当院での最近の症例を検討すると，男女比は約7：3であり，若年の非喫煙女性や高齢の女性での発症も決して珍しくなくなっている。基礎疾患や合併症として従来，陳旧性肺結核，塵肺，COPD等が代表的であったが[5]，今回の検討では消化性潰瘍や慢性肝障害の合併も多かった。

カンサシは非結核性抗酸菌の中では毒性が強く，混入が少なく，無治療では悪化する例が多いので，細菌学的診断基準が緩くかつ診断した例は治療するのが原則である[1)6)7)]。唯一化学療法で治せる非結核性抗酸菌症であると言っても過言ではなく，通常INH＋RFP＋EBを12～18カ月投与すれば，ほとんどの症例が再発なく改善すると言われている。一般に結核菌用の薬剤感受性検査を非結核性抗酸菌に応用しても臨床的には無益であると考えられているが，カンサシのRFP感受性だけは例外となっている。まれに存在するRFP耐性菌の場合，治療にはアミノグリコシド，CAM，ニューキノロン等も加えて治療する。ただし当院で1997～2004年に検出したカンサシ567株中RFP耐性であったのはわずか4株にすぎない。今回の検討では，CAMやLVFXを使用している例が41%存在した。その理由の大部分は，RFP耐性ではなく各種副作用のためINH＋RFP＋EBという標準的な治療ができないことであった。

CAMやニューキノロンのカンサシに対する試験管内での有効性はしばしば報告されているが，臨床データは乏しい。GriffithらはCAM＋RFP＋EBの間欠投与を18人の肺カンサシ症例に実施し，平均13カ月間の治療が完了した14例では，平均1カ月で排菌停止し，平均48カ月の観察期間中1例の再発もないと報告している[8)]。今回の検討も含め，両薬剤の臨床効果は十分期待できるものと推測される。

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4. 診療所における非結核性抗酸菌症の治療と管理

水谷内科呼吸器科クリニック　水谷　清二

1. はじめに

かつて非結核性抗酸菌症（NTM症）は抗酸菌塗抹または培養が陽性であるがために結核専門施設に患者が集中し，一般病院の呼吸器外来でさえ稀な疾患であった。しかし診断技術の進歩を背景に本症に関する理解が広がり，昨今では一般呼吸器外来ではよく知られた疾患の一つになっている。ヒトへの感染が否定的である本症であれば，今後は地域の中での診療需要が拡大するものと思われ診療所でも受け入れ態勢の確立が求められる。本稿では診療所における本症の治療と管理の問題を提起してみたい。

2. NTM症患者と結核患者の混合収容

過去のNTM症患者の多くは結核感染後の治癒巣での局所免疫低下を基礎に発病していた（結核後遺症型）。このため結核菌に対する細胞性免疫が維持されており，このタイプのNTM症患者が結核病棟に混合収容されても，結核院内感染が問題となることは稀であった。結核治療歴はなくとも老人は結核既感染者が多かった事実も混合収容を可能にしていた。しかし，昨今NTM症患者の発症年齢が低下しつつあり，本症患者を単に抗酸菌陽性を根拠として結核病棟に混合収容した場合での結核院内感染の発生につき懸念が生じている。NTM症感染が同時に結核感染に対しても免疫付与すればよいのであるが，十分とするデータはない。従って，現在の取り扱いは，NTM症と判明すれば一般病棟に入院とし，菌種不明の場合，結核病棟に入院させるか否かは臨床症状や核酸増幅法の結果次第とするのが現実的である。すなわち，早期菌種同定こそ重要である。

3. 診療所における本症診療機会の増加

患者は何らかの症状がある時，多くはまず身近な診療所を訪れる。病院勤務の経験でいえば，呼吸器専門病院での患者発見よりも診療所からの紹介のほうがはるかに多いのである。またCOPDが増加する趨勢のもと，NTM症は地域においても増加するものと予想される。本邦では多くの診療所においてレントゲン撮影が可能であり，呼吸器疾患の早期発見には優れた環境を国民に提供している。委託検査施設でも分子生物学的検査が広く採用されており，結核か否かの診断はより早期に可能になってきている。長引く咳を訴える患者に遭遇した場合，胸部レントゲン写真を積極的に撮影し，異常が認められれば，抗酸菌症を疑い，塗抹検査，核酸増幅法と培養検査を行うことが必要である。このことが結核病院に移送した場合でも，菌種同定までの時間を短縮し早期鑑別に寄与することとなる。なお塗抹陰性例での核酸増幅法の成績は塗抹陽性例に比較して不良であることを知らないと思わぬ誤診を引き起こすことになり，注意が必要である。すなわち核酸増幅法が陰性でも抗酸菌症の可能性は捨てきれない。

4. 結核予防法の動向

2005年3月に出された厚生労働省結核感染症課課長通達によれば，NTM症と診断された患者にあっては，たとえ入所命令で入院しても入院時まで遡って命令を取り消すとされている。個人経営者などは仕事を休止して入所したのにこれでは，経済的にも不利益をこうむることとなる。このためにも早期の鑑別診断が必要である。NTM症と判明したなら，症状軽微な例においては仕事をしながら，診療所での通院治療が可能となるからである。

5. 入院が必要なNTM症例

限定的であり，手術例，合併症の治療（血痰，喀血，肺炎，HIV感染など），副作用対策，服薬困難例が中心となる。すなわち，基本的にはNTM症では外来が治療の場となる。

6. 診療所医師へのNTM症取り組みへのアンケート

今後治療管理の主体となると期待される診療所医師の現時点でのNTM症への考えにつき，アンケートを行った。対象は筆者が所属する医師会を構成する内科医業を主とする医師とした。回答率は約30%であった。図1は過去数年間での抗酸菌症疑い例の経験の有無とその中で現実にNTM症患者の経験を尋ねたものである。84%で疑い例を経験していたが，経験有りとした中で，実際にNTM例であったものが57%あり，決して稀な疾患ではないことを示唆している。次に図2では早期診断に欠かせない核酸増幅法の依頼とMGIT法の導入について尋ねたものである。核酸増幅法については半数の51%で既に標準検査の中に採用されていた。しかしまだルーチンにしていない医療機関が34%あり，今後さらなる普及が待たれる。液体培地であるMGITの導入では68%で導入されておらず，今後本検査への理解の広がりが待たれるところである。しかし委託検査施設にあっては，MGIT法は検査コストが合わず，医師に対し積極的な周知への努力を怠っている可能性もあり，普及には検査コストの方面からの対策が必要と思われる。なおMGIT法で依頼すれば受付は行っている。図3左は現在のNTM症への実際の対応であり，右の図は今後の対応を尋ねたものである。現在は結核医療機関に紹介することを原則にしているものが82%あるが，しかし今後については，34%で自ら診察，管理を行いたいとしており，診療所の医師の中では本症診療に前向きの態度がうかがえる結果であった。

7. 診療所の利点/短所

なんといっても患者の自宅から近いことがあげられ

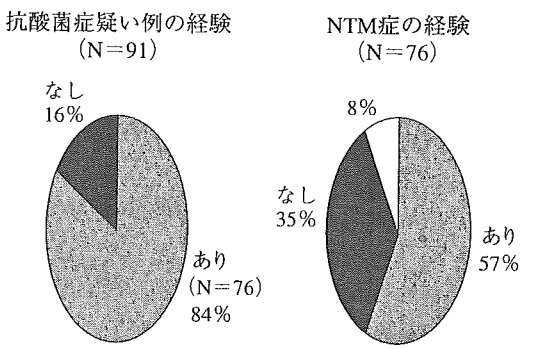

図1 診療所医師アンケート1
—過去数年間に抗酸菌症疑い例の経験は？—

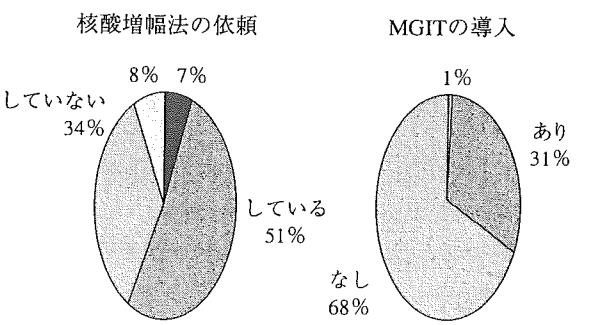

図2 診療所医師アンケート2

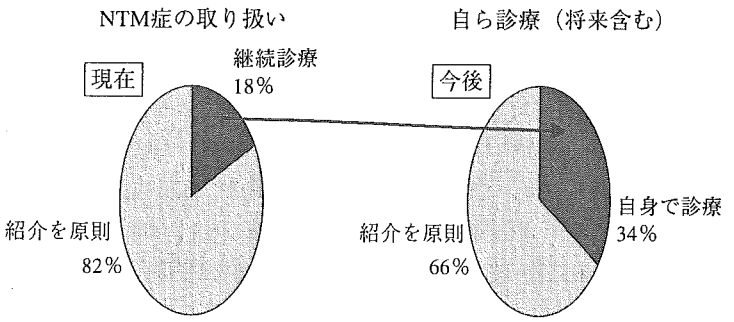

図3　診療所医師アンケート3

る。毎日でも主治医と連絡できることは患者の安心を保障することができる。SMなど注射も困難なく遂行でき，治療へのコンプライアンスを良好とすることができる。しかし他科との連携のためには複数の診療所への通院が強いられること，気管支鏡ができないこと，排痰など理学療法ができないことなど不利な点もある。筆者は専門外来を行っている他病院において自患者の気管支鏡を行っているが例外的であり，病院との連携が必要となる。

8. 初診から診断まで

抗酸菌症といっても空洞例などで結核かNTM症か，画像のみでは鑑別困難な症例が存在する。委託検査施設でも喀痰塗抹検査は24時間以内に報告可能になっているが，核酸増幅法の結果を翌日までに要求することは困難であり，この間自宅待機はできない。この場合は結核施設での鑑別を待つ以外ない。中葉舌区主体に陰影がある症例では，NTM症が多数を占めるため，待機してもよいが結核の可能性に留意しなくてはならない。検体は早朝痰ないし胃液検査の検体に限る。昼間の喀痰で塗抹陰性でも安心してはならない。

9. 診療所での採痰

本来第一線施設は常に結核を含めた抗酸菌症患者の来院を想定し，対策が必要であるが，狭い診療所内で採痰スペースすら確保することは容易ではない。筆者は開院時に狭いながらもHEPAフィルター入りの濾過装置を採痰室に設置した。安心して連続的に採痰できるし，結核のみならず感染症患者の隔離室として利用可能である。濾過装置が設置できない多くの診療所では，採痰は建物の外か換気扇の下で採取することとなる。NTM症と決すれば，院内採痰でよい。

最　後　に

アンケートの結果ではNTM症診療に前向きな診療所医師が増加しているように思われる。この背景には早期鑑別診断を可能とした医療の進歩とNTM症がヒトを介しての感染が否定されており，隔離不要であることへの理解がある。患者にとっては長期間通院を強いられる疾患であり，診療の場は家庭に近い診療所が好ましい。しかし診療所での診療には限界があり，入院適応患者の管理，気管支鏡検査，排痰訓練など理学療法も含む病診連携こそ重要である。本症にあっても病院と診療所が患者の利益を第一として診療の分担を担うべきである。開発された抗酸菌診断学や治療の進歩が，速やかに第一線を受け持つ診療所医師に伝えられなければならない。また検査を受け持つ委託検査施設に対しても，検査導入が容易になるよう医療経済上でも配慮が必要である。

5. 非結核性抗酸菌症に対する外科的治療

埼玉医科大学総合医療センター呼吸器外科　菊池　功次，柴　　修吾，福田　祐樹，
儀賀　理暁，山畑　　健，中山　光男

非結核性抗酸菌症は抗結核薬が無効な症例が多く，長い経過の中で徐々に肺組織が荒廃，進行する予後不良の疾患である。われわれはCAMを併用した抗結核薬による治療に抵抗性で，排菌の持続する症例に対しては，病巣が限局しているうちに切除することが肝要であると考えて，積極的に外科的治療を行ってきた。

本稿では埼玉医科大学総合医療センターで経験した非結核性抗酸菌症に対する外科的治療の適応と成績につい

て報告する。

対象とした症例は1997年4月から2005年3月までの8年間に当院で外科的治療を行った非結核性抗酸菌症9例である。性別は男性5例，女性4例で，年齢は38歳から68歳で，平均年齢は51歳である。非結核性抗酸菌の菌種は，MAC（*Mycobacterium avium* complex）が8例，*M. kansasii*が1例であった。MACの8例中1例は抗結核薬が奏効した後に症状の悪化とCT上の陰影の悪化により手術を行ったところ，浄化空洞にアスペルギローマが感染していた症例であった。患側は右側8例，左側1例である。

症状としては血痰が5例，咳嗽が2例，症状なしが2例で，血痰を訴える症例が最も多く見られた。

術前投薬歴はほとんどの症例でRFP，EB，CAMを併用していたが，1例は*M. kansasii*の症例で，もう1例は8年前の古い症例でCAMの併用を行っていない。その他の7例は原則として化学療法を最低でも10カ月程度行って手術適応を判断していたが1例のみ喀痰でクラスVが2度検出されたため肺癌の疑いで早い時期（4カ月）に手術を行っている。

手術適応理由は肺癌を疑って手術を施行した症例が1例，内科的治療に抵抗性の症例が8例で，このうち5例が排菌が持続した症例で，2例が陰影が残存して軽快しないため手術適応とした。残りの1例は浄化空洞にアスペルギローマが感染した症例である。

手術術式は区域切除術1例，部分切除術3例，肺葉切除術5例であった。肺葉切除術が5例と半数以上を占めていた。原則として肺機能を温存する術式（なるべく切除範囲を縮小する術式）を選択しているが，主病巣の周辺に散布巣がある場合にはなるべく肺葉切除を選択している。全例，手術直後の再燃を防ぐ目的で術後の化学療法は6カ月以上行っているが，治療終了後の再燃は3例にみられた。

手術適応となった症例を提示する。

症例は38歳の男性で，*M. avium*が検出されていた。左自然気胸に対し当院で手術を行ったが，術後9カ月，経過の観察中に右の肺尖に淡い腫瘤状の陰影が出現した。CT（図1左）では空洞と思われる病巣の周辺に浸潤影がみられた。MACと診断して，INH，RFP，EBにCAMを併用して9カ月治療したが，最終的に空洞性の病巣（図1右）が残って，排菌が止まらないため，右上葉の部分切除を行った。切除標本では乾酪壊死を伴う病巣および空洞性の病変と瘢痕の組織が見られた。術後は良好な経過をたどっていたが，2年3カ月で左肺尖に新しい陰影の出現を認め，再燃と診断，RFP，EB，CAMを投与し陰影の縮小を認めたため投薬を中止して経過観察中である。

次の症例は*M. kansasii*で手術を行った症例である。血痰を主訴として来院した53歳の男性で胸部CT（図2左）では右の肺尖のところに淡い浸潤影と空洞がみられた。抗結核薬を1年間行っても胸部CT上（図2右）空洞が残ってしまい，排菌も持続しているため，空洞を含む病巣を切除することにした。切除標本では右上葉に乾酪壊死を伴う空洞性の病変があり周囲に瘢痕が見られた。この症例は術後7年9カ月，再燃の兆候なく順調に経過している。

術後再燃症例を表に示した。術後の投薬期間は5～

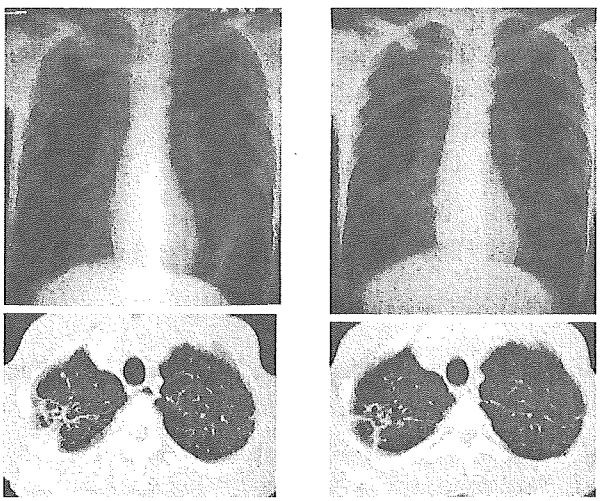

図1 治療前後の胸部X-PおよびCTスキャン

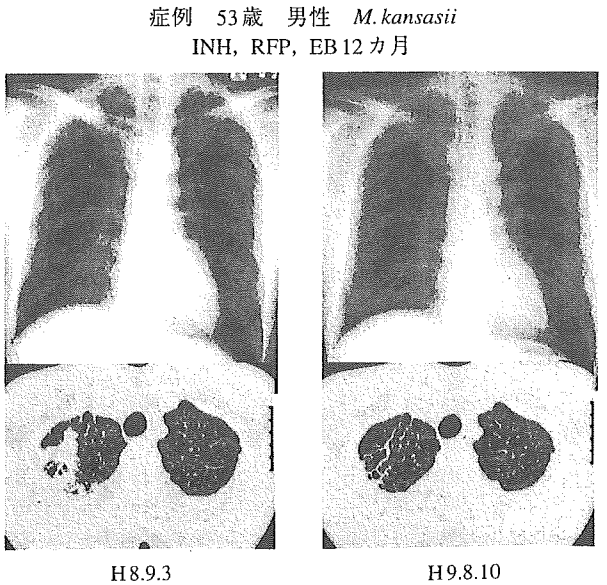

図2 治療前後の胸部X-PおよびCTスキャン

表　術後再燃症例（1997年4月～2005年3月）

	術式	術後投薬期間	術後再燃までの期間（部位）
①43歳　女	左上切	5カ月間	8カ月後（中葉，左S^{8}）
②38歳　男	右上葉部切	7カ月間	27カ月後（左右肺尖部）
③42歳　男	右S^{6}区切	19カ月間	24カ月後（右S^{10}）

※再化療により全例排菌停止，全例生存中

19カ月である。再燃までの期間は8カ月～27カ月間と2年経過して再燃している症例もあった。再燃後はCAMを加えた化学療法を行って，3例中2例で陰影も軽快している。

考　察

埼玉医科大学総合医療センター呼吸器外科で治療を行った非結核性抗酸菌症について検討を行った。

われわれが手術を行った非結核性抗酸菌症例9例中8例がMACであったが，1例のみ*M. kansasii*の手術を行った。*M. kansasii*の症例は1年間化学療法を行っても排菌が止まらないため手術適応とした。

*M. kansasii*に対しては通常抗結核薬が効果を示すことが多いため[1]，手術適応となる症例は少ないと思われるが本例のように1年間の投薬を行っても排菌が止まらない症例では手術を行ったほうが良いと考えている[2]。MACの8例中4例では内科的治療に抵抗性で排菌が止まらないため手術適応とした[3]。2例は陰影が縮小する傾向がないため手術適応とした。1例は肺癌を疑って手術を行った。残りの1例は薬剤が奏効して浄化空洞になったが，そこにアスペルギローマが感染して症状と陰影の悪化が認められたため手術を行った。

術後病巣の再燃については9例中3例にみられた。これは相良ら[4]が非結核性抗酸菌症55例のうち再燃が12例（21.8%）みられたとの報告と比較すると33%の再燃率でやや高かった。これは相良らの再燃率と比べとやや高かった。非結核性抗酸菌症の外科治療成績は，排菌の消失率，再燃の確率などを手術後10年以上の経過で評価しなければならない。ただ現在行われているように内科的治療と比較するのではなく，内科的治療で効果がでない症例と比較しなければならないと思う。外科的治療を行った症例で再燃のなかったのは6例，再燃した症例のうち，1例は新たな病巣の出現，1例が微小結節の増加・増大，1例が気管支拡張症に微小結節が出現したと考えられた。3例中2例でRFP，EB，CAMの投与で陰影も軽快し，薬剤の投与を中止して経過を追っているが，病巣の再悪化はみられていない。

非結核性抗酸菌症に対する外科的治療例では，術中，術後に手術に関連した合併症（術後膿胸，気管支瘻など）の発生はなく，良好な経過であった。非結核性抗酸菌症に対してCAMを併用した抗結核剤による内科的治療を1年程度行っても依然として活動性の病巣があったり，排菌が持続している症例では，あまり長く内科的治療に固執せず外科的治療の併用を検討すべきであると思われた。

ただし，最近呼吸器疾患に対する外科的治療が悪性疾患つまり肺癌に対してばかりが行われるようになって，結核を中心とした炎症性疾患に対する外科的治療に習熟した呼吸器外科医が以前と比べて極端に減少している。炎症性疾患に対する外科的治療に不慣れな呼吸器外科医がこのような非結核性抗酸菌症に対して外科的治療を行うと術中術後合併症の増加が危惧される。これを防ぐためには，炎症性呼吸器疾患に対する外科的治療に習熟した呼吸器外科医の育成が重要と考えている。

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結核 第81巻 第1号 2006年1月

指定発言：非結核性抗酸菌症に対する標準術式とは

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Ⅰ．はじめに

内科治療に抵抗性の肺非結核性抗酸菌症に対しては，治療効果を高める目的で肺切除療法が適応となる[1)~4)]。この場合病巣の拡がりによって肺部分切除術から肺全摘除術までの幅広い術式が選択される。末梢の小さな病巣であれば肺部分切除術の適応となりうる可能性があるが，当院ではそのような比較的軽度の肺非結核性抗酸菌症を見る機会が少なく，最低でも区域切除術を必要とするほど病巣が拡がっている症例がほとんどである[5)]。また仮に小さな病巣であっても肺部分切除術で良いかどうかは結論が出ていない。これとは対極に病巣が肺全体に多発している場合や一側肺が荒蕪肺になっている場合には肺全摘除術の対象となる。ここまで病巣が進行した症例でも肺切除療法の適応となるかどうかも議論の余地がある。そこで今回は当院で行った肺非結核性抗酸菌症に対する肺全摘除術の治療成績から肺非結核性抗酸菌症に対する標準術式とはいかなるものかを検討した。

Ⅱ．方法および対象

1983年から2002年までの20年間に53例の肺非結核性抗酸菌症症例に対して計55回の肺切除術を行った。そのうち肺全摘除術が施行された症例は11例であった。症例の内訳は男性3例，女性8例であり，年齢の中央値は57歳（43～69歳）であった。Body mass indexは15.5から22.2 kg/m^2（中央値16.6 kg/m^2）とやせ型が多かった。疾患の内訳は*Mycobacterium avium* complex（MAC）が10例，*M. abscessus*が1例であった。外科的切除の適応は内科治療の不成功が10例，緑膿菌の混合感染が1例であり，肺全摘除術の適応は多発空洞が6例（図1，2），荒蕪肺が5例であった。4例で対側肺に散布陰影が認められたが対側肺に軽度の病巣があったとしても，術側の病巣が主たる病巣である場合には手術適応とした。

Ⅲ．結　果

術側は右が5例，左が6例であり，手術時間は142～477分（中央値360分），術中出血量の中央値は555 m*l*（130～1245 m*l*）であった。気管支断端は広背筋弁で被覆した例が7例，肋間筋弁で被覆した例が2例であった。術死は認めなかった。術後合併症として気管支断端瘻が3例に発症し，いずれも右側であった。2例は広背筋弁使用例で術後2週と2.3カ月で発症し，1例は肋間筋弁使用例で術後3カ月で発症した。いずれの症例も瘻孔は小さく膿胸も併発しておらず，気管支断端の再縫合を行い3例とも治癒した。気管支断端瘻を伴わない膿胸が1例に発症し，洗浄と掻爬により治癒した。全例術後に菌が陰性化した。遠隔死亡は2例に認められた。1例は呼吸不全により術後11カ月で死亡した。もう1例は術後4年で呼吸不全により死亡し，この症例は術後再発した唯一の症例である。

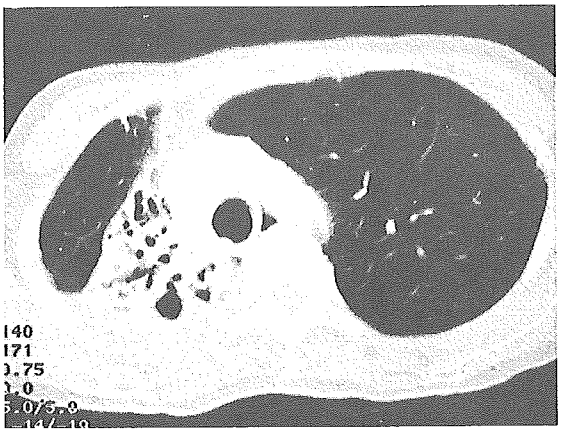

図1　多発空洞例の術前胸部CTスキャン
右上葉は破壊され一部空洞化している。

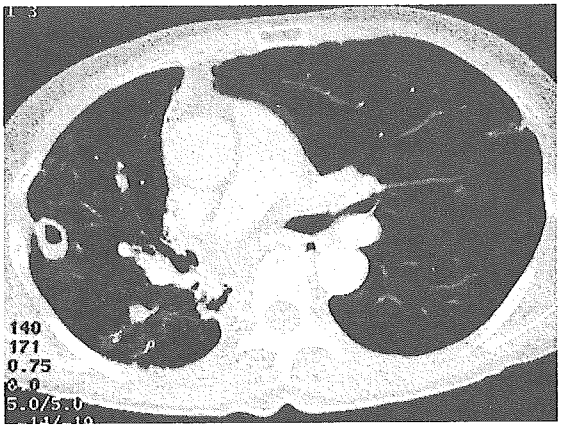

図2　多発空洞例の術前胸部CTスキャン
右下葉にも空洞が見られる。

Ⅳ．考　察

内科治療に抵抗性の非結核性抗酸菌症症例では外科治療が必要となる場合がある[1)~5)]。病巣が一肺葉に限局し

ていれば肺葉切除術の適応となるが，空洞が多発している場合や一側肺が荒蕪肺となっている場合には肺全摘除術の適応となる[6)7)]。肺葉切除に比べて肺全摘除が行われる場合は少なく，Corpeらの報告では124例のMAC症例中肺全摘除が行われたのは9例のみであった[1)]。Pomerantzらの報告では38例の非結核性抗酸菌症症例に対して41回の肺切除が行われ，うち肺全摘除例は17例であった[2)]。Nelsonらは28例のMAC症例に対して肺切除を行い，うち8例が肺全摘除例であった[3)]。われわれの過去20年間の経験では53例の非結核性抗酸菌症症例に対して肺切除を行い，11例が肺全摘除例であった[7)]。

肺全摘除後の合併症として問題になるのは気管支断端瘻である。われわれの症例でも3例に発症し，いずれも右側であった。2例は広背筋弁使用，1例は肋間筋弁使用にもかかわらず発症した。いずれの症例も瘻孔は小さく膿胸も併発しておらず，気管支断端の再縫合を行い3例とも治癒した。筋弁で被覆することにより気管支断端瘻に付随する合併症のリスクは軽減できたといえる。

1例術後呼吸不全で死亡したが，この症例は術前の肺活量が1.73 L，一秒量が1.12 Lと肺機能の低下していた症例であった。術後気管支断端瘻を併発して再手術を行ったこともあり長期間にわたる人工呼吸管理が必要となり最終的にはARDSで死亡した。

肺全摘除術を必要とする症例はかなり病巣が進行している症例であり外科治療の限界に近いと考えられるが，全例術後に菌が陰性化しており肺全摘除術は排菌を停止させる点で有効な治療法であるといえる。

V. まとめ

肺非結核性抗酸菌症に対する右肺全摘除術は合併症のリスクが高く特に気管支断端瘻が依然として問題である。筋弁による気管支断端被覆にもかかわらず気管支断端瘻が発生したが，筋弁で被覆することにより気管支断端瘻に付随する合併症のリスクは軽減できた。われわれの結果は肺全摘除術を必要とするほど進行した肺非結核性抗酸菌症の症例であっても十分な化学療法と肺全摘除術を行うことにより治癒可能な例が存在することを示している。肺切除療法の意味とは外科的に空洞や荒蕪肺といった菌が大量に存在する部分を切除することであり，この目的が達せられるのであれば肺全摘除術まで標準術式となる。

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The 80th Annual Meeting Symposium

TREATMENT OF NON-TUBERCULOUS PULMONARY MYCOBACTERIOSIS

Chairpersons: [1]Mitsunori SAKATANI and [2]Yoshiki NAKAJIMA

Abstract The non-tuberculous mycobacteriosis (NTM) is not a unitary disease. It is a general term for the broncho-pulmonary diseases caused by any mycobacterium other than *M. tuberculosis*. We don't call the pulmonary "pseudomoniosis" for the diffuse bronchiectasis caused by *Pseudomonas aeruginosa*, though conditions of the disease looks like NTM. The name of NTM represents that the causative bacteria belong to the same species with *M. tuberculosis* which causes serious pulmonary infectious disease.

The pulmonary diseases caused by *M. kansasii* or *M. szulgai* are usually treated by RFP, EB and INH, the same regimens with tuberculosis, which generally lead to sufficient results for patients. But for MAC diseases, the number of patients is top of NTM in Japan, recent treatment with new-macrolides and some anti-tuberculous drugs generally does not bring about the desired effect. The plenty clinical experiences for NTM in HIV positive patients have lead to such new regemens in the USA. For NTM caused by rare Mycobacterium detected very seldom, clinical experiences and knowledge are definitely insufficient.

1. Present state of therapy for pulmonary MAC disease (drug therapy): Yoshihiro KOBASHI (Division of Respiratory Diseases, Department of Medicine, Kawasaki Medical School, Kurashiki, Okayama Prefecture)

Co-operative study of Research Committee of Mycobacterium in the Chugoku and Shikoku areas revealed that the combined therapy according to the guidline is clinically appropriate for pulmonary *Mycobacterium avium* complex (MAC) disease.

2. Relapse and chemotherapy duration of pulmonary *Mycobacterium avium* complex infection: Atsuyuki KURASHIMA (Division of Clinical Research, National Organization Tokyo National Hospital, Kiyose, Tokyo)

Reviewing the 71 relapses out of 1170 pulmonary MAC infection cases, he indicated that 11.3% relapsed during the chemotherapy continuation, 23.9% after the reduction of chemotherapy, 64.8% after the termination of chemotherapy. In the last group, there is no correlation to the relapse period after the end of treatment with the preceding chemotherapy duration. It is supposed that the main cause of these relapses are enviromental re-infection.

3. Chemotherapy for pulmonary *M. kansasii* disease: Katsuhiro SUZUKI (Clinical Research Center, Nationl Organization Kinki-chuo Chest Medical Center, Sakai, Osaka)

Analysis of 938 pulmonary mycobacteriosis in 2003 revealed that 244 (26%) patients suffered from NTM, in which 66 (27% of NTM) were *M. kansasii* disease. The 48 (73%) patients were male. From 2001 to 2004, in the 190 *M. kansasii* patients treated by anti-tuberculous drugs, H/R/E was prescribed for 84 cases (44%), other 41% of prescriptions included CAM and (or) LVFX. Almost all patients were converted into bacilli negative in about 30 days with any prescription.

4. Treatment and management for NTM patients in a private clinic: Seiji MIZUTANI (Mizutani Respiratory Clinic, Nerima ward, Tokyo)

Analysing clinical experiences, he emphasized that most NTM patients with some symptoms visits private clinics in the first place. In Japan, diagnosis of NTM with radiological and bacteriological examinations is not difficult, and most NTM patients can be controlled as the outpatients of the clinics.

5. Surgical Treatment for non-tuberculous mycobacteriosis: Kouji KIKUCHI (Division of Chest Surgery, Medical Center, Saitama Medical School, Iruma county, Saitama Prefecture)

The 9 NTM cases surgically treated were analysed, 8 were MAC cases and 1 was *M. kansasii* case. The main reasons for sugical resection were, continuous hemoptisis, continuous productive cough, or exacerbation on chest X-ray features. The NTM bacilli were positive in 8 cases, another one was bacilli negative, though X-ray shadows increased. After the surgery, expectoration of bacilli converted to negative in 5 cases.

Special commentaries: Can pneumonectomy be an acceptable procedure for non-tuberculous mycobacterial infection?: Yuji SHIRAISHI (Division of Chest Surgery, Fukujuji Hospital, Kiyose, Tokyo)

The 11 NTM patients were analysed, who underewent pneumonectomy. The median blood loss was 555 m*l* and there was no operative mortality. Bronchpleural fistula or empyema occured in 4 patients. The bacilli negative conditions were achieved in all patients after surgery.

The NTM is not a legal epidemic disease and Japanese Tuberculosis Prevention Act doesn't cover this disease. The medical treatment insurance system doesn't contain the NTM in the list of applicable diseases in Japan. Though these some problems with increasing numbers of patients remain in clinical practice, chairpersons hope that this symposium will be a milestone for the generalized progress of treatment and management of NTM in Japan.

Key words: Pulmonary MAC disease, *M. kansasaii* disease, New-macrolides, Antituberculous drugs, Surgical operation

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DNA vaccine using hemagglutinating virus of Japan-liposome encapsulating combination encoding mycobacterial heat shock protein 65 and interleukin-12 confers protection against *Mycobacterium tuberculosis* by T cell activation

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Abstract

We investigated the immunogenicity and protective efficacy of DNA vaccine combinations expressing mycobacterial heat shock protein 65 (Hsp65) and interleukin-12 (IL-12) using gene gun bombardment and the hemagglutinating virus of Japan (HVJ)-liposome method. A mouse IL-12 expression vector (mIL-12 DNA) encoding single-chain IL-12 proteins comprised of p40 and p35 subunits were constructed. In a mouse model, a single gene gun vaccination with the combination of Hsp65 DNA and mIL-12 DNA provided a remarkably high degree of protection against challenge with virulent *Mycobacterium tuberculosis*; bacterial numbers were 100-fold lower in the lungs compared to BCG-vaccinated mice. To explore the clinical use of the DNA vaccines, we evaluated HVJ-liposome encapsulated Hsp65 DNA and mIL-12DNA (Hsp65 + mIL-12/HVJ). The HVJ-liposome method improved the protective efficacy of the Hsp65 DNA vaccine compared to gene gun vaccination. Hsp65 + mIL-12/HVJ induced $CD8^+$ cytotoxic T lymphocyte activity against Hsp65 antigen. Most importantly, Hsp65 + mIL-12/HVJ vaccination resulted in a greater degree of protection than that evoked by BCG. This protective efficacy was associated with the emergence of IFN-γ-secreting T cells and activation of proliferative T cells and cytokines (IFN-γ and IL-2) production upon stimulation with Hsp65 and antigens from *M. tuberculosis*. These results suggest that Hsp65 + IL-12/HVJ could be a promising candidate for a new tuberculosis DNA vaccine, which is superior to BCG vaccine.

Keywords: Tuberculosis; DNA vaccine; HVJ-liposome

1. Introduction

Tuberculosis is a major global threat to human health, with about 2 million people dying each year from *Mycobacterium tuberculosis* infections. The only tuberculosis vaccine currently available is an attenuated strain of *Mycobacterium bovis* BCG. BCG continues to be widely administered to children in developing countries, yet its efficacy remains controversial, particularly against the pulmonary form of the disease in adults. In recent years, the increasing frequency of drug-resistant *M. tuberculosis* isolates has further complicated the clinical management of this disease. Clearly, a more effective vaccine for the control of tuberculosis is urgently needed.

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It is well established that protective immunity to *M. tuberculosis* depends on both $CD4^+$ and $CD8^+$ T cells [1–6]. Because DNA vaccination results in the generation of cellular immune responses, including those of a Th-1-type response, and protection in animal models of infectious diseases [7,8]. In fact, several human clinical trials have recently been initiated to test the efficacy of DNA vaccines against emerging and re-emerging infectious diseases including hepatitis B [9], malaria [10,11] and HIV infections [12]. DNA vaccination has also shown potential for the development of tuberculosis vaccines in the mouse model [13–16]. However, in a guinea pig model, which is arguably one of the most biologically relevant systems available for studying human pulmonary tuberculosis, DNA vaccines has not proven more efficacious than BCG [17]. The efficacy of any experimental tuberculosis vaccine remains to be evaluated in human clinical trials and, thus, a vaccine against tuberculosis is still anxiously awaited.

Mycobacterial heat shock protein 65 (Hsp65) is a potential target for protective immunity and has been extensively studied [18]. Several groups have reported that *hsp65* DNA vaccines can induce strong protective immune responses in mice against virulent *M. tuberculosis* infections [19–21]. Protection is attributed to the establishment of a cellular immune response dominated by Hsp65-specific T cells that both produce IFN-γ and are cytotoxic towards infected cells. Furthermore, Lowrie et al. have reported that this vaccine reduces bacterial loads in mice infected with *M. tuberculosis* when given therapeutically after infection [22]. Interleukin-12 (IL-12) is a cytokine with a major role in the induction of IFN-γ-dominated immune responses to microbial pathogens. Orme and colleagues have demonstrated the importance of IL-12 in generation of the protective response to tuberculosis [23]. Co-administration of the IL-12 gene, which participates in the induction of IFN-γ dominated immune responses to microbial pathogens, with various tuberculosis DNA vaccines including the *hsp65* DNA [20,24] and 35 K MW DNA [25] may boost the efficacy of these DNA vaccines to levels achieved with BCG in the mouse model, although inhibitory effect rather than synergistic effect on immunotherapy was observed in mice co-administrated with *hsp65* DNA vaccine plus the *IL-12* gene.

In order to explore the preclinical use of tuberculosis DNA vaccine combinations of the *IL-12* DNA with the *hsp65* DNA, we chose the viral-based hybrid antigen delivery system hemagglutinating virus of Japan (HVJ)-liposome because this delivery system results in a high transfection efficacy, repeated gene transfection without reduction of gene transfer efficiency in vivo, and no apparent toxicity. These characteristics of HVJ-liposomes support the feasibility of its clinical application not only for cancer gene therapy but also for DNA vaccinations. In a recent study, highly efficient transfection of muscle cells was observed for several weeks when pcDNA3 plasmid containing the human tumor antigen genes, *MAGE-1* and *MAGE-3*, were encapsulated in HVJ-liposomes and injected intramuscularly into mice [26]. Effective induction of $CD4^+$ T cell responses by a hepatitis B core particle-based HIV vaccine was achieved by subcutaneous administration of HVJ-liposomes in mice [27]. HVJ-liposomes were also very effective as a mucosal vaccine against HIV infection [28]. Thus, it is likely that HVJ proteins may be responsible for inducing a robust immune response. No side effects from repetitive injections of HVJ-liposomes into mice, rats or monkeys were observed.

We designed this study to clarify the clinical feasibility of HVJ-liposome-mediated DNA vaccines for tuberculosis. First, we clarify that co-administration of IL-12 DNA with Hsp65 DNA via gene gun delivery enhanced protection in mice compared with Hsp65 DNA alone. Second, we show that vaccination with HVJ-liposome encapsulated Hsp65 DNA resulted in better protection than did gene gun vaccination. Third, we demonstrate that HVJ-liposome encapsulated Hsp65 DNA and IL-12 DNA induce enhanced protective immunity in the mouse model compared to that seen with BCG. This protective efficacy was associated with the emergence of IFN-γ-secreting T cells upon stimulation with Hsp65 and purified protein derivative. These results suggest that Hsp65 + IL-12/HVJ could be a promising candidate for a new tuberculosis DNA vaccine, which is superior to BCG vaccine. We also discuss in this paper the prospects of this HVJ-liposome-based DNA vaccine for testing in primate models [29] and, ultimately, in a clinical setting.

2. Materials and methods

2.1. Bacteria

M. tuberculosis strain H37Rv and *M. bovis* BCG Tokyo were kindly provided by Dr. I. Sugawara (JATA, Tokyo, Japan). *M. bovis* BCG Tokyo was maintained in synthetic Sauton medium (Wako Chemicals, Osaka, Japan). For the mouse infection studies, a single colony of *M. tuberculosis* H37Rv was grown in Middlebrook 7H9 (DIFCO Laboratories, Detroit, MI: lot 137971 XA MD) medium supplemented with albumin–dextrose complex and grown at 37 °C until approximately mid-log phase. Aliquots were stored at −80 °C and thawed at 10 days before use, grown to mid-log phase in 7H9 medium.

2.2. Reagents and antibodies

Purified protein derivative (PPD: lot T-3-4) was obtained from JAPAN BCG Co., Ltd. (Tokyo, Japan). Killed *M. tuberculosis* H37Ra (lot 13971XA) was obtained from DIFCO Laboratories. Fetal calf serum (FCS: lot AGC6341) was obtained from Hyclone (Logan, UT). Anti-L3T4, anti-Lyt2.2 monoclonal antibodies and anti-Thy1.2 antibody were kindly provided by Dr. K. Kuribayashi (Mie University, Tsu, Japan) and Dr. E. Nakayama (Okayama University, Okayama, Japan) [30].

2.3. Animals

Inbred and specific pathogen-free female BALB/c mice were purchased from Clea Japan Inc. (Tokyo, Japan). Mice were maintained in isolator cages, manipulated in laminar flow hoods and used between 8 and 10 weeks of age. Infected animals were housed in individual micro-isolator cages in a biosafety level (BL) 3 animal facility of the NHO Kinki-chuo Chest Medical Center.

2.4. Cell lines

COS-7 cells were kindly provided by Dr. H. Endoh (Jichi Medical School, Tochigi, Japan). COS-7 cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics. A mouse mastocytoma cell line (P815: DBA/2 origin) was kindly provided by Dr. C.S. Henney (Fred Hutchinson Cancer Research Center, Seattle) [31]. A mouse macrophage cell line (J774.1: BALB/c origin) was kindly provided by Dr. P. Ralph (Sloan Kettering Cancer Inst., New York, NY) [32]. The P815 and J774.1 cells were maintained in RPMI 1640 medium (Flow Laboratories, Inc., Mclean, VA) supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) and 5×10^{-5} M 2-mercaptoethanol [33,34].

2.5. Plasmid construction

The *hsp65* gene was amplified from *M. tuberculosis* H37Rv genomic DNA by PCR using a set of primers, phsp65-F1 and phsp65-R1, and cloned into the *Bam*HI/*Not*I sites of pcDNA3.1 (+) (Invitrogen, San Diego, CA) to generate pcDNA-hsp65. pcDNA-hsp65 was designated as Hsp65 DNA in this text. For the construction of the *hsp65* gene fused with the mouse Igκ secretion signal sequence, the PCR product was cloned into the *Bam*HI/*Not*I sites of pcDNA-CS87 [35] to generate pcDNA-Ighsp65. pcDNA-Ighsp65 was designated as IgHsp65 DNA in this text. For the construction of the mouse IL-12 (mIL-12) *p40* and *p35* single-chain gene, the *mIL12p35*and *mIL12p40* genes were cloned from pcDNA-p40p35 [35] by PCR using sets of primers, pmIL12p35-F1 and pmIL12p35-R1, and pmIL12p40-F1 and pmIL12p40-R1, respectively, and cloned into pcDNA3.1 (+) to generate pcDNA-mIL12p40p35-F. pcDNA-mIL12p40p35-F was designated as mIL-12 DNA in this text. As a control, pcDNA-EGFP vector expressing the *EGFP* gene was used. Sequences of oligonucleotide primers used are available as request.

2.6. Protein production and antibody preparation

Recombinant Hsp65 (rHsp65) protein was expressed in *E. coli* BL21 (λDE3) and purified by affinity chromatography on Ni-NTA columns (Qiagen).

2.7. Transfection

DNA transfection of COS-7 cells was performed with the PolyFect Transfection Reagent (Qiagen) according to the manufacturer's instructions. After 24 h, supernatant and cells were harvested separately. Immunoprecipitation of cell lysates and supernatants with antibodies were performed as described previously [36]. Rat anti-mouse IL-12p70 (BD Biosciences Pharmingen, San Diego, CA) and mouse anti-rHsp65 polyclonal antibody were used for immunoprecipitation. For IL-12 bioassay, COS-7 cells (1×10^6 cells/plate) were plated into 60-mm cell culture plates and transfected with 2.5 μg of pcDNA 3.1, pcDNA-mIL12p40 + p35, or pcDNA-mIL12p40p35-F using the PolyFect Transfection Reagent. At 48 h after transfection, culture supernatants were collected and stored at −70 °C until use. Various volumes of the supernatants were added to the mouse spleen cells (2×10^6 cells/ml). Murine culture supernatants after 60 h incubation were collected and the level of mouse IFN-γ measured using sandwich ELISA kits (BD Opt EIA™ Set. BD Biosciences Pharmingen), according to manufacturer's instructions.

2.8. Vaccination

2.8.1. Gene gun vaccination

Gold particles coated with plasmid DNAs and their cartridges were prepared as described previously [35]. The abdomen was shaved and gold particles coated with plasmid DNA (1 μg plasmid DNA per shot) was delivered once into the abdomen using a Helios Gene gun (Nippon Bio-Rad Laboratory, Tokyo, Japan) at a helium discharge pressure of 300 psi. A separate group was vaccinated once subcutaneously with 1×10^6 colony-forming units (CFU) of *M. bovis* BCG Tokyo strain.

2.8.2. HVJ-liposome vaccination

HVJ-liposomes were prepared as described previously [37]. The HVJ–liposome complex was aliquoted with 10% DMSO and stored at −70 °C until use. HVJ-liposomes without plasmid DNA was used and designated as Empty/HVJ in this text. Groups of BALB/c mice were vaccinated three times at 3-week intervals with 100 μl of HVJ-liposome solution containing 50 μg of pcDNA-IgHsp65 and/or 50 μg of pcDNA-mIL12p40p35-F in the tibia both anterior muscles. A separate group was vaccinated once with 1×10^6 CFU *M. bovis* BCG Tokyo by subcutaneous injection at four different sites (left upper, right upper, left lower, right lower back) at the same time. HVJ-liposome DNA vaccines encapsulating pcDNA-IgHsp65, pcDNA-mIL12p40p35-F, or combination of pcDNA-IgHsp65 and pcDNA-mIL12p40p35-F was designated as IgHsp65/HVJ, mIL-12/HVJ, and IgHsp65 + mIL-12/HVJ, respectively, in this text.

2.9. Challenge infection of vaccinated animals and bacterial load determination

Mice were challenged by the intravenous route with 5×10^5 CFU of *M. tuberculosis* H37Rv 3 weeks after the third vaccination as described previously [38]. At 5 and 10 weeks after *M. tuberculosis* H37Rv challenge, the lungs, spleens, and livers were aseptically homogenized by using homogenizer in saline, and serial dilutions of the organ homogenates were plated on Ogawa agar (Kyokuto, Tokyo, Japan) or 7H11 Middlebrook agar (Kyokuto). Plates were sealed up and incubated at 37 °C and the number of CFU was counted 2 or 4 weeks later. Results are converted to $\log_{10}$ values and $\log_{10}$ [mean ± standard deviation (S.D.)] for CFU/organ/animal were calculated for each experimental group.

2.10. Histological analysis

The lungs were obtained from the mice, fixed with 10% buffered formalin, and embedded in paraffin. Each block was cut into 4 μm-thick sections and stained using hematoxylin and eosin. Semi-quantitative morphometric analysis of pathological slides was performed by our modified method of Dascher et al. [39] using a micrometer-attached microscope (Microphot-FXA, Nikon, Japan) [39,40]. The longer axis and minor axis of each granuloma in the field (×4 magnification) were measured. Longer axis to minor axis of each granuloma were multiplied and added up. Three random fields from each tissue section of mice and six random fields of guinea pigs were evaluated, and the average score of the fields was designated as the granuloma index ($\times 10^{-2}$ mm^2). This method for the evaluation of granuloma area is significantly correlated with the granuloma area by other scanning method of hematoxylin and eosin section.

2.11. Tuberculosis-specific cytotoxic test using ^{51}Cr release

Eight weeks after the final vaccination, CTL activity of spleen cells and mesenteric lymph node cells from vaccinated mice was assessed by using the ^{51}Cr-release assay. P815 mastocytoma cells, which have the same major histocompatibility complex (MHC) (H-2^d) as BALB/c mice, were transfected with pcDNA-hsp65 and used as Hsp65 protein-expressing target cells. J774.1 macrophage cells were pulsed with *M. tuberculosis* (killed H37Ra) for 24 h and used as target cells. A total of 2×10^6 cells/ml effector splenic cells were treated with anti-CD8 antibody, anti-CD4 antibody or anti-Thy1.2 antibody followed by complement as described above. ^{51}Cr release was assessed using the ^{51}Cr-release assay [31,33] at the effector:target (E:T) ratio of 50:1. Spontaneous lysis (with medium alone) and maximum lysis (^{51}Cr release after three cycles of freeze-thaw) were set up for background and targets. Percent specific lysis was determined as:

$$\left[\frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})}\right] \times 100.$$

2.12. Proliferative responses of lymphocytes

Vaccinated mice were sacrificed immediately prior to challenge, and 1×10^5 single spleen cells were cultured in a 96-well flat bottom plate (Linbro) with rHsp65 protein (10 μg/ml) or PPD (20 μg/ml) for 60 h at 37 °C, and then pulsed with 1 μCi of [^{3}H]thymidine per well for the final 12 h of incubation [30]. Cells were harvested onto glass wool fiber filters, and [^{3}H]thymidine incorporation was measured using a Liquid Scintillation Counter LSC-6100 (ALOKA Co. Ltd., Tokyo, Japan).

2.13. Production of cytokines (IL-2 and IFN-γ)

Mouse cytokines were measured in quantitative ELISAs for IL-2 and IFN-γ as described previously [38]. Briefly, spleen cells from vaccinated mice were cultured at a concentration of 5×10^6 cells/ml in 200 μl of medium at various antigen concentrations. Culture supernatants were collected 48 h later and the levels of IFN-γ and IL-2 measured using sandwich ELISA kits (BD Opt EIA^{TM}), according to manufacturer's instructions.

2.14. ELISPOT assay

The spleens were removed aseptically from vaccinated mice three weeks after the third vaccination. Antigen-specific IFN-γ-producing cells were determined by ELISPOT as described previously [41]. Briefly, ELISPOT plates (MultiScreen IP Filtration plate MAIPS45; Millipore, Bedford, MA) were coated with anti-mouse IFN-γ MAb R4-6A2 (BD Biosciences Pharmingen). Spleen cells from vaccinated mice were suspended to 1×10^7 cells/ml (1×10^6 cells/well). In some experiments, the spleen cells from mice vaccinated with IgHsp65 + mIL-12/HVJ were pre-incubated with anti-CD8 antibody or anti-CD4 antibody (1:50 dilution) for 15 min at 4 °C and then incubated with rabbit complement (1:10 dilution) (Cedarlane, Hornby, Ont., Canada) for 45 min at 37 °C as described previously [30,33]. The cells were placed in five wells into antibody-coated wells, and rHsp65 protein (10 μg/ml) or PPD (10 μg/ml) was added to each well. After 20 h of incubation at 37 °C, cells were removed by washing the plates, and the site of cytokine secretions was detected using biotynylated anti-mouse IFN-γ MAb XMG1.2 (BD Biosciences Pharmingen) and streptavidin-alkaline phosphatase conjugate (BD Biosciences Pharmingen). The enzyme reaction was developed with BCIP-NBT substrate (Vector Laboratories, Inc., Burlingame, CA). Spot-forming cells (SFCs) were enumerated using KS ELISPOT system (Carl Zeiss, Hallbergmoos, Germany).

2.15. Statistical analysis

Tukey–Kramer's HSD tests were used to compare $\log_{10}$ value of CFU between groups following challenge and T cell responses between groups in ELISPOT assay. Student's *t* tests were performed to compare T cell responses between groups in T cell proliferation assay and granuloma formation between groups following challenge. A *P*-value of <0.05 was considered significant.

3. Results

3.1. In vitro expression of Hsp65 and IL-12

The DNA vaccines encoding mature and secreted forms of Hsp65 were constructed as Hsp65 DNA and IgHsp65 DNA, respectively. Hsp65 DNA contains the full-length *M. tuberculosis hsp65* gene. IgHsp65 DNA contains the full-length *M. tuberculosis hsp65* gene fused to the mouse Igκ signal sequence. Each construct is driven by CMV promoter and terminated at a bovine growth hormone polyadenylation sequence. Hsp65 DNA or IgHsp65 DNA was transfected into COS-7 cells and cell lysates and supernatants were analyzed for the *hsp65* gene expression. As shown in Fig. 1A, the mature form was detected as a single band in cell lysates (lane 2), whereas the secreted form was detected as a doublet band in cell lysates (lane 3). The doublet of slightly higher molecular weight than the mature form is most likely due to incomplete cleavage of the Igκ signal peptide in COS-7 cells because only a single band corresponding to the mature form was seen when HeLa cells or HepG2 cells were transfected with pcDNA-IgHsp65 (data not shown).

Based on the results of study reporting with high levels of IL-12 expression [42], we constructed a mouse IL-12 expression vector, mIL-12 DNA. The vector encodes mouse single-chain IL-12 protein comprised of p40 and p35 subunits linked by Gly_6Ser polypeptide linkers. As shown Fig. 1A, COS-7 cells transfected with mIL-12 DNA transiently expressed the mouse single-chain IL-12 protein with molecular weight of 80 kDa (lane 8). Quantitative analysis using ELISA showed that the COS-7 cells transfected with the mIL-12 DNA secreted four-fold higher levels of mIL-12p70 (125 ng/ml) than those transfected with from pcDNAmIL-12p40 + p35 (30 ng/ml), which previously constructed as a murine expression vector with IL-12 p40 and p35 expression cassettes in tandem array [35] (data not shown). Consistent with the mIL-12p70 expression level, the supernatant form the mIL-12 DNA transfectant cells induced 3.2-fold higher levels of IFN-γ from murine T lymphocytes than that from pcDNAmIL-12p40 + p35 transfectant cells (Fig. 1B). Thus, the mIL-12 DNA construct expresses biologically active IL-12, indicating that the single-chain IL-12 DNA is an effective DNA vaccine adjuvant capable of inducing primary Th-1 responses.

3.2. Evaluation of the best combination of Hsp65-based DNA vaccines with mIL-12 DNA for vaccine efficacy via gene gun

We compared the protective abilities of two versions of Hsp65-based DNA vaccine (Hsp65 DNA versus IgHsp65 DNA), and combinations with mIL-12 DNA (Hsp65 DNA versus Hsp65 DNA plus mIL-12 DNA, or IgHsp65 DNA versus IgHsp65 DNA plus mIL-12 DNA). Mice vaccinated with Hsp65 DNA, IgHsp65 DNA, and the combination with

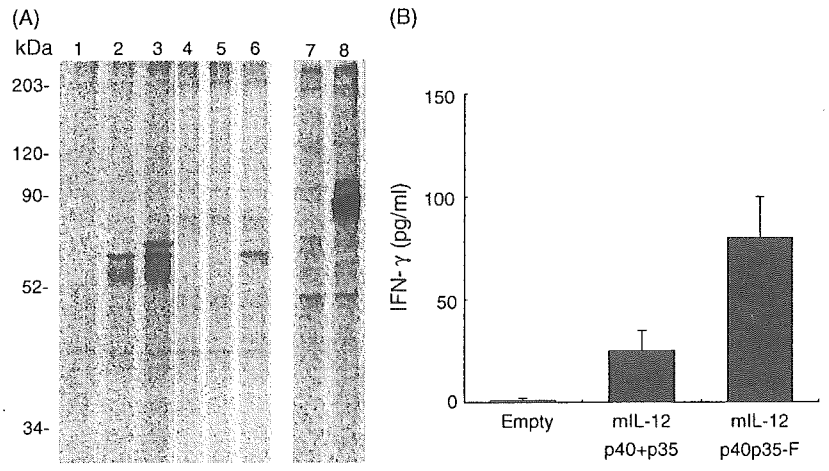

Fig. 1. Expression and biological analysis of Hsp65 and mIL-12. (A) In vitro expression analysis of Hsp65 and mIL-12 from cells transiently transfected with DNA vaccines. COS-7 cells were transfected with EGFP DNA (lanes 1, 4 and 7), Hsp65 DNA (lanes 2 and 5), IgHsp65 DNA (lanes 3 and 6), and mILp40p35-F DNA (lane 8). Following metabolically labeling with [^{35}S]methionine, cell lysates (lanes 1, 2, 3, 7 and 8) and supernatants (lanes 4–6) were immunoprecipitated with mouse anti-Hsp65 polyclonal antibody (lanes 1–6) or rabbit anti-murine IL-12p70 antibody (lanes 7 and 8). (B) The biological activities of IL-12 expressed in transfected cell supernatants in vitro. Culture supernatants from COS-7 cells transfected with pcDNA3.1 (no insert empty vector), pcDNA-mIL12p40 + p35 or pcDNA-mIL12p40p35-F were added to the mouse spleen cells (2×10^6 cells/ml) at the final concentration of 4% (v/v) and incubated for 60 h. The levels of mouse IFN-γ were measured using sandwich ELISA kits as described in Section 2.

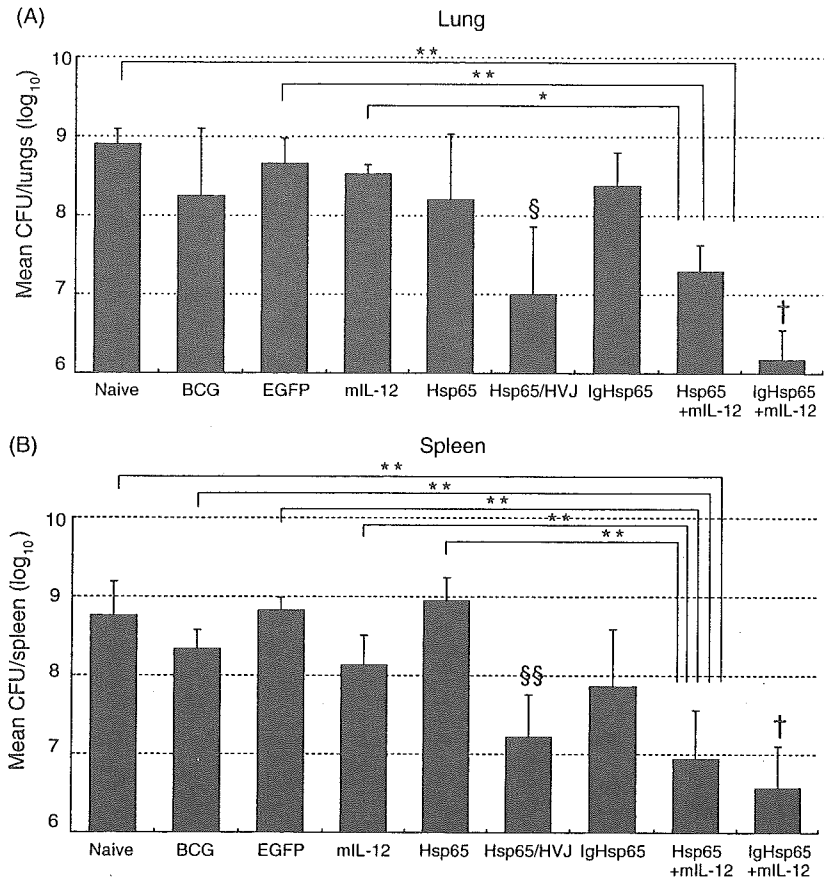

Fig. 2. The effect of a combination of mIL-12 expression vector and Hsp65-based DNA vaccines and comparison of different vaccines on the protective efficacy against challenge with *M. tuberculosis*. Groups of mice were vaccinated once with Hsp65 DNA, IgHsp65 DNA and a combination of mIL12 DNA via gene gun or three times with Hsp65/HVJ via intramuscular route and challenged intravenously with *M. tuberculosis* H37Rv as described in Section 2. Ten weeks after challenge, protection was measured by enumerating bacterial loads (CFU) in the lungs and spleen from vaccinated mice. Reduction of bacterial load was expressed as the mean $\log_{10}$ difference in CFU in the organs of the naive and vaccinated mice. The statistical significance of differences between individual groups in the number of CFU was determined by Tukey–Kramer's HSD test ($n = 4$–5). * and **, the statistical significance of differences ($P < 0.05$ and $P < 0.01$) compared to Hsp65 DNA + mIL-12 DNA group, respectively; †, the statistical significance of differences ($P < 0.01$) of IgHsp65 DNA + mIL-12 DNA group compared to the naive, BCG, EGFP DNA, mIL12 DNA, Hsp65 DNA and IgHsp65 DNA groups; §, the statistical significance of differences of Hsp65/HVJ group compared to BCG group ($P < 0.05$) in the lungs; §§, the statistical significance of differences of Hsp65/HVJ group compared to Hsp65 DNA ($P < 0.01$) and BCG ($P < 0.05$) groups in the spleen.

mIL-12 DNA via gene gun were challenged intravenously with *M. tuberculosis* H37Rv. The bacterial loads of the naive and vaccinated mice were compared 10 weeks after challenge (Fig. 2). Consistent with the previous report by Lima et al. [43], gene gun vaccination with Hsp65 DNA alone did not result in significant protective immunity as assessed by the bacterial load in the lungs or spleen. Vaccination with IgHsp65 DNA, which encodes the additional mouse Igκ signal sequence upstream of the *hsp65* gene, did not significantly improve the protective efficacy in the bacterial load in the lungs, although there was a modest decrease in the bacterial load in the spleen. In contrast, the combination with mIL-12 DNA markedly improved the protective efficacy both in the lungs and spleen ($P < 0.01$). In particular, vaccination of IgHsp65 DNA plus mIL-12 DNA conferred the greatest reduction of the bacterial load both in the lungs and spleen. Similar to IgHsp65 DNA plus mIL-12 DNA, the increased protection in the lungs and spleen was also observed in mice vaccinated with Hsp65 DNA plus mIL-12 DNA compared to IgHsp65 DNA alone and mIL-12 DNA alone. Thus, a strong synergistic effect on protection was achieved when Hsp65 DNA was co-administrated with IL-12 DNA. It is notable that the prophylactic effect of IgHsp65 DNA plus mIL-12 DNA in the lungs was more than 100-fold greater than that of BCG. These vaccinations of IgHsp65 DNA plus mIL-12 DNA and Hsp65 DNA plus mIL-12 DNA also exerted the significant reduction in the liver compared to the naive ($P < 0.05$) and control EGFP DNA groups ($P < 0.01$), whereas there was no significant difference of the naive group compared with Hsp65 or mIL-12 group (data not shown). In mice vaccinated with IgHsp65 DNA plus mIL-12 DNA, increased protection in the lungs were also observed at 5 weeks after challenge, which was equivalent to that obtained by vaccination with BCG (data not shown).

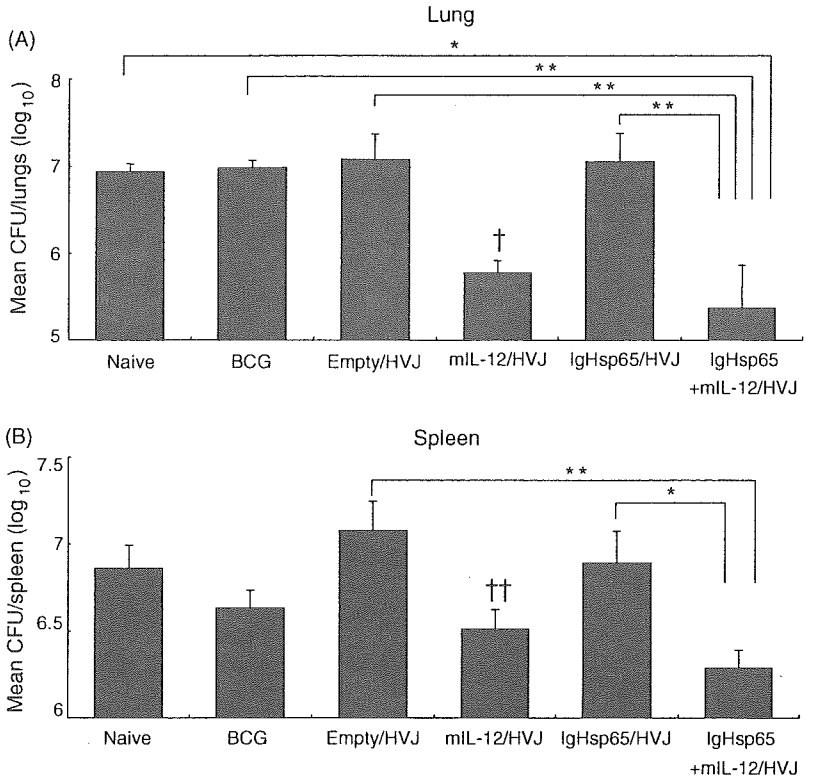

Fig. 3. Mouse protection studies using HVJ-liposome vaccines. Groups of mice vaccinated with HVJ-liposome DNA or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, protection was measured by enumerating the bacterial loads (CFU) in the lungs (A) and spleen (B) from vaccinated mice. Results are expressed as the mean $\log_{10} \pm$ S.D. of CFU. The statistical significance of differences between individual groups in the number of CFU was determined by Tukey–Kramer's HSD test ($n = 4$–5). $^{*}P < 0.05$; $^{**}P < 0.01$; †, the statistical significance of differences ($P < 0.05$) of mIL-12/HVJ group compared to BCG, Empty/HVJ, and Hsp65/HVJ groups in the lungs; ††, the statistical significance of differences ($P < 0.05$) of mIL-12 DNA group compared to Empty/HVJ group in the spleens.

3.3. Comparison of the protective efficacy of gene gun versus HVJ-liposome delivery of Hsp65 DNA vaccines

We next compared methods of DNA vaccine delivery on vaccine efficacy at 10 weeks after challenge. Hsp65/HVJ vaccination and challenge experiments were conducted simultaneously with gene gun experiments. As shown in Fig. 2, Hsp65/HVJ vaccination significantly reduced the bacterial loads as compared to Hsp65 gene gun immunization in the spleen ($P < 0.01$). IgHsp65 gene gun immunization significantly reduced the bacterial loads as compared to Hsp65 gene gun immunization in the spleen ($P < 0.05$, data not shown). Therefore, we used IgHsp65/HVJ for further experiments.

3.4. Protective efficacy of HVJ-liposome DNA vaccines

At 5 and 10 weeks after intravenous challenge of *M. tuberculosis* H37Rv, the number of CFU in the lungs, spleen, and liver were determined. Fig. 3 shows the results of bacterial loads 5 weeks after challenge. Vaccination with mIL-12/HVJ group resulted in significant protective immunity in the bacterial as compared to BCG, Empty/HVJ and Hsp65/HVJ groups in the lung ($P < 0.05$) and as compared to Empty/HVJ group in the spleen ($P < 0.05$). Vaccination with IgHsp65 + mIL-12/HVJ induced better protective immunity in the bacterial load both in the lungs and spleens than IgHsp65/HVJ alone and mIL-12/HVJ alone. Thus, the synergistic effect of IgHsp65 DNA and mIL-12 DNA resulted in improving the protective efficacy. At 10 weeks after challenge, the same reduction was also observed in these organs from mice vaccinated with IgHsp65 + mIL-12/HVJ (data not shown). Body weights of vaccinated mice were similar in all vaccinated groups. Tissue weight of lungs, liver, and spleen in the IgHsp65 + mIL-12/HVJ group were slightly lower than that from the naive mice (data not shown). In this experiment, BCG vaccination did not provide significant reduction of the bacterial load compared to the naive group. This may be due to the single-dose of vaccination used usually, the use of BCG Tokyo strain requires a three-dose vaccination to achieve 10 to 30-fold reduction of the bacterial loads compared to a non-vaccinated group. Although, 5 weeks after challenge, no reduction of bacterial loads was observed in IgHsp65/HVJ group compared with the naive control group, we confirmed the increased protection 10 weeks after challenge compared with the naive control group

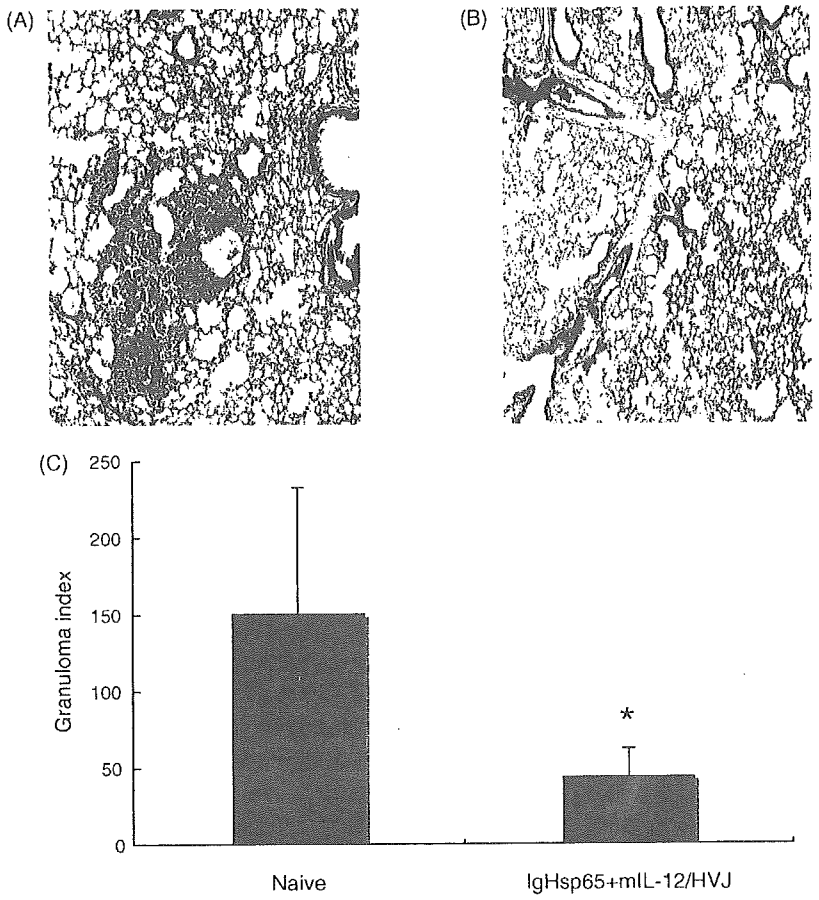

Fig. 4. Histopathological analysis of vaccinated mice 10 weeks after *M. tuberculosis* challenge. Representative photomicrographs of lung tissue sections harvested from the naive control group (A) and from the IgHsp65 + mIL-12/HVJ group (B) are shown (10 weeks after *M. tuberculosis* challenge, hematoxylin and eosin staining, ×10 objective). There were much infiltration of mononuclear cells and extensive parenchymal destruction by large, poorly demarcated granuloma in the lung from the naive control group. In the IgHsp65 + mIL-12/HVJ group, the lungs were less inflamed and only a few granuloma was observed. (C) Granuloma index of the naive control group and the IgHsp65+mIL-12/HVJ group in the lungs. Results are expressed as the mean ± S.D. of triplicates of five mice per group. The statistical significance of differences between the groups was determined by Student's *t*-test. $^{*}P < 0.05$ as compared with the naive control group.

at the same experiments (data not shown). These results indicate that co-vaccination with IL-12 DNA was effective for inducing protective immunity at as early as 5 weeks after challenge.

3.5. IgHsp65 + mIL-12/HVJ vaccination markedly reduced granuloma formation in the lung

In addition to the reduction of bacterial loads, the effects of vaccination on the mice were assessed by histological analysis. The granulomatous lesions in the lungs from IgHsp65 + mIL-12/HVJ mice were significantly less in number and size than from the naive control group (Fig. 4A and B). Quantitative evaluation of the granulomatous lesions clearly shows that IgHsp65 + mIL-12/HVJ vaccinated mice group exhibited significant reduction in granuloma index in the lungs, compared to the naive group ($P < 0.05$) (Fig. 4C). Thus IgHsp65 + mIL-12/HVJ vaccine provided significant protection against the pulmonary pathology caused by *M. tuberculosis* infection.

3.6. HVJ-liposome DNA vaccines generated T-helper response and cytokine production

To investigate lymphocyte proliferative and cytokine responses induced by HVJ-liposome DNA vaccines, spleen cells from vaccinated mice were re-stimulated with antigen in vitro. As shown in Fig. 5, substantial lymphocyte proliferation was observed in response to rHsp65 protein in spleen cells from mice vaccinated with IgHsp65/HVJ or IgHsp65 + mIL-12/HVJ but not with the naïve control. IgHsp65 + mIL-12/HVJ vaccination induced significantly better proliferative response to rHsp65 protein than did IgHsp65/HVJ vaccination ($P < 0.01$). In addition to lymphocyte proliferative responses, vaccination with IgHsp65 + mIL-12/HVJ induced elevated levels of IFN-γ and

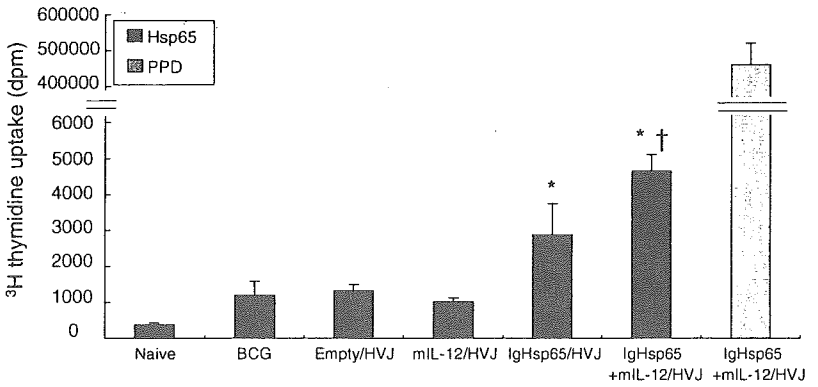

Fig. 5. The effect of vaccination with HVJ-liposome DNA on T cell proliferation. Proliferative responses of splenic lymphocytes from mice vaccinated with IgHsp65/HVJ, mIL-12/HVJ, IgHsp65 + mIL-12/HVJ, BCG, or Empty/HVJ. Incorporation of [^{3}H]thymidine in response to rHsp65 protein (black bars) or PPD (gray bar) was measured as described in Section 2. Results are expressed as the mean ± S.D. of triplicates of three mice per group. The statistical significance of differences between individual groups in T cell proliferation was determined by Tukey–Kramer's HSD test. The statistical significance of differences ($P < 0.01$) compared to the naive and BCG groups are indicated as (*) and (†), respectively.

IL-2 in response to rHsp65 protein, but not with the naive control or BCG group (Fig. 6). In response to PPD, vaccination with IgHsp65 + mIL-12/HVJ markedly increased both IFN-γ and IL-2 production as compared to the BCG group. Moderate but significant levels of IFN-γ and IL-2 were also induced in Hsp65/HVJ vaccination in response to Hsp65 protein and PPD. Thus, the synergistic effect of IgHsp65 DNA and mIL-12 DNA resulted in the strongest response not only to T cell proliferation but also to cytokine production.

3.7. HVJ-liposome DNA vaccines generated cytotoxic $CD8^+$ T cells

Because $CD8^+$ CTLs have been considered critical effectors of protective immunity to *M. tuberculosis*, it was of interest to determine whether a tuberculosis specific response could be induced in the vaccinated mice. We characterized $CD8^+$ T cells specific for Hsp65, PPD or killed *M. tuberculosis* by using a conventional ^{51}Cr release assay in the

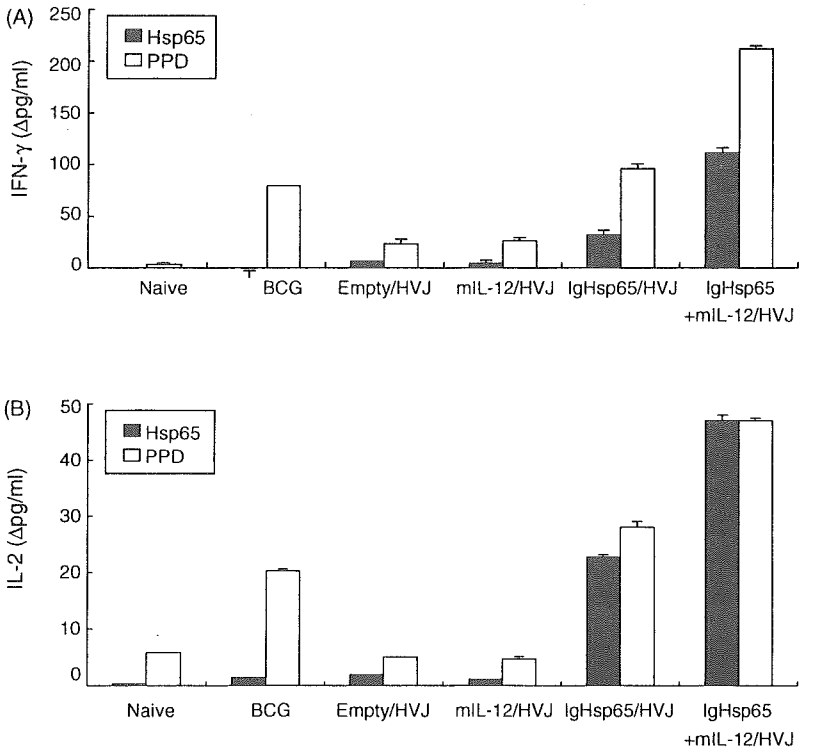

Fig. 6. IFN-γ (A) and IL-2 (B) production in spleen cell culture supernatants from vaccinated mice following stimulation with rHsp65 protein and PPD. Spleen cell cultures were stimulated with rHsp65 protein (black bars) or PPD (white bars) for 48 h, and the levels of IFN-γ and IL-2 production were determined by ELISA. Results are expressed as the mean ± S.D. of duplicates of three mice per group with antigens minus the mean ± S.D. of triplicates of three mice per group with medium alone.

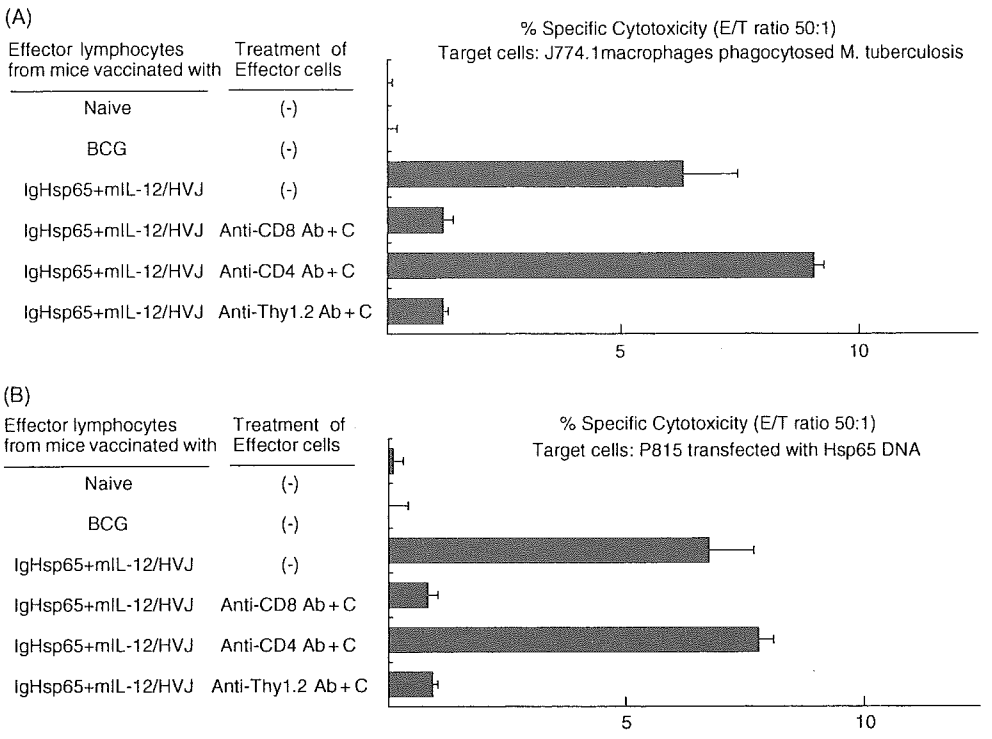

Fig. 7. Induction of $CD8^+$ CTL specific for Hsp65 protein and *M. tuberculosis* by vaccination with IgHsp65 + mIL-12/HVJ. Spleen cells from the naive, BCG-, and IgHsp65 + mIL-12/HVJ-vaccinated mice were obtained 8 weeks after the final vaccination. Cytotoxicity was assayed as release of radioactivity from ^{51}Cr-labeled J774.1 macrophages that had phagocytosed *M. tuberculosis* (killed H37Ra) (A) or from ^{51}Cr-labeled P815 target that had been transfected with Hsp65 DNA (B) using a conventional ^{51}Cr release assay at E:T ratio of 50:1. The effector cells were pre-incubated with anti-CD8, anti-CD4 or anti-Thy1.2 antibody, followed by treatment with complement. Percent specific lysis was determined as: [(experimental release–medium control release)/(maximum release–medium control release)] × 100. Ab: antibody; C: complement; (−), non-treatment.

absence of re-stimulation. As shown in Fig. 7, high levels of Hsp65- and *M. tuberculosis*-CTL specific lysis against J774.1 macrophages phagocytosed *M. tuberculosis* and P815 mastocytomas transfected with Hsp65 DNA were detected in mice vaccinated with IgHsp65 + mIL-12/HVJ, whereas little CTL response was detectable in either the naive or BCG-vaccinated mice. In vitro depletion of $CD8^+$ T cells eliminated the specific lysis. Depletion of $CD4^+$ T cells had no effect. Stronger (more than twenty percent) cytotoxicity against Hsp65 was detected in the spleen cells from mice 2 weeks after the last vaccination with IgHsp65 + mIL-12/HVJ (data not shown). These results indicate that IgHsp65 + mIL-

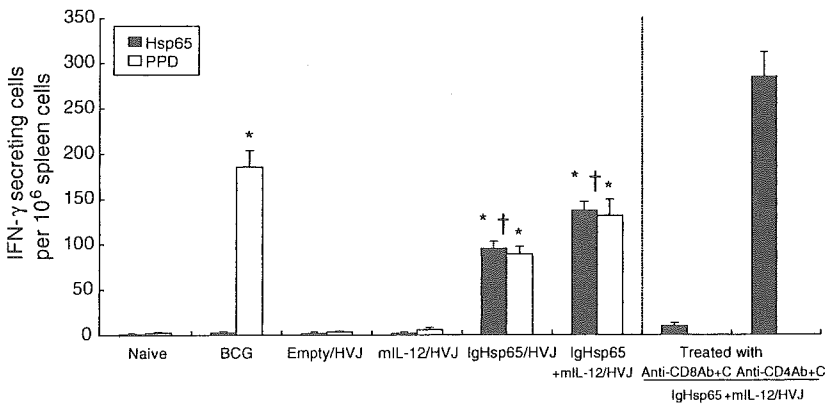

Fig. 8. ELISPOT assay for IFN-γ antigen-specific responses in the spleens of vaccinated mice following stimulation with rHsp65 protein and PPD. Spleen cell cultures were stimulated with rHsp65 protein or PPD for 20 h or pre-incubated with anti-CD8 antibody or anti-CD4 antibody followed by treatment with complement and then stimulated with rHsp65 protein for 20 h. The number of IFN-γ-secreting cells specific for rHsp65 protein (black bars) or PPD (white bars) per million cells were determined individually by ELISPOT assay. Results are expressed as the mean ± S.D. of five-wells of three mice per group. The statistical significance of differences between individual groups in the number of IFN-γ-secreting cells was determined by Tukey–Kramer's HSD test. The statistical significance of differences ($P < 0.01$) compared to the naive and BCG groups are indicated as (*) and (†), respectively.

12/HVJ vaccine induced long-term immune response with strong $CD8^+$ CTL activity.

3.8. ELISPOT assay

In order to determine whether enhanced protection was associated with increased IFN-γ production, the frequency of IFN-γ-secreting cells was enumerated by ELISPOT. Vaccination with IgHsp65/HVJ and IgHsp65 + mIL-12/HVJ resulted in a marked increase of IFN-γ secreting cells following stimulation with rHsp65 protein (Fig. 8). Moreover, the increase of IFN-γ secreting cells was also seen in IgHsp65/HVJ and IgHsp65 + mIL-12/HVJ groups following stimulation with PPD. These results indicate that vaccination with IgHsp65/HVJ and IgHsp65 + mIL-12/HVJ activated antigen-specific T cells producing IFN-γ. Depletion of $CD8^+$ cells from responder cells by treatment with anti-CD8 antibody and complement almost abrogated the IFN-γ producing cells. In contrast, an increase in the number of IFN-γ producing cells was observed in the responder cells when treated with anti-CD4 antibody and complement. BCG vaccination resulted in significant increase of IFN-γ secreting cells following stimulation with PPD but not rHsp65 protein. These data indicate that the protective efficacy of IgHsp65 + mIL-12/HVJ is strongly associated with the emergence of IFN-γ-secreting cells upon stimulation with Hsp65. Taken together, vaccination with IgHsp65 + mIL-12/HVJ capable of augmenting T cell activation and frequency of IFN-γ-secreting cells proves to reduce bacterial burden and pathology in the lungs—all to an extent greater than those achieved by vaccination with BCG.

4. Discussion

In the first stage of this study, we evaluated the protective efficacy of Hsp65 DNA vaccines via gene gun vaccination. One of the significant findings of the present study is that a single gene gun vaccination with the combination of IgHsp65 DNA and mIL-12 DNA led to a remarkably high degree of protection against intravenous challenge infection with virulent *M. tuberculosis*; bacterial numbers declined exponentially in internal organs and were 100-fold lower in the lungs than in BCG-vaccinated mice. Consistent with previous studies [43], gene gun vaccination with Hsp65 DNA alone did not promote reduction in bacterial burden compared to the naive mice. However, co-vaccination of Hsp65 DNA or IgHsp65 DNA plus mIL-12 DNA significantly improved the protective efficacy compared to either Hsp65 DNA alone or IgHsp65 DNA alone. Since the importance of IL-12 in the control of mycobacterial infections has been well documented, these results are consistent with other studies describing an adjuvant effect of IL-12 gene when administrated in combination with various tuberculosis DNA vaccines [20,24,25]. The mIL-12 DNA, which express both p40 and p35 chains as a single molecule, is able to induce four-fold higher levels of IFN-γ from mouse T lymphocytes than mIL12p40 + p35, which has previously been constructed as a murine expression vector with IL-12 p40 and p35 expression cassettes in tandem array [35]. Culture supernatants from the mIL-12 DNA-transfected COS-7 cells were effectively induced IFN-γ from mouse spleen cells. Thus, the improved expression levels of IL-12 DNA and the biologically active IL-12 explain the enhanced protection observed.

The second stage of this study demonstrated the protective efficacy of HVJ-liposome DNA vaccines in mouse and guinea pig models. We originally developed HVJ-liposomes, a viral/nonviral hybrid vector, as a gene transfer vector for cancer gene therapy. HVJ-liposome gene transfer method can deliver DNA directly and efficiently into host cells in vivo by means of the HVJ virus cell fusion machinery. We found that HVJ-liposome-mediated gene transfer was 30–100 times more efficient in gene expression in skeletal muscle than naked DNA transfer (unpublished data) and over three times more efficient in delivering intact oligodeoxyribonucleotide within the nuclei of transfected cells than Lipofectin®, a different cation liposome [44]. In addition to its high transfection efficiency, there are numerous safety advantages of HVJ-liposomes including: (i) no apparent toxicity or inflammation and (ii) repeated gene transfection without reduction of transfection efficiency. In fact, no significant adverse effects were induced in monkeys by intravenous injection of HVJ-liposomes [45]. Using this novel vector, we observed the enhancement of protection conferred by Hsp65 DNA compared to gene gun vaccination. This result is encouraging for the development of a novel tuberculosis DNA vaccine that is applicable both for prophylactic and therapeutic uses with no side-effects after repeated injections.

The most significant finding of this study is that vaccination with IgHsp65 + IL-12/HVJ provided greater protective efficacy than vaccination with BCG. In the mouse model, IgHsp65 + mIL-12/HVJ preferentially triggered a Th1 type T helper response, characterized by elevated levels of IFN-γ and IL-2, and augmentation of lymphocyte proliferation. After challenge, vaccination with IgHsp65 + mIL-12/HVJ resulted in a greater degree of protection than that evoked by BCG. This protective efficacy was associated with the emergence of IFN-γ-secreting T cells directed against Hsp65 and PPD. $CD8^+$ CTL activity against macrophage target cells, which had previously phagocytosed *M. tuberculosis* or expressed Hsp65 protein, was still observed in the spleen cells from mice vaccinated with IgHsp65 + mIL-12/HVJ at 8 weeks after the final vaccination, IgHsp65 + mIL-12/HVJ vaccine capable of augmenting long-term immune response with anti-tuberculosis CTL activity proves IgHsp65 + mIL12/HVJ to be a promising tuberculosis vaccine candidate.

Although the *hsp65* DNA vaccines have been shown to have significant promise as a new prophylactic vaccine against tuberculosis [19,21,46], negative outcomes have also been reported [47,48]. In the case of vaccination with *hsp65* DNA alone, our results are consistent with the previous report

that vaccination with *hsp65* DNA alone did not provide significant protective effect in the bacterial load in the lung either in the mouse model or in the guinea pig model [43,47]. However, as described above, the combination with mIL-12 DNA expressing biologically active IL-12 and the use of HVJ-liposome as a DNA vaccine delivery system remarkably improved the protective efficacy. In addition, our preliminary results of a guinea pig model in the collaborative study with Dr. D. McMurray (Texas A&M University) show that vaccination with IgHsp65 + guinea pig IL-12 (gpIL-12)/HVJ provided better protection against the pulmonary pathology caused by aerosol challenge with *M. tuberculosis* than did BCG vaccination (data not shown). For immunotherapeutic use, *hsp60*/lep DNA vaccine (*hsp65* DNA derived from *Mycobacterium leprae*) has been shown to be effective in a Cornel-type model [22], although others have argued that this vaccine induced progressively severe pulmonary necrosis in the model [48]. In support of the effectiveness, when administered to mice or SCID-PBL/hu mice [49] already infected with *M. tuberculosis*, neither IgHsp65 + mIL-12/HVJ vaccine nor IgHsp65 + human IL-12 (hIL-12)/HVJ vaccine, respectively, resulted in exacerbation of the granulomatous response in the lungs (unpublished data). Moreover, therapeutic administration of IgHsp65 + mIL-12/HVJ resulted in significant reduction of bacterial loads (paper in submission). The pathological parameter of protection included reductions in the mean lung granulomatous lesion score in our study. In parallel with the protective efficacy of HVJ-liposome vaccines on bacterial loads, histopathological analysis shows that mice vaccinated with IgHsp65 + mIL-12/HVJ had fewer and smaller lesions in the lung and significantly less lung granuloma than the naive mice. These results suggest that severe toxicities (Koch phenomenon) could not be induced by this vaccine. One possible explanation for these diverging results may be different *hsp65* DNA construct (secreted form versus cytoplasmic form; derived from *M. tuberculosis* versus *M. leprae*), different mIL-12 DNA construct (p40p35 fusion form versus p40-p35 tandem form), and different vaccine delivery (HVJ-liposome versus gene gun or naked DNA).

In conclusion, we demonstrate the development of a novel HVJ-liposome DNA vaccine encapsulating Hsp65 DNA plus IL-12 DNA. These results suggest that Hsp65 + IL-12/HVJ could be a promising candidate for a new tuberculosis DNA vaccine, which is superior to the currently available BCG vaccine. The goal of our study is to develop a new tuberculosis vaccine superior to BCG. To this aim, we believe that the protective efficacy and protective immune responses for vaccine candidates should be addressed in larger animals, such as non-human primates, before proceeding to human clinical trials. Although other DNA vaccine candidates that appear to protect against virulent *M. tuberculosis* in mice better than BCG have failed to provide better protection than BCG in guinea pigs against aerosol challenge of a low dose of virulent *M. tuberculosis* [47,50,51], some of them are being prepared to enter early human clinical trials [52]. More recently, we evaluated the IgHsp65 + hIL-12/HVJ vaccine in the cynomolgus monkey model [29], which is currently the best non-human primate animal model of human tuberculosis. Monkeys were subsequently challenged with virulent *M. tuberculosis* by the intra-tracheal route after the third vaccination. This challenge dose normally causes death from acute respiratory infection within 4–6 months. In this particular experiment, monkeys vaccinated with IgHsp65 + hIL-12/HVJ induced Hsp65-specific T cell proliferation and improvement of chest X-P findings, resulting in an increased survival for over a year, superior to BCG group [29]. Thus, we are taking advantage of the availability of multiple animal models (mouse, guinea pig, and monkey) to accumulate essential data of the HVJ-liposome DNA vaccine, including the vaccine efficacy and safety, for up-coming Phase I clinical trials.

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