ than that with MDP1 alone, demonstrating that DNA augments cell-mediated immune responses to MDP1 (Fig. 7*C*).

Simultaneous administration of MDP1 and DNA confers protection against M. tuberculosis *infection in mice*

The ability to produce IFN-γ by MDP1 prompted us to explore whether MDP1 could induce protection in vivo against challenge with a virulent strain of *M. tuberculosis*. C3H/He mice were immunized with MDP1 alone or with MDP1 plus 5 ng of DNA. As controls, mice were given RIB alone, DNA alone, BCG, or Ag85B, which is a major vaccine candidate (11). After a 3-wk interval, mice were boosted with the same Ag; 3 wk later, mice were challenged with *M. tuberculosis* Kurono. After 14 and 28 days, mice were killed, and the numbers of bacteria in the lungs and spleens were determined. These data showed that immunization with Ag85B, DNA, and MDP1 failed to protect (Fig. 8, *A–C*). In contrast, BCG and coadministration of MDP1 and DNA significantly reduced the bacterial load in the lungs ($p < 0.005$ and $p = 0.0119$ on day 14, and $p = 0.008$ and $p = 0.0316$ on day 28, respectively). A protective effect of immunization of MDP1 plus DNA, but not MDP1 alone, was also observed in the spleens ($p = 0.021$; Fig. 8*C*). As shown in Fig. 8*D*, immunization with both MDP1 and DNA resulted in a modest, but significant, decrease in bacterial burden in BALB/c mice as well ($p < 0.005$). Although the effect was less than that of BCG, MDP1 confers substantial protection against *M. tuberculosis* challenge only when it is administered with DNA.

MDP1 augments TLR9-dependent immunostimulation by CpG DNA

Immunostimulatory effects of DNA are dependent on unmethylated CpG motifs (27) that signal via TLR9 (28). Our data revealed that a very small amount of DNA stimulates immune responses against MDP1, in contrast with previous reports (32–34). Therefore, we hypothesized that MDP1 might enhance the immunostimulatory activity of CpG DNA. To test this hypothesis, we evaluated the effect of MDP1 on CpG-ODN-mediated immune activation in vitro. Spleen cells from both C57BL/6 and TLR9 KO C57BL/6 mice were stimulated with oligo B containing CpG DNA sequence in the presence or the absence of rMDP1. Me-oligo B, which has the same structure, except that its cytosine is methylated, and LPS, which signals via TLR4 (47, 48), were used as controls. After 24 h, levels of the proinflammatory cytokines TNF-α and IL-6 in the culture supernatants were determined by ELISA. Oligo B alone (1 μM) did not induce the production of TNF-α (Fig. 9). In contrast, the mixture of rMDP1 and oligo B dramatically stimulated TNF-α production (Fig. 9). This effect was undetectable in splenocytes from TLR9 KO mice or with the combination of Me-oligo B and rMDP1. Similar results were seen for

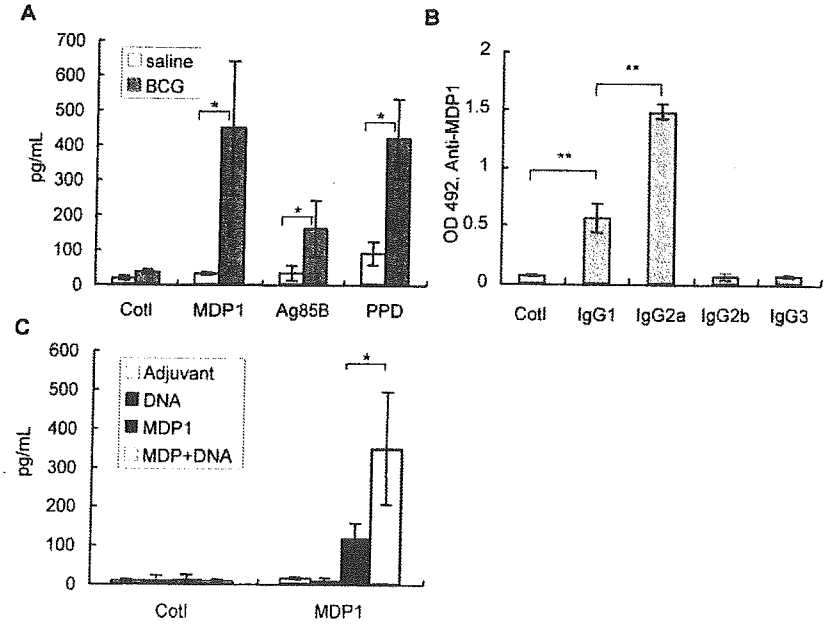

FIGURE 7. Development of Th1-type immune responses against MDP1 after challenge with BCG (*A* and *B*) or MDP1 (*C*). *A*, Amounts of IFN-γ in culture supernatants from lymph node cells were determined by ELISA. Lymph nodes were derived from C3H/He mice immunized with saline (□) or BCG (▨) and incubated for 5 days with 10 μg/ml MDP1, Ag85B, and PPD as indicated. Cotl, without Ag stimulation. The production of IFN-γ was measured by ELISA. *B*, MDP1-specific IgG isotypes (IgG1, IgG2a, IgG2b, and IgG3) in sera of C3H/He mice immunized with BCG detected by ELISA. Cotl, controls without secondary Ab. *C*, Lymph node cells derived from C3H/He mice immunized with adjuvant alone (□), DNA (■), MDP1 (▧), and MDP1 plus DNA (▣) were cultured in the presence (MDP1) or the absence (Cotl) of 10 μg/ml MDP1 for 5 days, and the amounts of IFN-γ in the culture supernatants were determined by ELISA. *, $p < 0.05$; **, $p < 0.01$ (by ANOVA).

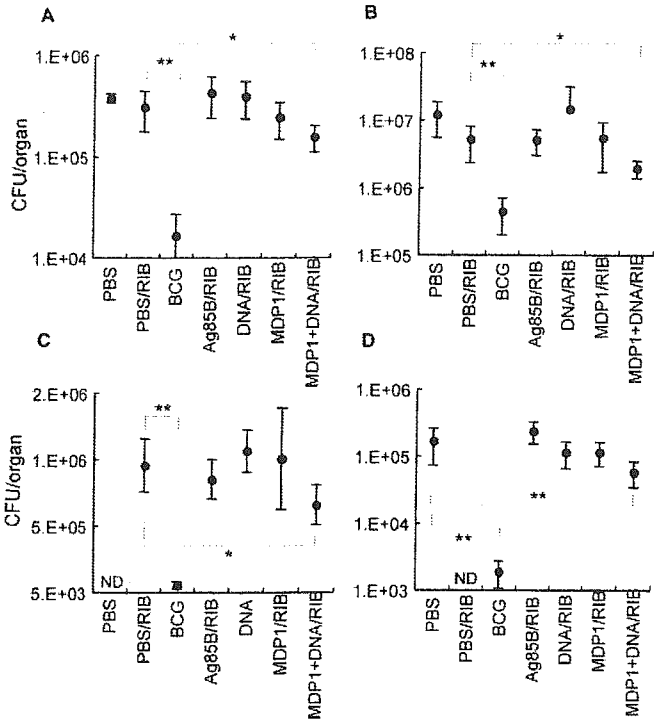

FIGURE 8. Coadministration of MDP1 and DNA confers protection against *M. tuberculosis* challenge. C3H/HeJ (*A–C*) and BALB/c (*D*) mice were immunized with Ags, as indicated below the horizontal axis, and challenged i.v. with 10^6 CFU of *M. tuberculosis* Kurono strain. Fourteen (*A*) and 28 (*B–D*) days after the challenge, bacterial numbers in lung (*A*, *B*, and *D*) and spleen (*C*) were determined by counting CFUs. *, $p < 0.05$; **, $p < 0.005$ (by ANOVA).

IL-6 production, although 0.5 μM rMDP1 itself induced a low level of IL-6 production (Fig. 9). Similar data were obtained when natural MDP1 was used under the same conditions (data not shown). These data clearly demonstrate that MDP1 activates TLR9-dependent immunostimulation by CpG ODN.

Discussion

In the present study we have evaluated the antigenicity of MDP1, a DNA-binding protein specific to mycobacteria. Anti-MDP1 IgG was produced in C3H/He mice challenged with either BCG (Fig. 1*A*) or *M. tuberculosis* (data not shown). Marked cell proliferation occurred when splenocytes from *M. tuberculosis*-infected mice were stimulated with 10 μg/ml MDP1 in vitro. Uptake of [^{3}H]thymidine was higher compared with stimulation with the gold standard, PPD (our unpublished observations). Thus, in agreement with a human study (25), MDP1 is also highly immunogenic in mice.

However, administration of purified MDP1 failed to produce anti-MDP1-IgG in C3H/He mice (Fig. 1). This lack of antigenicity was reversed by adding mycobacterial DNA when immunizing with MDP1 (Fig. 1). Similarly, DNA enhanced the production of MDP1-specific IgG in other mouse strains (Fig. 2). B cell epitope mapping (Fig. 5*B*) and Ab reaction-inhibition assay (Fig. 6) implied association of MDP1 with genomic DNA in live BCG. These results suggest that the strong immunogenicity of MDP1 in mycobacterial infection is responsible for colocalization of DNA.

Studies to determine the optimal dose of DNA showed that 5 ng of DNA was enough to activate MDP1-specific IgG production (Fig. 1*B*). This dose is unusually low compared with other studies in which 5–500 μg of DNA or ODN/mouse was used for immune activation (32–34, 36–38). We confirmed that 5 ng of DNA did not enhance the production of IgG against other proteins, including DNA-binding proteins (Fig. 4). Thus, a very small amount of DNA-stimulated Ig production appears to be a specific feature of MDP1.

We determined B cell epitopes on MDP1 by using synthetic peptides. B cell epitopes differed among mouse strains. Surprisingly, the epitopes were different when DNA was coadministered with MDP1, even within the same mouse strain (Fig. 5). Thus, DNA not only stimulates MDP1-specific IgG production, but also modifies the recognition site of IgG. This suggests that the three-dimensional structure of MDP1 differs depending on whether DNA is present or absent, and this difference is recognized by the immune system of the host. This conformational change might be involved in the disparate antigenicities of this protein.

To investigate the role of MDP1 in host protection, we examined the activity of MDP1 in the induction of IFN-γ that is critical for host defense against *M. tuberculosis* infection in mice (42, 43). When stimulated in vitro with 10 μg/ml MDP1, lymph node cells derived from BCG-immunized C3H/He (Fig. 7*A*) and C57BL/6 (data not shown) mice produced a significant amount of IFN-γ. Analysis of IgG isotype in BCG-immunized mice revealed the production of MDP1-specific IgG2a, which was indicative of a Th1-type immune response (Fig. 7*B*) (44). Administration of purified MDP1 also expanded the population of IFN-γ-producing cells (Fig. 7*C*) and stimulated Th1-associated IgG2a production (Fig. 3). Again, simultaneous injection of MDP1 and DNA stimulated adaptive immunity and enhanced IFN-γ production (Fig. 7*C*). This was confirmed when mice were infected with *M. tuberculosis*, and MDP1 was found to decrease bacterial load only when coadministered with DNA (Fig. 8). Thus, MDP1 can be a novel vaccine target, although it is effective only when administered simultaneously with DNA. Because *M. tuberculosis* is transmitted by the aerogenic route, future studies are needed to explore the efficacy using the aerosol challenge model.

As discussed above, our data show that MDP1 has a unique feature as an Ag, in that its antigenicity is profoundly enhanced by even a small amount of DNA. This raises an important question as to how this immune stimulation is coordinated. At least six nucleotides are necessary for immune activation by ODN (49). Because DNA is highly sensitive to degradation by DNases, a large amount of DNA is required for immune activation (50). We found that

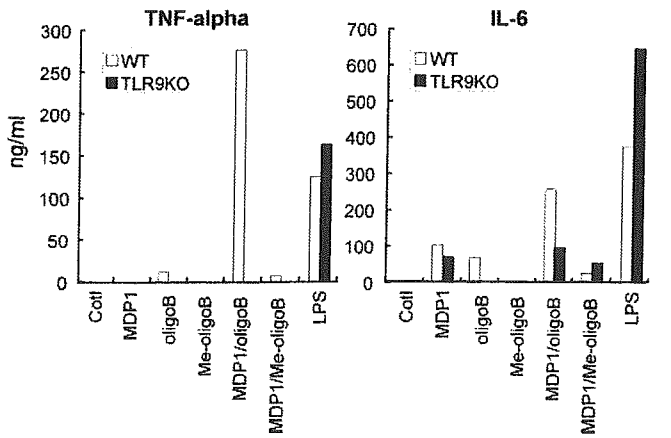

FIGURE 9. MDP1 enhances CpG-mediated production of proinflammatory cytokines in vitro. Splenocytes were stimulated with 0.5 μM MDP1 alone, a mixture of MDP1 and ODNs (1 μM), or *E. coli* LPS (100 ng/ml) for 24 h in vitro. Levels of TNF-α (*right*) and IL-6 (*left*) were determined by ELISA. The ELISA units represent the average of duplicate samples and are representative of two experiments performed.

MDP1 blocks degradation of DNA by DNases in vitro (unpublished observations), and this DNA-protective activity of MDP1 is one possible explanation.

Another possible explanation is the cell-binding activity of MDP1. To exert immunostimulatory activity, CpG DNA must attach to the macrophage surface and be internalized, with subsequent maturation of the phagosome (51). In our preliminary work, biotin-labeled ODN was more quickly bound to the macrophage surface and internalized when it was added with MDP1 (our unpublished observations). We have demonstrated that MDP1 binds to glycosaminoglycans and to A549 human lung epithelial cells through hyaluronic acid (16). After adding 0.5 μM MDP1, >95% of A549 cells became MDP1 positive in 60 min (16). In addition, it has been shown that HupB/MDP1 binds to C3 (52). Complement receptors are major receptors for *M. tuberculosis* on macrophages (53, 54). Collectively, it is reasonable to assume that MDP1 binds to macrophages through surface glycosaminoglycans or complement receptors. This cell-binding activity of MDP1 is advantageous for carrying DNA to/into macrophages, resulting in subsequent immunostimulation.

Immunization with MDP1 plus mycobacterial DNA significantly reduced the bacterial burden compared with treatment with Ag85B (Fig. 8). To develop effective vaccines against tuberculosis, additional studies are necessary to assess vaccine efficacy using MDP1 in conjunction with CpG-ODNs that can induce a Th1 response (32–34). Although the Ag85 complex has been widely studied as a major component of tuberculosis vaccines (11, 55), we did not observe a protective effect (Fig. 8). These conflicting results may be due to the mouse strains used in this experiment, because Ag85A and 85B induce protective immunity against mycobacterial infection in C57BL/6 mice (55, 56). The protective effect of Ag85B is conspicuous in guinea pigs as well (11). Guinea pigs are relatively susceptible to *M. tuberculosis* infection, whereas the mouse has low to moderate susceptibility (57, 58). In addition, guinea pigs, but not mice, develop cavitary lesions and caseous necrosis similar to human tuberculosis. It will be important to examine the protective effect of coadministration of MDP1 and DNA in a guinea pig model.

A key step in initiating adaptive immunity is the presentation of pathogen-derived peptides on class II MHC molecules by APCs. APC functions are up-regulated after recognition of pathogen-associated molecular patterns, including CpG DNA motifs (28). Therefore, we examined the effects of MDP1 on CpG ODN-mediated immune activation. We found that MDP1 magnified CpG-DNA effects, such as the production of the proinflammatory cytokines TNF-α and IL-6 (Fig. 9). As far as we know, MDP1 is the first protein identified that remarkably enhances CpG-mediated immune stimulation. Proinflammatory cytokines are critical for APC activation and promote the maturation of professional APCs. Immunostimulation induced by the interaction between MDP1 and CpG DNA might be involved in inducing strong adaptive immune responses against MDP1, which lead to protection (Fig. 8).

MyD88 is an adaptor molecule critical for the CpG-DNA-TLR9 signaling pathway (59, 60). Recently, it was shown that MyD88 KO mice are highly susceptible to *M. tuberculosis* (61) and *M. avium* (62), although mice with genetic mutations of TLR2 and TLR4 displayed comparable resistance as wild-type mice challenged with *M. tuberculosis* (63) and *M. avium* (62). These studies suggest that resistance to mycobacterial infection is regulated by multiple MyD88-dependent signals in addition to those attributed to TLR2 (64) or TLR4. As we show in this study, MDP1 stimulates TLR9-dependent immune responses by CpG ODN (Fig. 9), and the MDP1-DNA complex can induce protective immunity (Fig. 8). TLR9 signaling stimulated by MDP1-mycobacterial DNA complexes might be involved in MyD88-dependent antimycobacterial immunity.

The immunostimulatory activity of DNA was initially discovered in a DNA-rich fraction derived from BCG, referred to as MY1 (65, 66). Those studies demonstrated that the antitumor activity of MY1 was diminished by DNase treatment. MY1 is a mycobacterial nucleoid (65, 66). It is conceivable that MDP1 is involved in the activity of MY1.

The immunostimulatory activity of DNA has huge potential for immunotherapy against infectious, neoplastic, and allergic diseases (50, 67–69). To our knowledge, MDP1 is the first protein discovered that remarkably augments CpG-mediated immune stimulation (Fig. 9). MDP1 has great potential as an adjuvant for CpG-ODN-based immune interventions.

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Disclosures

The authors have no financial conflict of interest.

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