別紙4

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
岡田全司	Synthesis and SAR of Caprazamycin derivatives CPZEN-45: as a promising drug candidate for treating XDR-TB.	ACS Medical Chemistry Letters.			in press
岡田全司	Novel vaccine against tuberculosis using prime-boost method.	Clin. Develop Immunol.			in press
岡田全司	Novel therapeutic vaccine: granulysin vaccine against tuberculosis.	Human Vaccine.			in press
岡田全司	Development of therapeutic and prophylactic vaccine against tuberculosis using monkey and granulysin transgenic mice models.	Human Vaccine.			in press
岡田全司	Trends in tuberculosis infection among foreigners in Japan according to work status.	Kekkaku			2010
岡田全司	Comparison of rifabutin susceptibility and rpoB mutations in multi-drug-resistant Mycobacterium tuberculosis strains by DNA sequencing and the line probe assay.	J Infect Chemother.	16(5)	360-363	2010
岡田全司	Detection of molecular epidemiology of Mycobacterium gordonae isolates.	Kekkaku	85(7)	609-614	2010
岡田全司	A Novel Therapeutic and Prophylactic Vaccine (HVJ-Envelope/Hsp65 DNA+ IL-12 DNA) against Tuberculosis Using The Cynomolgus Monkey Models.	Procedia in Vaccinology.	2(1)	34-39	2010
岡田全司	Tuberculosis vaccine development: The development of novel (preclinical) DNA vaccine.	Human Vaccine	6(4)	297-308	2010
岡田全司	Anti-tuberculosis immunity by cytotoxic T cells granulysin and the development of novel vaccines (HSP-65 DNA+ IL-12 DNA).	Kekkaku	85(6)	531-538	2010
岡田全司	Immunity against Mycobacterium tuberculosis (introduction).	Kekkaku	85(6)	501-508	2010

加藤誠也	Phylogeographical particularity of the Mycobacterium tuberculosis Beijing family in South Korea based on international comparison with surrounding countries.	J Med Microbiol	59	1191-1197	2010
豊田恵美子	外国人結核対策への取り組み -結核低蔓延国における外国人に対する健診実施状況-	結核			投稿中
野内英樹	Decreased granulysin and increased IFN-levels in plasma of patients with newly diagnosed and relapse tuberculosis.	submitted			
服部俊夫	Transactivation of human osteopontin promoter by human T cell leukemia virus type 1-encoded Tax protein.	Leuk Res	34	763-768	2010
服部俊夫	A sensitive HIV-1 envelope induced fusion assay identifies fusion enhancement of thrombin.	Biochem Biophys Res Commun.	39	1780-1784	2010
服部俊夫	The increase of plasma galectin-9 in a patient with insulin allergy: a case report.	Clin Mol Allergy	8	12	2010
服部俊夫	High numbers of Interferon-γ-Producing T Cells and Low Titers of Anti-Tuberculous Glycolipid Antibody in Individuals with Latent Tuberculosis.	Tohoku J Exp Med	220	21-25	2010
服部俊夫	Rev-derived peptides inhibit HIV-1 replication by antagonism of Rev and a co-receptor, CXCR4.	Int J Biochem Cell Biol.	42(9)	1482-1488	2010
服部俊夫	Procyanidin B1 purified from Cinnamon cortex suppresses hepatitis C virus replication.	Antivir Chem Chemother.	20(6)	239-248	2010
服部俊夫	Genotypes and characteristics of Clustering and drug-susceptibility of Mycobacterium tuberculosis isolates in Heilongjiang Province, China.	J Clin Microbiol	49(4)	1354-1362	in press
服部俊夫	Antibody to tubercular glycolipid antigen; TBGL-IgG and TBGL-IgA responses in pulmonary tuberculosis patients and healthy individuals from Thailand.	a TB-endemic country			投稿中
服部俊夫	Identification of CD44 as a downstream target of noncanonical NF-kB pathway activated by Human T-cell leukemia virus type 1-encoded.	Tax protein			投稿中
服部俊夫	Evaluation of the antibacterial and anticancer activities of some South African medicinal Plants.	BMC Complementray and Alternative Medicine	11	14	2011

高鳥毛敏雄	Tuberculosis infection among homeless persons and caregivers in a high-tuberculosis-prevalence area in Japan: a cross-sectional study.	BMC Infectious Diseases			2011
高鳥毛敏雄	米国、イギリス、ドイツにおける結核医療の提供体制	結核	85(2)	98-101	2010
慶長直人	Identification of tuberculosis-associated proteins in whole blood supernatant.	BMC Infect Dis.	11(1)	71	2011
慶長直人	Genetic predisposition to diffuse panbronchiolitis.	Respirology.			2011 in press
慶長直人	Molecular cloning of two novel mucin-like genes in the disease-susceptibility locus for diffuse panbronchiolitis.	Hum Genet.	129(2)	117-128	2011
慶長直人	[Biomarkers to assess different aspects of tuberculosis--from development to relapse].	Kekkaku.	85(11)	823-828	2010
慶長直人	Association analysis of susceptibility Candidate region on chromosome 5q31 for tuberculosis.	Genes Immun.	11(5)	416-422	2010
竹田　潔	Prophylactic and therapeutic implications of Toll-like receptor ligands.	Med. Res. Rev.			in press
竹田　潔	Commensal microbiota induce LPS hyporesponsiveness in colonic macrophages via the production of IL-10.	Int. Immunol.	22	953-962	2010
竹田　潔	Activation of myeloid dendritic cells by deoxynucleic acids from Cordyceps sinensis via a Toll-like receptor 9-dependent pathway.	Cell Immunol.	263	241-250	2010
竹田　潔	Current views of Toll-like receptor signaling pathways.	Gastroenterol Res Pract.			2010
竹田　潔	A novel inducible dendritic cell ablation model in mice.	Biochem. Biophys. Res. Commun.	397	559-563	2010
竹田　潔	The innate immune response to Trypanosoma cruzi infection.	Microbes Infect.	12	511-517	2010
竹田　潔	Therapeutic activation of STAT3 by Interleukin-11 ameliorates cardiac fibrosis after myocardial infarction.	Circulation	121	684-691	2010
中島俊洋	Novel Prophylactic Vaccine Using a Prime-Boost Method and Hemagglutinating Virus of Japan-Envelope Against Tuberculosis.	Clin Dev Immunol.			2011

中島俊洋	Development of therapeutic and prophylactic vaccine against Tuberculosis using monkey and transgenic mice models.	Hum Vaccin.	7	108-114	2011
露口一成	Comparison of rifabutin susceptibility and rpoB mutations in multi-drug-resistant Mycobacterium tuberculosis strains by DNA sequencing and the line probe assay.	J Infect Chemother.	16(5)	360-363	2010
露口一成	第84回総会シンポジウム　V.日本における多剤耐性結核　1.多剤耐性結核の疫学、診断	結核	85(2)	126-128	2010
露口一成	当センターにおける *Mycobacterium gordonae* の分子疫学的解析	結核	85(7)	609-614	2010

添付資料

就業状況別の在留外国人結核の推移とその背景

[1]星野 斉之 [1]大森 正子 [2]岡田 全司

要旨：〔目的〕先進国では外国人結核が課題である。日本の現状を検討することを目的とした。〔方法〕在留外国人の就業状況別患者数と罹患率の推移を解析した。〔結果〕1998年以降の労働者と学生の患者数は増加傾向を示し，要因として母数としての労働者と学生の増加が示唆された。家事従事者の患者数に一定の傾向はなく，永住者数は増加傾向だったが，配偶者等の数は横ばいであり，永住者数の影響は小さいと考えられた。罹患率の推移は，労働者は不変で，学生と家事従事者は低下傾向にあり，罹患率の変化が患者数増加の要因ではなかった。なお，罹患率低下の要因として，長期在留者の増加や出身国の罹患率の低下が示唆された。〔考察〕在留外国人の罹患率は低下傾向にあるが，同じ就業状況の日本人の罹患率の数倍を示しており，患者の早期発見は重要な課題である。具体的には，学生，労働者（特に臨時・日雇い），家事従事者に対する定期健診の普及や有症状時における医療機関受診の勧奨が挙げられる。また，出身国の結核蔓延状況の改善が，在留外国人の罹患率に影響する可能性があるので，周辺国への対策支援による日本国内の外国人の結核対策への寄与が期待される。

キーワーズ：結核，外国人，就業状態，罹患率

はじめに

外国人（外国出生または外国国籍）の登録結核患者数は，米国や西欧の一部（英国，スウェーデン，オランダ，デンマーク，ベルギー，スイス，ノルウェー等）では年間登録患者の半数以上を占めており，高い罹患率，低い治療成功率や高い薬剤耐性率などの課題が指摘されている[1~3]。日本でも同様に，高い罹患率，受診の遅れ，低い治療成功率などが結核対策上の課題として報告されてきた[4~6]。日本では，結核登録者情報調査（2006年までは結核発生動向調査）に1998年から国籍に関する入力項目が入り，外国国籍の者（以下，在留外国人）の結核登録者の状況が把握できるようになり，2007年からは出身国別の分析も可能になった。在留外国人の登録結核患者数は，730人（1998年）から次第に増加を続け，945人（2008年）に達している。また，治療成績では，日本国籍の者に比して死亡率は低いが，脱落率が高い傾向などが指摘されている[7]。本報告では，1998年以降の在留外国人結核患者数の推移を就業状況別に検討し，その背景を解析した。

方　法

就業状況（労働者，学生，家事従事者等）別の在留外国人結核患者数と，該当する就業状況にある在留外国人の人数を各種の統計から入手して推移の検討を行った。就業状況別の在留外国人結核患者数は，結核登録者調査年報から入手した。外国人労働者数の推計〔就労目的外国人（専門的・技術的分野），技能実習生，留学生のアルバイト，日系人労働者，不法就労者等の和〕，は外国人労働者数の推移（http://www2.ttcn.ne.jp/honkawa/3820.html）から得た。

学生（留学生と就学生），配偶者，定住者，永住者等の人数は，在留外国人統計（http://www.immi-moj.go.jp/toukei/index.html）から得た。また，近年における在留外国人結核患者の就業状況別の状況を検討するために，結核登録者調査年報から，2007年から2008年の外国籍

[1]結核予防会結核研究所，[2]国立病院機構近畿中央胸部疾患センター臨床研究センター

連絡先：星野斉之，結核研究所，〒204-8533 東京都清瀬市松山3-1-24（E-mail：hhoshino@jata.or.jp）

(Received 18 Dec. 2009/Accepted 15 Jun. 2010)

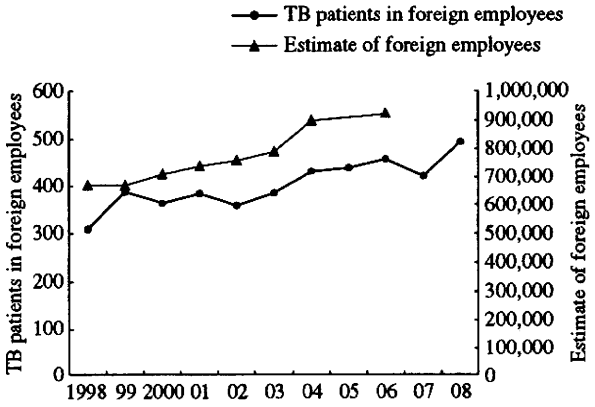

Fig. 1-1 Number of TB patients among foreign employees and estimate of foreign employees

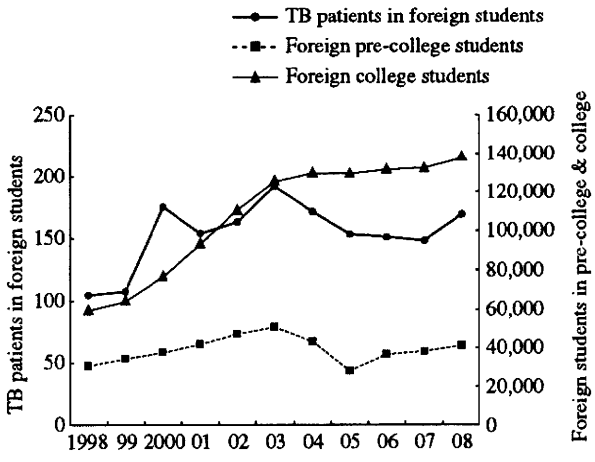

Fig. 1-2 Number of TB patients in foreign students, foreign pre-college students, and foreign college students

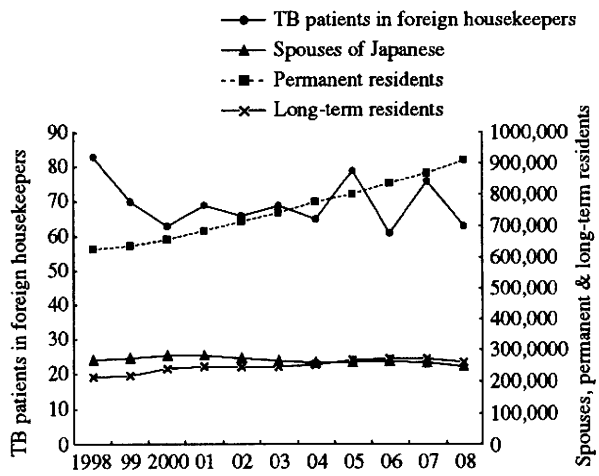

Fig. 1-3 Number of TB patients in foreign housekeepers, spouses of Japanese, permanent and long-term residents

結核患者の情報を用いて，就業状況別に，性比，在留年数，発見方法，国籍を検討した。また，上記の就業状況別の結核患者数と推計した在留外国人数を用いて罹患率を計算し，その推移を分析した。なお，家事従事者の母数の推計については，外国人の労働力率が66.8%（http://www.stat.go.jp/data/kokusei/2005/gaikoku/index.htm）なので，在留外国人統計における配偶者等と定住者と永住者の和の33.2%を分母に用いた。

結　果

Fig. 1に就業状況別（労働者，学生，家事従事者）の在留外国人結核患者数の推移と，それぞれの母数と考えられる就業状態の外国人数（推計外国人労働者，就学生および留学生，配偶者，定住者，永住者）の推移を示す。労働者では推計労働者数の増加に伴って結核患者数も増加を示した（Fig. 1-1）。学生については，就学生（主に日本語学校生）の増減と結核患者数の増減は対応していたが，留学生とは対応していなかった（Fig. 1-2）。家事従事者では結核患者数は一定の傾向はなく，増加を続ける永住者とは対応しなかった（Fig. 1-3）。

Tableに2007～2008年の2年間に登録された在留外国人結核患者について就業状況別に性比，在留年数，発見方法，国籍の分布を示す。就業状況別の在留期間を見ると，常勤労働者と臨時日雇いは在留5年以内の者が多く，学生は在留5年以内が大半を占め，家事従事者は在留5年以上が多く，無職・その他も在留5年以上が多い。発見方法では，医療機関受診が全体の71.3%を占めて最も多い。就業状況別では，学生では学校健診が最も多く次いで医療機関受診であり，労働者では医療機関が最も多く次に職場健診となっている。家事従事者や無職・その他は医療機関受診が大半を占めている。外国人結核患者における就業状況別の発見方法の推移を見るために，1998年，2003年，2007～08年間で比較すると，学生では，学校健診発見が常に最も多く（43.8%，43.8%，50.9%），次いで医療機関受診が多い（32.4%，35.4%，36.2%）。常勤労働者では医療機関受診（73.3%，70.3%，72.5%），ついで職場健診（16.8%，20.9%，20.5%）であった。臨時・日雇いでは医療機関受診（78.5%，85.8%，76.5%），ついで職場健診（9.3%，6.6%，16.2%）であり，常勤労働者より医療機関受診が多かった。家事従事者では医療機関受診が大半を占めており（84.2%, 86.4%, 95.7%），住民健診発見例は毎年数例にとどまった。各就業状況について調査期間中に発見方法の分布に大きな変動はなかった。

2007～2008年登録者に関する国籍別分布では，常勤労働者では，中国（26.8%），フィリピン（21.3%），インドネシア（8.7%），ブラジル（7.4%）の順であり，臨時・

日雇いではフィリピン（25.8%），中国（25.4%），ブラジル（10.8%），インドネシア（7.7%）と若干順位が変わった。学生では中国（50%），次いで韓国（16.3%），家事従事者では，フィリピン（55.4%），次いで中国（15.8%）である。無職・その他でもフィリピンが最も多く（32.2%），次いで中国（17.3%），韓国（16.7%）が続いた。

また，就業状況別の全結核罹患率の推移を検討した（Fig. 2）。推計罹患率（人口10万対）の推移では，労働者（分母は推計外国人労働者数）については46.3（1998年）から49.5（2006年）と不変であったが，学生（分母は在留外国人統計の就学生と留学生の和）では116.2（1998年）から94.0（2008年）と低下していた。また，家事従事者（分母は在留外国人統計における配偶者等と定住者と永住者の和の33.2%）の推計罹患率は22.7（1998年）から13.4（2008年）と低下していた。なお，喀痰塗抹陽性肺結核罹患率では，労働者は19.7（1998）から19.0（2006）であり，学生は16.6（1998）から13.9（2008）であり，家事従事者は8.5（1998）から3.6（2008）であり，学生と家事従事者の低下傾向と労働者の停滞傾向は同様に見られた。

考　察

就業状況別の患者数や罹患率について，在留外国人人口の増減，患者発見方策の変化，出身国の罹患率の変化，在留期間の分布などの影響を検討し，今後の方策について考察した。

在留外国人数

在留外国人の増加に伴って外国人結核患者数が増加する現象は，米国や英国でも見られている[2)3)8)]。日本の在留外国人登録者数[9)]は，1998年末の151.2万人から2008年末では221.7万人に増加した。就業状況別に見てもFig. 1-1から1-3で示したように，労働者，留学生，就学生，永住者が増加した。また，国勢調査（http://www.stat.go.jp/data/kokusei/2005/index.htm）における外国人数でも114.0万人（1995年）から155.6万人（2005年）と増加しており，就業状況別でも，労働力人口は19.2万人，通学者は1.2万人，家事従事者は4.4万人増加した。1998年以降について就業状況別の在留外国人結核患者数と在留外国人数の関係について見ると，労働者については，在留外国人数の増加と結核患者数の増加が対応している。また，学生については，就学生数に結核患者数の推移が対応している。以上より，1998から2008年における労働者と学生における結核患者数の増加の要因に，在留外国人数の増加があると考えられる。なお，家事従事者については，結核患者数に一定の傾向はなく，外国人配偶者と定住者数は不変だった。よって，母集団の一部である永住者の増加の結核患者数への影響は小さいと思わ

Table　Characteristics of tuberculosis patients with alien citizenship

	Total	Sex ratio M : F	Residence ≦5 years	Residence >5 years	Mode of case-detection: Out-patient department	School health exam.	Company health exam.	Contact survey	Others/ unknown	Citizenship of country: China	Philippines	Korea	Indonesia	Brasil	Others
Regular employee	619	327 : 292	360	259	449	1	127	24	18	166	132	41	54	46	180
(%)	100	52.8 : 47.2	58.2	41.8	72.5	0.2	20.5	3.9	2.9	26.8	21.3	6.6	8.7	7.4	29.1
Temporary/daily employee	260	118 : 142	157	103	199	1	42	2	16	66	67	15	20	28	64
(%)	100	45.4 : 54.6	60.4	39.6	76.5	0.4	16.2	0.8	6.2	25.4	25.8	5.8	7.7	10.8	24.6
Self-employed	38	21 : 17	16	22	27	0	2	5	4	4	10	9	0	1	14
(%)	100	55.3 : 44.7	42.1	57.9	71.1	0	5.3	13.2	10.5	10.5	26.3	23.7	0	2.6	36.8
Attending schools	326	165 : 161	261	65	118	166	2	14	26	163	7	53	6	2	95
(%)	100	50.6 : 49.4	80.1	19.9	36.2	50.9	0.6	4.3	8.0	50.0	2.1	16.3	1.8	0.6	29.1
Housekeepers	139	3 : 136	44	95	133	0	1	2	3	22	77	12	5	3	20
(%)	100	2.2 : 97.8	31.7	68.3	95.7	0	0.7	1.4	2.2	15.8	55.4	8.6	3.6	2.2	14.4
Children < 6 year-old	6	3 : 3	5	1	4	0	0	2	0	0	3	0	1	0	2
(%)	100	50 : 50	83.3	16.7	66.7	0	0	33.3	0	0	50.0	0	16.7	0	33.3
Jobless & others	323	108 : 215	134	189	283	1	7	10	22	56	104	54	10	10	89
(%)	100	33.4 : 66.6	41.5	58.5	87.6	0.3	2.2	3.1	6.8	17.3	32.2	16.7	3.1	3.1	27.6
Unknown	76	35 : 41	28	48	61	0	2	2	11	10	29	5	1	2	29
(%)	100	46.1 : 53.9	36.8	63.2	80.3	0	2.6	2.6	14.5	13.2	38.2	6.6	1.3	2.6	38.2
Total	1787	780 : 1007	1005	782	1274	169	183	61	100	487	429	189	97	92	493
(%)	100	43.6 : 56.4	56.2	43.8	71.3	9.5	10.2	3.4	5.6	27.3	24.0	10.6	5.4	5.1	27.6

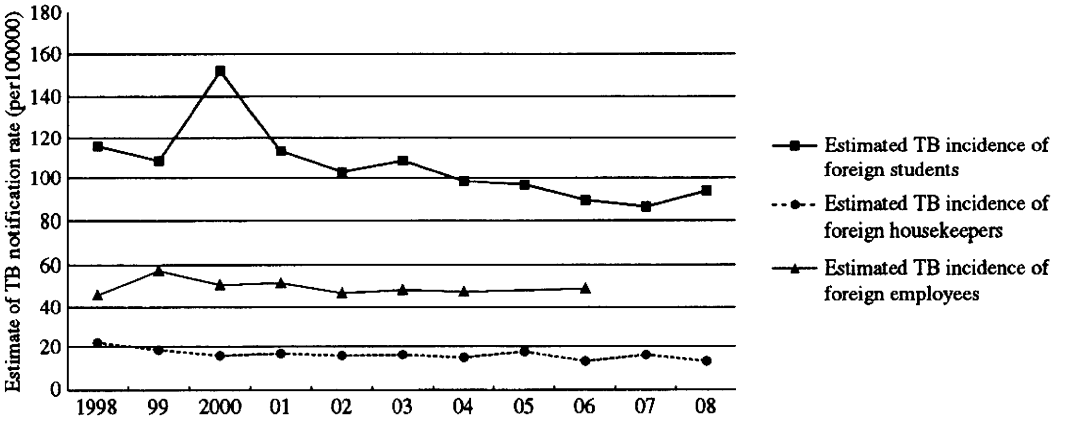

Fig. 2 Trend of estimated TB notification rate of foreigners by labor status

れる。今後，就業状況別の在留外国人人口の動向に留意しながら，各就業状況別の外国人結核対策の整備や強化を行うことが重要と考えられる。

患者発見活動の動向

学生では入学時に健康診断が行われており，労働者では常勤労働者について就職年度とその後は毎年度に健康診断が行われている。家事従事者が対象に含まれる住民対象の結核健診は，2004年までは15歳以上，2005年からは65歳以上を対象として，胸部X線検査が行われてきた。また，医療機関における結核発見は，調査期間中に大きな変化はなく行われてきた。結果に示した発見方法の分布の推移から，常勤労働者と学生については患者発見状況に大きな変化はなく，結核患者数の増加の要因ではないと考えられた。家事従事者について，住民健診による患者発見数が低い理由としては，該当する年齢層の低健診受診率（女性の25～34歳では45.8%）（平成16年国民生活基礎調査 http://www.mhlw.go.jp/toukei/saikin/hw/k-tyosa/k-tyosa04/3-7.html）があり，健診対象年齢の変更は，外国人家事従事者の発見状況には影響しなかったと思われる。以上より，調査期間中における患者発見状況に大きな変動はなく，発見患者数や罹患率の変化の要因ではないと考えられる。なお，全結核罹患率の就業状況間の違いは，健診発見割合の違い（高いほうから学生＞労働者＞家事従事者の順番）が影響している可能性がある。また，塗抹陽性肺結核罹患率で見ると，学生と労働者の差が縮まることも，健診発見の影響を示唆する知見であると考えられる。以上より学生の高い罹患率の背景には健康診断による積極的な患者発見の影響があり，家事従事者の低い罹患率には健康診断を受診する機会の少なさが影響している可能性が示唆された。

出身国の罹患率の推移

主要な出身国の罹患率について，母国の推計罹患率（人口10万対）の推移（1990年と2007年）を世界保健機関の年次報告[10]から検討した。中国（116から98へ低下），フィリピン（393から290へ低下），ブラジル（84から48へ低下），韓国（165から90へ低下），インドネシア（343から228へ低下）であり，日本への入国者数が多い国はすべて推計罹患率が低下傾向にある。これらの国の結核蔓延状況の改善が，在留外国人における結核罹患率低下の要因の一つである可能性が示され，日本の周辺国への対策支援が，間接的に日本国内の結核状況の改善に貢献する可能性が示唆された。米国については，出身国の結核対策への支援が，本国の結核蔓延状況の改善に寄与するというモデルも報告されている[11]。罹患率は低下傾向にあるが，日本人の同じ就業状況の罹患率の数倍を示しており[12]，それぞれの就業状況における患者発見の推進の努力は，今後も重要な方策である。具体的には，学生や労働者（特に臨時・日雇い）への定期健康診断の普及や，家事従事者が有症状時に早期医療機関受診できる体制整備，そして長期滞在予定者に対するQFT検査による潜在結核感染者の積極的な発見・治療の有効性の検討が考えられる。なお，家事従事者や労働者の罹患率が出身国（中国，フィリピン，ブラジル）の罹患率よりも低いが，その要因の1つとして，来日前の健康診断等による入国者の選別の影響が考えられる。しかし，その実態（健診の実施状況やその質）把握はできなかった。

出身国別の患者分布

1998～2008年における出身国の分布は2007年以降しか得られないので，1993年の在留外国人結核患者の調査結果[13]と比較すると，出身国の罹患率が比較的高い中国，フィリピンの結核患者数および割合が増加し，罹患率が比較的低い韓国やブラジルの人数は微増にとどまった。また，国勢調査を用いて就業状況別出身国別人数の変化（1995年と2005年）を見ると，労働者では中国の増加，ブラジル，フィリピンの漸増，韓国・朝鮮の漸減が，通学者では中国の増加と韓国・朝鮮の低下が，家事従事

者では中国とフィリピンの増加と韓国・朝鮮の低下が見られた。以上より，どの就業状況でも罹患率が比較的高い国が増加し，罹患率が比較的低い国は減少しているので，学生と家事従事者の罹患率の低下に出身国の分布の変化が影響している可能性は低い。なお，労働者における罹患率の停滞は，出身国罹患率の低下と患者分布の変動（中国出身者の増加と韓国・朝鮮出身者の減少）が相殺している可能性はある。

在留期間の影響

在留期間の差異による影響については，米国の研究では，入国後の期間が長くなるほど罹患率が低下することが観察されており，その要因として入国前の感染と発症が指摘されている[14][15]。現在日本に在住する外国人の在留期間を把握することは難しいが，永住許可に関する実務的な条件に，10年以上日本に継続して滞在することが含まれるので，永住者（特別永住者を除く）の推移から推察が可能である。在留外国人統計を用いて1998年末と2008年末を比較すると，永住者の人数，割合とも増加している。また，国別に在留外国人中の永住者（特別永住者を除く）割合を見ると，中国（11.6%から21.7%），韓国·朝鮮（11.6%から21.7%），フィリピン（11.6%から21.7%）と3国とも増加している。また，家事従事者の母数となる配偶者と定住者と永住者（特別永住を含む）の総計における永住者割合でも，56.8%（1998年末）から64.4%（2008年末）に増加していた。また，入国時年齢の分布がほぼ不変であることから，1998年末と2008年末で在留外国人の年齢分布を，在留外国人統計を用いて比較すると，全体では40歳以上が31.5%から34.2%に増加しており，在留外国人の多い3国については，中国は22.2%から20.4%に漸減しているが，韓国は47.1%から56.0%に増加し，フィリピンは8.9%から30.2%と大幅に増加していた。また，国勢調査の外国人統計（15歳以上対象）で1995年，2000年，2005年を比較すると，外国人家事従事者では，40歳以上の者の割合が37.3%，41.9%，45.9%と増加していたが，学生と労働者ではそのような傾向は見られなかった。なお，学生については，日本語学校生は1.5万人（1998年）から3.5万人（2008年）に2.0万人増加したが，留学生数（その60%が日本語学校を修了して進学する者）数は，5.1万人（1998年）から12.4万人（2008年）に7.3万人増加しており（留学生の増加数および伸び率http://www.jasso.go.jp/statistics/intl_student/ref07_01.html），外国人学生の滞在期間は延びている可能性がある。また，結果で見たように，5年以上滞在している結核患者の割合でみると，家事従事者では68.3%を占めており，学生（同19.9%）や常勤労働者（同41.8%）に比して高かった。以上より，外国人家事従事者については，長期に在留する外国人割合の増加と，長期在留による結核罹患率の低下が，罹患率低下の要因になっていると思われる。また，星野らの報告でも，在留外国人の家事従事者の結核罹患率は年齢が上がるほど低下しており，これらの考察の傍証となると思われる[13]。なお，長期在留者の増加の影響は，外国人学生では可能性が示されるにとどまり，労働者では否定的であった。

ま と め

1. 在留外国人の結核患者数は増加傾向にあり，在留外国人数（主に労働者と学生）の増加が主な要因と考えられた。今後，就業状況別の在留外国人人口の動向に対応して，外国人結核対策を強化することが望まれる。
2. 就業状況別の罹患率では，学生，労働者，家事従事者の順であり，健康診断の受診状況，在留期間の違いが要因として示唆された。
3. 学生と家事従事者の罹患率を1998年と2008年で比較すると，低下しており，母国の罹患率の低下傾向，在留期間の長い者の増加が要因として考えられた。
4. 罹患率は低下傾向にあると言っても，日本人の同じ就業状況の罹患率の数倍を示しており，それぞれの就業状況における患者発見の推進の努力は，今後も重要な方策となると思われる。特に，罹患率の高い外国人学生や労働者（特に臨時・日雇い）に対する定期の健康診断の実施や外国人家事従事者が有症状時に早期に医療機関を受診できる体制作り，そして長期滞在予定者に対するQFT検査による潜在結核感染者の積極的な発見・治療の有効性の検討等が望まれる。
5. 出身国の結核蔓延状況の改善が，入国者の結核罹患率の低下に寄与している可能性がある。周辺国（中国，韓国，フィリピン等）への結核対策の技術的支援が，日本国内の在留外国人の結核対策に寄与する可能性がある。

謝　辞

本調査は，平成21年度厚生労働省厚生労働科学研究費補助金（新型インフルエンザ等新興・再興感染症研究事業）「輸入感染症としての多剤耐性結核の対策・制御に関する研究」（岡田全司班）より助成を頂きました。深謝致します。

文　献

1）WHO Collaborating Center for the Surveillance of Tuberculosis in Europe Surveillance of Tuberculosis in Europe－Euro TB Report on tuberculosis cases notified in 2006. 2008.
2）Cain KP, Benoit SR, Winston CA, et al.: Tuberculosis among foreign-born persons in the United States. JAMA.

2008 ; 300 : 405−412.
3) CDC Trend in Tuberculosis − United States, 2008. MMWR. 2009 : 58 ; 249−253.
4) 吉山 崇, 石川信克, 星野斉之, 他：在留外国人結核症の最近の疫学動向. 結核. 1999 ; 74 : 667−675.
5) 石川信克: 外国人結核の背景と対策. 結核. 1995 ; 70 : 691−703.
6) 星野斉之：在日外国人結核の疫学的状況. 資料と展望. 2001 ; 38 : 1−10.
7) 結核予防会：在日外国人の結核. 「結核の統計 2009」. 東京, 平成21年10月, 18.
8) French CE, Antoine D, Gelb D, et al.: Tuberculosis in non-UK-born persons, England and Wales, 2001−2003. Int J Tuberc Lung Dis. 2007 ; 11 : 577−584.
9) 財団法人入管協会：概説「平成21年版 在留外国人統計」. 東京, 平成21年9月, 1−25.
10) WHO : WHO REPORT 2008 Global TB Control Surveillance, Planning, Financing 2009.
11) Achwartzman K, Oxlade O, Graham R, et al.: Domestic returns from investment in the control of tuberculosis in other countries : N Engl J Med. 2005 ; 353 : 1008−1020.
12) 星野斉之, 大森正子, 内村和広, 他：就業状況別結核罹患率の推定と背景の検討. 結核. 2007 : 82 : 685−695.
13) 厚生労働省保健医療局結核感染症課, 結核予防会結核研究所：平成 8 年度在日外国人結核実態調査報告. 資料と展望. 1998 ; 27 : 47−63.
14) Zuber PLF, McKenna MT, Binkin NJ, et al.: Long-term risk of tuberculosis among foreign-born persons in the United States. JAMA. 1997 ; 278 : 304−307.
15) Patel S, Parsyan AE, Gunn J, et al.: Risk of progression to active tuberculosis among foreign-born persons with latent tuberculosis. Chest. 2007 ; 131 : 1811−1816.

Original Article

TRENDS IN TUBERCULOSIS INFECTION AMONG FOREIGNERS IN JAPAN ACCORDING TO WORK STATUS

[1]Hitoshi HOSHINO, [1]Masako OHMORI, and [2]Masaji OKADA

Abstract [Purpose] TB among foreigners is presently a serious issue in some developed countries and could become so in Japan. The purpose of this report is to assess the epidemiological situation of TB among foreigners in Japan.

[Materials and Methods] The trend of TB reporting among foreigners in Japan was examined with regard to work status.

[Results] The number of reported TB cases among employees and students in Japan increased between 1998 and 2008, but that among housekeepers was level throughout the same period. The increase among employees and students might be due to the increased numbers of foreign employees and students. In the case of housekeepers, the increase in the number of permanent residents did not lead to an increase in TB among these housekeepers. Estimates of TB reporting rates decreased during the study period, so the changes in reporting rates would not have caused the increase in TB cases. This downward trend may have been caused by an increase in longer-term residents and a decrease in TB incidence in home countries. Even though the TB reporting rate is decreasing, the rates in those countries are much higher than in Japan in the same work categories.

[Discussion] To control the spread of TB, it is important to identify high-risk individuals. The Japanese TB control program should further strengthen mass health examination programs for foreign housekeepers and employees (especially temporary and daily employees), case-finding based on individuals' access to hospitals or clinics when suffering from TB symptoms, and flexible and periodic adjustment of TB control activities for foreigners according to future changes in the number and distribution of foreigners in Japan. Furthermore, improving the TB epidemiological situation in home countries might contribute to the downward trend of TB reporting rates among foreigners in Japan. Therefore, Japanese assistance in TB control activities in surrounding countries such as China, South Korea, and the Philippines might contribute to TB control activities for foreigners in Japan.

Key words: Tuberculosis, Foreigners, Labor status, Incidence

[1]Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association (JATA), [2]National Hospital Organization Kinki-chuo Chest Medical Center

Correspondence to: Hitoshi Hoshino, Research Institute of Tuberculosis, JATA, 3−1−24, Matsuyama, Kiyose-shi, Tokyo 204−8533 Japan. (E-mail: hhoshino@jata.or.jp)

Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2011, Article ID 549281, 11 pages
doi:10.1155/2011/549281

Research Article

Novel Prophylactic Vaccine Using a Prime-Boost Method and Hemagglutinating Virus of Japan-Envelope against Tuberculosis

Masaji Okada,[1] Yoko Kita,[1] Toshihiro Nakajima,[2] Noriko Kanamaru,[1] Satomi Hashimoto,[1] Tetsuji Nagasawa,[2] Yasufumi Kaneda,[3] Shigeto Yoshida,[4] Yasuko Nishida,[1] Hitoshi Nakatani,[1] Kyoko Takao,[1] Chie Kishigami,[1] Shiho Nishimatsu,[1] Yuki Sekine,[1] Yoshikazu Inoue,[1] David N. McMurray,[5] and Mitsunori Sakatani[1]

[1] *Clinical Research Center, National Hospital Organization, Kinki-Chuo Chest Medical Center, 1180 Nagasone, Kitaku, Sakai, Osaka 591-8555, Japan*
[2] *Ikeda Laboratory, GenomIdea Inc., 1-8-31 Midorigaoka, Ikeda, Osaka 530-0043, Japan*
[3] *Division of Gene Therapy Science, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan*
[4] *Department of Medical Zoology, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-machi, Tochigi 329-0498, Japan*
[5] *System Health Science Center, College of Medicine, Texas A&M University, College Station, TX 77843-1114, USA*

Correspondence should be addressed to Masaji Okada, **okm@kch.hosp.go.jp**

Received 8 September 2010; Revised 6 January 2011; Accepted 16 January 2011

Academic Editor: Nicholas West

Objective. *Mycobacterium tuberculosis* infection is a major global threat to human health. The only tuberculosis (TB) vaccine currently available is bacillus Calmette-Guérin (BCG), although it has no efficacy in adults. Therefore, the development of a novel vaccine against TB for adults is desired. *Method.* A novel TB vaccine expressing mycobacterial heat shock protein 65 (HSP65) and interleukin-12 (IL-12) delivered by the hemagglutinating virus of Japan- (HVJ)- envelope was evaluated against TB infection in mice. Bacterial load reductions and histopathological assessments were used to determine efficacy. *Results.* Vaccination by BCG prime with IgHSP65 + murine IL-12/HVJ-envelope boost resulted in significant protective efficacy (>10,000-fold versus BCG alone) against TB infection in the lungs of mice. In addition to bacterial loads, significant protective efficacy was demonstrated by histopathological analysis of the lungs. Furthermore, the vaccine increased the number of T cells secreting IFN-γ. *Conclusion.* This vaccine showed extremely significant protection against TB in a mouse model, consistent with results from a similar paper on cynomolgus monkeys. The results suggest that further development of the vaccine for eventual testing in clinical trials may be warranted.

1. Introduction

Tuberculosis (TB) is a major global threat to human health, with about 2 million people dying every year from *Mycobacterium tuberculosis* infection. The only TB vaccine currently available is an attenuated strain of *Mycobacterium bovis*, bacillus Calmette-Guerin (BCG), although its efficacy against adult TB disease is unclear. Furthermore, multidrug-resistant TB (MDR-TB) and extremely drug-resistant TB (XDR-TB) are becoming big problems worldwide. For these reasons, a prophylactic and therapeutic vaccine against TB is sought. TB vaccines are classified into 4 main groups: (1) DNA vaccines, (2) recombinant BCG vaccines, (3) subunit vaccines, and (4) attenuated vaccines.

It is well established that protective immunity to *M. tuberculosis* depends on both $CD4^+$ and $CD8^+$ T cells [1–6]. DNA vaccines induce cellular immune responses, including the Th-1-type cellular immune response, and they prevent infections in animal models [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 [10–12], and HIV infections [13]. DNA vaccines have also shown their potential as TB vaccines in mouse

models [14–17]. However, in a guinea pig model, which is one of the most biologically relevant systems available for studying human pulmonary TB, DNA vaccines have not been proven more efficacious than BCG [18]. The efficacy of any experimental TB vaccine must be evaluated in human clinical trials, and a vaccine against TB is still anxiously awaited.

We have been developing a novel TB vaccine that is a DNA vaccine expressing mycobacterial heat shock protein 65 (HSP65) and interleukin-12 (IL-12), delivered by the hemagglutinating virus of Japan- (HVJ)- liposome or -envelope (HVJ-E) (HSP65 + IL-12/HVJ) [19–22]. The former vaccine was 100-fold more efficacious than BCG in a murine model on the basis of the elimination of *M. tuberculosis* [19]. In the present study, we demonstrated that the combination of BCG prime with HSP65 + IL-12/HVJ-E vaccine-boost was 10,000-fold more efficacious than BCG alone in a murine TB prophylactic model.

2. Materials and Methods

2.1. Bacteria. *M. tuberculosis* strains 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's medium (Wako Chemicals, Osaka, Japan). For the mouse infection studies, a single colony of *M. tuberculosis* H37Rv was grown in Middlebrook 7H9 medium (DIFCO Laboratories, Detroit, MI; lot 137971 XA MD) supplemented with albumin-dextrose complex and grown at 37°C until approximately midlog phase. Aliquots were stored at −80°C and thawed 10 days before use. Each bacterium was grown to midlog phase in 7H9 medium.

2.2. Animals. Inbred and specific pathogen-free female BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). Mice were maintained in isolator cages, manipulated in laminar flow hoods, and used between 8 and 10 weeks of age. All animal experiments were approved by the National Hospital Organization Kinki-chuo Chest Medical Center Animal Care and Use Committee. All vaccinations and experiments on isolated tissues were performed on anesthetized animals with sevoflurane. Infected animals were housed in individual microisolator cages in Biosafety Level (BL) 3 animal facility of the NHO Kinki-chuo Chest Medical Center.

2.3. Plasmid Construction. The *HSP65* gene was amplified from *M. tuberculosis* H37Rv genomic DNA, and cloned into pcDNA3.1 (+) (Invitrogen, San Diego, CA) to generate pcDNA-hsp65 (designated as HSP65 DNA) as described previously [19]. The *hsp65* gene was fused with mouse Igκ secretion signal sequence, and pcDNA-Ighsp65 (designated as IgHSP65 DNA) was generated. For construction of the mouse IL-12 (mIL-12) *p40* and *p35* single-chain genes, *mIL12p35* and *mIL12p40* genes were cloned from pcDNA-p40p35 [21], fused and cloned into pcDNA3.1 (+) to generate pcDNA-mIL12p40p35-F (designated as mIL-12 DNA).

2.4. HVJ-E Vaccination. HVJ-E was prepared as described previously (**Figure 1**) [19–23]. The HVJ-E complex was aliquoted and stored at −70°C until use. Groups of BALB/c mice were vaccinated 3 times at 3-week intervals with 100 μL of HVJ-E solution containing 50 μg of pcDNA-IgHSP65 and 50 μg of mIL12 DNA. These DNA vaccines were injected into both anterior muscles in the tibia. Mice were vaccinated using 1×10^6 colony-forming units (CFU) *M. bovis* BCG Tokyo by subcutaneous injection at 4 different sites (left-upper, right-upper, left-lower, and right-lower back). HVJ-E DNA vaccine containing pcDNA-IgHSP65 and -mIL12 DNA was designated as IgHSP65 + mIL-12/HVJ-E in this text.

2.5. 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 4 weeks after the third vaccination as described previously (**Figure 2**) [19, 24]. 0.2 mL of saline containing 5×10^5 CFU of H37Rv *Mycobacterium tuberculosis* were injected into tail vein of mice. At 5 and 10 weeks after *M. tuberculosis* H37Rv challenge, lungs, spleens, and livers were aseptically homogenized by using a homogenizer in saline, and serial dilutions of the organ homogenates were plated on 7H11 Middlebrook agar (Kyokuto. Tokyo, Japan). Plates were sealed and incubated at 37°C, and the number of colonies was counted 2 weeks later. Results were converted to $\log_{10}$ values. The $\log_{10}$ [mean ± standard deviation (S.D.)] values for CFU/organs/animals were calculated for each experimental group. Weight of lungs, liver, or spleen was measured by a balance (Sartorius Co. LP620S).

2.6. Histological Analysis. The lungs obtained from the mice were fixed with 10% buffered formalin and embedded in paraffin. Each block was sliced into 4-μm-thick sections and stained using hematoxylin and eosin. Semiquantitative morphometric analysis of pathological slides was performed by a method modified over that of Dascher et al. (2003) using a micrometer-attached microscope (Microphot-FXA, Nikon, Japan) [19, 25, 26]. The longer axis and minor axis of each granuloma in the field (×40 magnification) were measured and then multiplied and summed. Three random fields from each tissue section of mice were evaluated. 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 was significantly correlated with the granuloma area determined by a hematoxylin and eosin section scanning method.

2.7. ELISPOT Assay. The spleens were removed aseptically from vaccinated mice 3 weeks after the third vaccination. Antigen-specific IFN-γ-producing cells were determined by enzyme-linked immunosorbent spot (ELISPOT) as described previously [19]. Briefly, ELISPOT plates (MultiScreen IP Filtration plate MAIPS45; Millipore, Bedford, MA) were coated with antimouse IFN-γ MAb R4-6A2 (BD Biosciences Pharmingen. San Diego, CA). Spleen cells from vaccinated mice were suspended at 1×10^7 cells/mL (1×10^6 cells/well). The cells were placed into 6 antibody-coated wells, and rHSP65 protein (10 μg/mL) or PPD

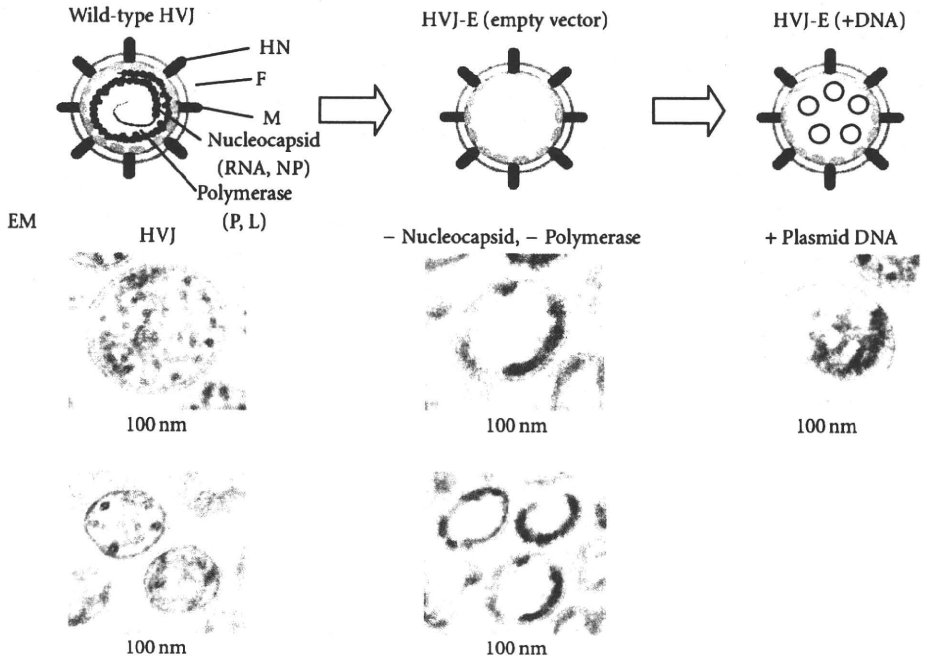

FIGURE 1: Hemagglutinating virus of Japan- (HVJ)- envelope vaccination: pcDNA3.1/HSP65DNA + IL-12DNA were incorporated into an HVJ-envelope empty vector (nonviral vector). Graphical representations of the HVJ-envelope empty vector in the presence or absence of DNA are shown. Electronic microscopy (EM) photographs of the HVJ-envelope empty vector are also shown.

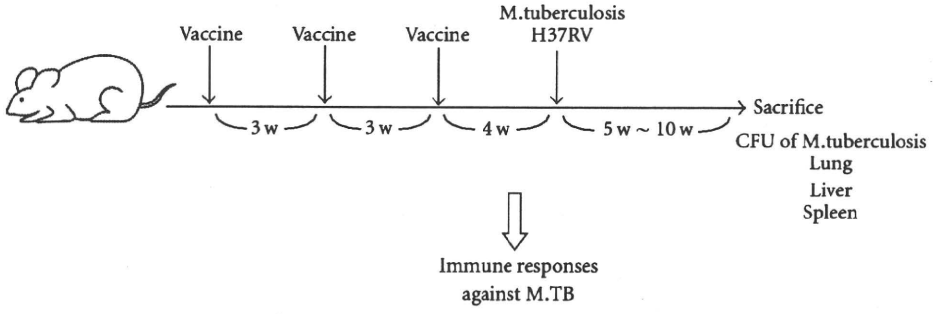

FIGURE 2: Groups of mice were vaccinated 3 times every 3 weeks using the prime-boost method and challenged intravenously with *M. tuberculosis* H137Rv as described in the Materials and Methods section. Five or 10 weeks after challenge with TB, protection was measured by enumerating bacterial loads (CFU) in the lungs, liver, and spleen of the vaccinated mice.

(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 biotinylated antimouse 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 the KS ELISPOT system (Carl Zeiss, Hallbergmoos, Germany).

2.8. Statistical Analysis. Dunnett's tests (multiple comparisons) were used to compare $\log_{10}$ values of CFUs between groups following challenge and used to compare T-cell responses between groups in the ELISPOT assay. A P-value of $< .05$ was considered significant.

3. Results and Discussion

3.1. Results

3.1.1. Prophylactic Efficacy Using Prime-Boost Method. The IgHSP65 + mIL-12/HVJ-E and BCG vaccines were administered using the prime-boost method as shown in **Table 1**.

At 5 and 10 weeks after intravenous challenge of *M. tuberculosis* H137Rv, the number of CFUs in the lungs, spleen, and liver were determined. **Figure 3(a)** shows the result of bacterial loads 5 weeks after challenge.

Table 1: BCG-HVJ-E/HSP65 DNA + IL-12 DNA Prime/Boost Experiment. Groups of mice were vaccinated 2 or 3 times with IgHSP65 + mIL-12/HVJ-E vaccine and/or BCG by using the prime-boost method. IgHSP65 + mIL-12/HVJ-E vaccine was injected intramuscularly, and BCG was injected subcutaneously. 4 weeks after the last immunization, *M. tuberculosis* H37Rv was challenged intravenously. 5 weeks and 10 weeks after TB challenge, protection was measured by enumerating bacterial loads (CFU) in the lungs, liver, and spleen from vaccinated mice. One week before the TB challenge, the immune responses of cytotoxic T cells, proliferation of T cells, and cytokines (IFN-γ, IL-2, IL-6) production were assayed.

Group	First immunization	Second immunization	Third immunization
1	—	—	—
2	—	—	BCG
3	HSP65 + IL-12/HVJ-E	HSP65 + IL-12/HVJ-E	HSP65 + IL-12/HVJ-E
4	BCG	HSP65 + IL-12/HVJ-E	HSP65 + IL-12/HVJ-E
5	HSP65 + IL-12/HVJ-E	HSP65 + IL-12/HVJ-E	BCG

13 mice per group.
3 mice for the *in vitro* assay prior to challenge (IFN γ ELISPOT, etc.).
10 mice for the protection study (half of the mice were used for necropsy at 5 weeks after challenge and half at 10 weeks).

Vaccination by BCG prime + IgHSP65 + mIL-12/HVJ-E boost showed significant protective effects on the bacterial loads in the lungs as compared to BCG alone ($P < .01$). The prime-boost method using DNA and BCG vaccines showed extremely strong protective efficacy (>10,000-fold versus BCG alone) regardless of the order of administration (**Figure 3(a)**). Vaccination with BCG vaccine alone decreased TB CFUs in the lungs by 1 log unit as compared to nonvaccinated mice.

Vaccination with IgHSP65 + mIL-12/HVJ-E and BCG by the prime-boost method also showed significant protective efficacy on the bacterial loads in the liver as compared to BCG (>100-fold, $P < .05$; **Figure 3(b)**). The combination of 2 vaccines and administration by the prime-boost method also exerted a significant protective effect on the bacterial load in the spleen as compared to naive control group (10-fold higher, $P < .05$; **Figure 3(c)**).

Body weight of vaccinated mice was similar in all vaccinated groups. Tissue weights of spleens and livers in the prime-boost groups were lower than those of naive group (Figures **4** and **5**).

We also confirmed the greater enhancement of protective effects in the BCG-DNA vaccine combination groups than those in the naive control group or BCG-alone group 10 weeks after challenge (data not shown). These results indicate that treatment using 2 vaccines by the prime-boost method was more effective than BCG alone.

3.1.2. Histological Analysis. In addition to the reduction of bacterial loads, the efficacies of each vaccine were assessed by histological analysis. The number and size of granulomatous lesions in the lungs were significantly lower and smaller, respectively, in the mice vaccinated by the BCG prime-DNA boost group than in the naive control mice and BCG control mice groups (**Figure 6**). Quantitative evaluation of the granulomatous lesions clearly showed that the BCG prime with IgHSP65 + mIL-12/HVJ-E boost significantly reduced the granuloma index in the lungs as compared to naive and BCG groups ($P < .05$; **Figure 7**). Thus, vaccination by the prime-boost method has the capability to reduce pulmonary lesions caused by *M. tuberculosis* infection.

3.1.3. Immunological Analysis. Furthermore, BCG prime with IgHSP65 + mIL-12/HVJ-E boost augmented the proliferation and IFN-γ production of HSP65 antigen-specific T cells in the K-S ELISPOT Assay. The efficacy of BCG prime with IgHSP65 + mIL-12/HVJ-E boost was higher compared with BCG Tokyo alone or IgHSP65 + mIL-12/HVJ-E prime with BCG boost (**Figure 8**).

These data indicate that the protective efficacies of BCG prime with IgHSP65 + mIL-12/HVJ-E boost are strongly associated with the number and activity of IFN-γ-secreting and HSP65-specific T cells. Taken together, combinational vaccination with BCG and IgHSP65 + mIL-12/HVJ-E by the prime-boost method is capable of augmenting T-cell activation. In addition, increase of IFN-γ-secreting cells is involved in the reduction of bacterial burden and lesions in the lungs. The efficacies of the prime-boost method are greater than those achieved by vaccination with BCG alone.

3.2. Discussion. In this study, we evaluated the protective efficacy of IgHSP65 + mIL-12/HVJ-E vaccine, using the prime-boost method. One of the significant findings was that the combination of IgHSP65 + mIL-12/HVJ-E and BCG led to a remarkably high degree of protection against intravenous challenge infection with virulent *M. tuberculosis*; bacterial numbers exponentially declined in 3 organs, especially in the lungs (10,000-fold lower than that of mice vaccinated with BCG alone; **Figure 3(a)**).

The pathological parameters of protection included reductions in the mean lung granulomatous lesion score in our study. The protective efficacies of BCG with IgHSP65 + mIL-12/HVJ-E administered by the prime-boost method were indicated on the basis of histopathological methods as well as bacterial loads. Histopathological analysis showed that mice vaccinated with BCG prime with IgHSP65 + mIL-12/HVJ-E boost had fewer and smaller lesions in the lungs and significantly less lung granuloma than naive mice and mice treated with BCG alone. These results suggest that severe toxicities (Koch phenomenon) were suppressed by the combination of two kinds of vaccines.

The data in the present study also show that the protective efficacy of BCG prime with IgHSP65 + mIL-12/HVJ-E boost is strongly associated with the emergence of IFN-γ-secreting T cells upon stimulation with HSP65. In the previous study, we demonstrated that *in vivo* function of CD8-positive T cells as well as CD4-positive T cells is involved in prophylactic efficacy of the IgHSP65 + mIL-12/HVJ-E in mice [**22**].

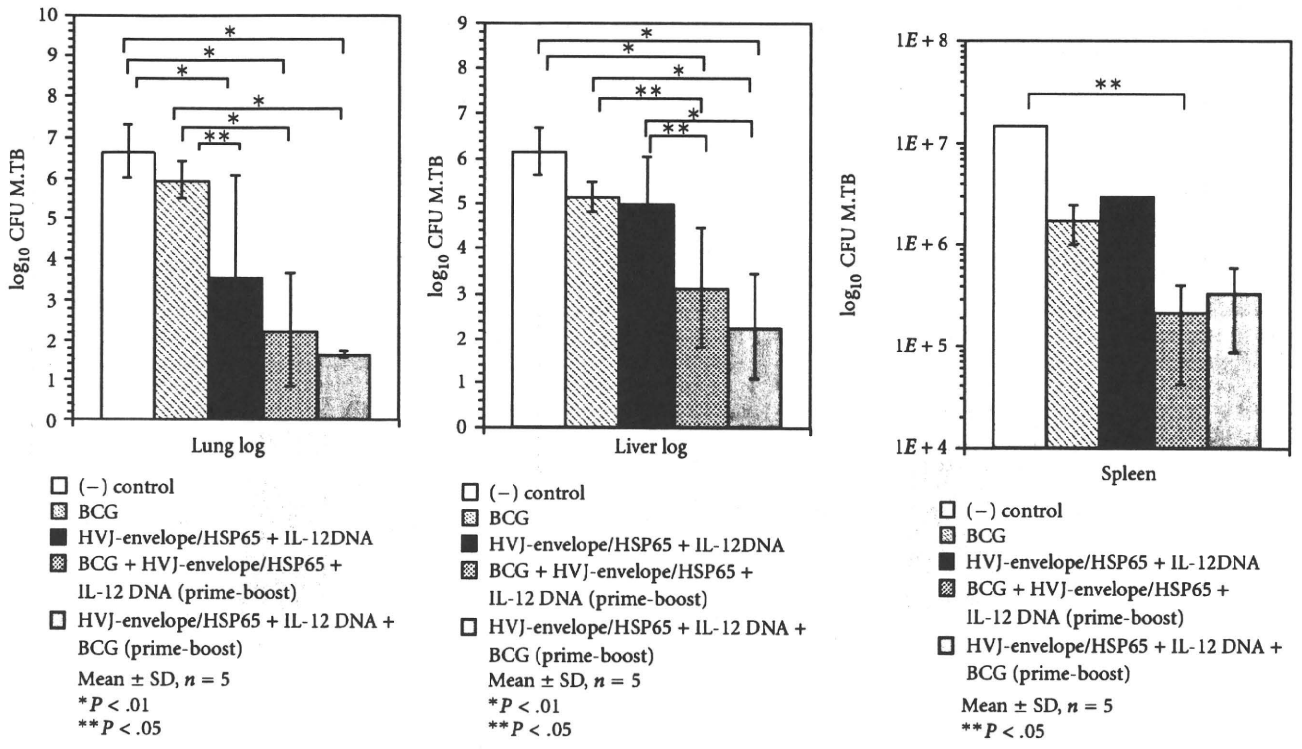

FIGURE 3: (a) Mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-envelope (HVJ E) DNA and/or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, protection was measured by enumerating the bacterial loads (CFU) in the lungs. Results are expressed as the mean $\log_{10} \pm$ S.D. of CFU. The statistical significance of differences between individual groups in the CFU number was determined by Dunnett test ($n = 5$); $^*P < .01$ and $^{**}P < .05$; the statistical significance of differences ($P < .01$) of the G1 (naive) group compared to the G3 group (DNA/DNA/DNA), G4 group (BCG/DNA/DNA), or the G5 group (DNA/DNA/BCG). The statistical significance of differences ($^{**}P < .05$) of the G2 group (BCG-alone group) compared to the G3 group (DNA/DNA/DNA), that of differences ($P < .01$) of the G2 group compared to the G4 group (BCG/DNA/DNA), or the G5 group (DNA/DNA/BCG). (b) Mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-E DNA and/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 liver. Results are expressed as the mean $\log_{10} \pm$ S.D. of CFU. The statistical significance of differences between individual groups in the CFU number was determined by Dunnett test ($n = 5$), $^*P < .01$; the statistical significance of differences ($P < .01$) of the G1 (naive) group compared to the G4 group (BCG/DNA/DNA), or the G5 group (DNA/DNA/BCG). The statistical significance of differences ($P < .05$) of the G2 group (BCG-alone group) compared to the G4 group (BCG/DNA/DNA). The statistical significance of differences($P < .01$) of the G2 group compared to the G5 group (DNA/DNA/BCG). The statistical significance of differences ($P < .05$) of the G3 group (DNA/DNA/DNA) compared to G4 (BCG/DNA/DNA). That of differences ($P < .01$) of the G3 group compared to the G5 group. (c) Mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-E DNA and/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 spleen. 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 Dunnett test ($n = 5$); $^{**}P < .05$; the statistical significance of differences ($P < .05$) of the G1 (naive) group compared to the G4 group (BCG/DNA/DNA).

In this study, we used the murine model of TB, which may not reflect the pathologic status of human TB. As to the difference of the infection route, our previous results in a guinea pig model used in a collaborative study with Dr. D. McMurray (Texas A&M University) showed that vaccination with HSP65 + guinea pig IL-12/HVJ resulted in better protection against pulmonary pathology caused by aerosol challenge with *M. tuberculosis* than BCG vaccination (data not shown).

In addition, we have recently confirmed that the prime-boost method was also effective in a cynomolgus monkey model [**20–22**]. We evaluated our HSP65 + human IL-12/HVJ (HSP65 + hIL-12/HVJ) in the monkey model infected by an intratracheal instillation (aerogenic route), which is currently the best animal model of human TB. Vaccination with HSP65 + hIL-12/HVJ resulted in better protective efficacy than that with BCG alone on the basis of the erythrocyte sedimentation rate test, chest X-ray findings, and immune responses. In addition, vaccination with HSP65 + hIL-12/HVJ resulted in increased survival for over a year. This was the first report of successful DNA vaccination against M. tuberculosis in a monkey model [**21**].

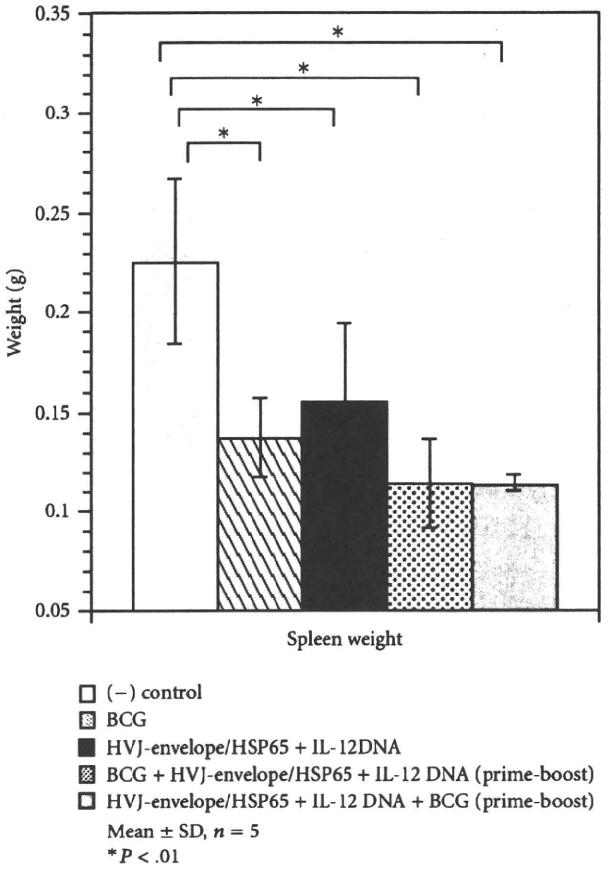

FIGURE 4: Tissue weight in mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-E DNA and/or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, spleen weight was measured. Results are expressed as the mean ± S.D. in grams (g). The statistical significance of differences between individual groups in the weight was determined by Dunnett test ($n = 5$), $^{*}P \leq .01$; the statistical significance of differences ($P < .01$) of the G1 (naive) group compared to the G2 group (BCG-alone group), G3 group (DNA/DNA/DNA), G4 group (BCG/DNA/DNA), or G5 group (DNA/DNA/BCG).

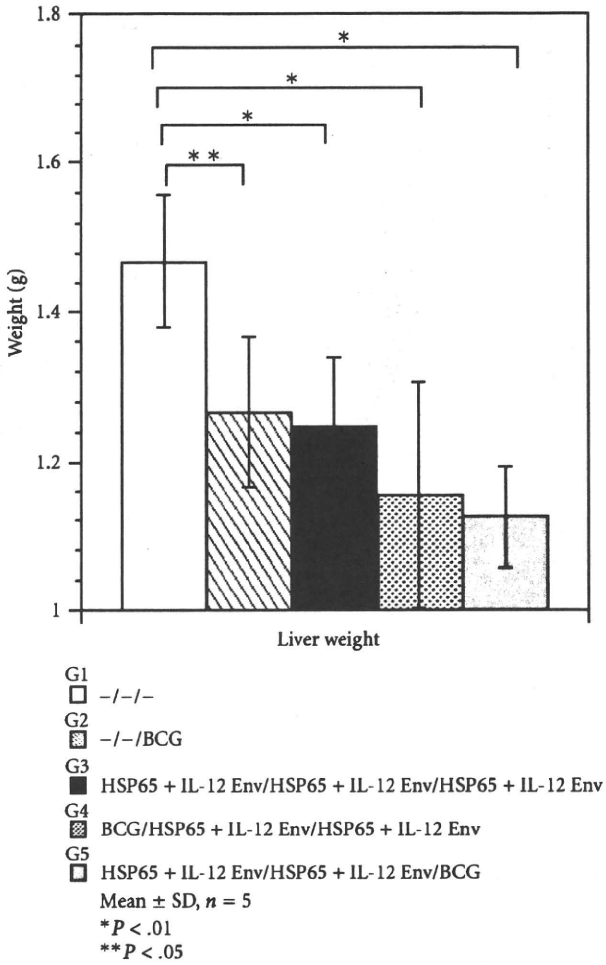

FIGURE 5: Tissue weight in mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-E DNA and/or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, liver weight was measured. Results are expressed as the mean ± S.D. in grams (g). The statistical significance of differences between individual groups in the weight was determined by Dunnett test ($n = 5$), $^{*}P < .01$; the statistical significance of differences ($P \leq .01$) of the G1 (naive) group compared to the G3 group (DNA/DNA/DNA), G4 group (BCG/DNA/DNA), or G5 group (DNA/DNA/BCG). $^{**}P < .05$; that of differences ($P < .05$) of the G1 group compared to the G2 group (BCG alone group).

Most importantly, protective efficacy was augmented when BCG and HSP65 + hIL-12/HVJ were administered by the prime-boost method. Survival rates of BCG alone, saline control, HSP65 + hIL-12/HVJ-prime with BCG-boost, and BCG-prime with HSP65 + hIL-12/HVJ-boost groups were 33%(2/6), 50%(3/6), 50%(2/4), and 100%(4/4) at 12 months after the infection (aerogenic route), respectively [21]. We also evaluated immune responses in the monkey model of TB. Antigen-specific IFN-γ-production and proliferation of peripheral blood lymphocyte (PBL) were enhanced by the vaccination using the prime-boost method.

We also demonstrated efficacies in the monkey model when the boost was performed after a long-term period (4 months) from the prime. The prolongation of the survival was observed in the BCG-prime and HSP65 + IL-12/HVJ-booster group [27]. Improvement of ESR, increase of the body weight and augmentation of IFN-γ production, and proliferation of PBL were also observed in the BCG-prime and HSP65 + IL-12/HVJ-booster group.

Taken together, these results clearly demonstrated that BCG-prime with HSP65 + hIL-12/HVJ-boost could provide extremely strong protective efficacy against M. tuberculosis in a cynomolgus monkey model (intratracheal infection route), which is currently the best animal model of human TB [21].

The prime-boost method was reported in a study of the MVA85A vaccine, which is a modified vaccinia virus Ankara (MVA) strain expressing antigen 85A. In phase I studies in humans, this vaccine has induced high immune responses in previously BCG-vaccinated individuals [28].

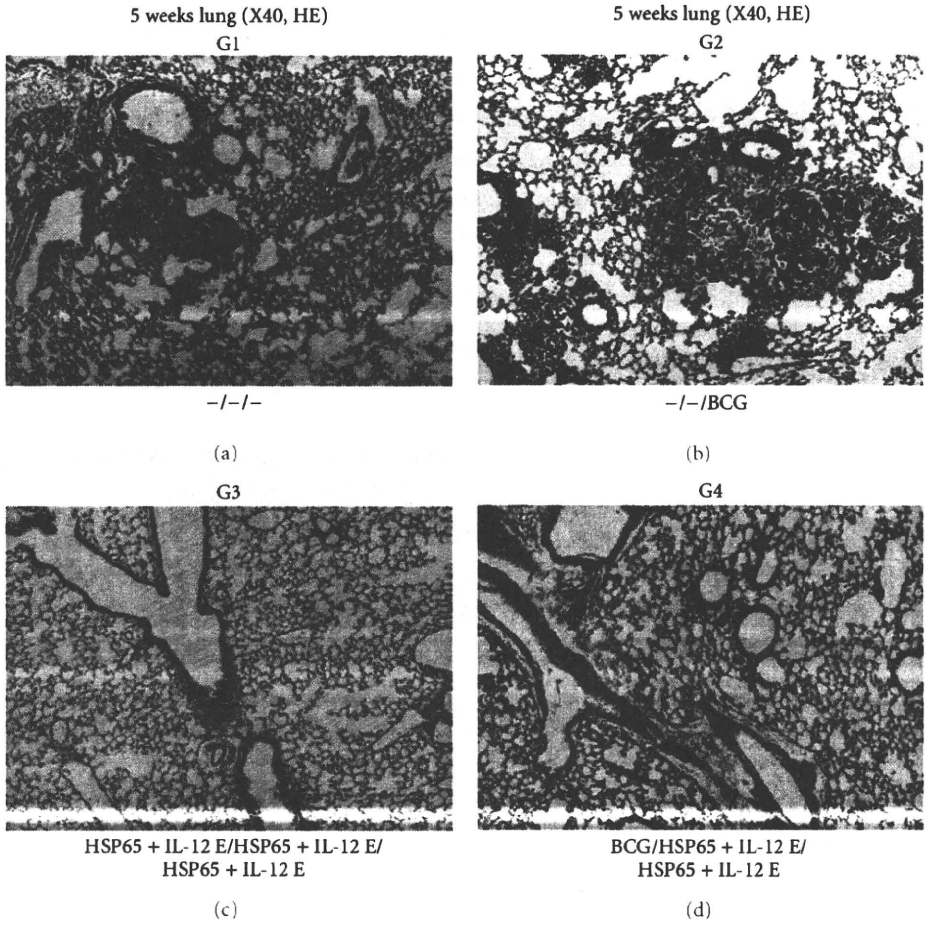

FIGURE 6: Histopathological analysis of vaccinated mice 5 weeks after *M. tuberculosis* challenge. Representative photomicrographs of lung tissue sections harvested from the G1 naive control group, G2 (BCG alone) group, G3 group (DNA/DNA/DNA), and G4 group (BCG/DNA/DNA) are shown (5 weeks after *M. tuberculosis* challenge, hematoxylin and eosin staining, × 4 objective). There was much infiltration of mononuclear cells and extensive parenchymal destruction by large, poorly demarcated granuloma in the lungs from the G1 (naive control) group and G2 (BCG alone) group. In the G3 (DNA/DNA/DNA) group and G4 (BCG/DNA/DNA) group, there was less inflammation, and only a few granulomas were observed.

Boosting BCG vaccination with MVA85A downregulates the immunoregulatory cytokine TGF-β1 [**29**]. Aeras-402 DNA (DNA that expressed 85A, 85B, and TB10.4) vaccine using adenovirus vector is intended for use as a boosting vaccine in BCG-primed individuals [**30**]. Several vaccines have been used with a prime-boost strategy to complement immune responses [**31**].

DNA vaccines are a relatively new approach to induce immunities for the protection of infectious diseases [**14, 19, 22, 32–34**]. Prophylactic and therapeutic DNA vaccines were established by using several kinds of vectors such as HVJ-liposome, HVJ-E, adenovirus vector, adenoassociated virus vector, and lentivirus vector [**19–22, 35, 36**]. In order to explore the preclinical use of a tuberculosis DNA vaccine combination of *IL-12* DNA with *hsp65* DNA, we chose the HVJ-based delivery system (HVJ-liposome and HVJ-E). These systems have high transfection efficiency and are available for repeated *in vivo* gene transfection without reduction of gene transfer efficiency or apparent toxicity. These characters 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 gene expression in 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 in mice [**37**]. 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 [**38**]. HVJ-liposomes were also very effective as a mucosal vaccine against HIV infection [**39**]. Thus, it is likely that HVJ proteins may be responsible for the induction of a robust immune response. No side effects were observed when repetitive injections of HVJ-liposomes were performed in mice, rats, or monkeys. We have previously developed an HVJ-E using inactivated Sendai virus, as a nonviral vector for drug delivery [**40–42**]. It can be used for efficient delivery of DNAs, siRNAs, proteins, and anticancer drugs into cells

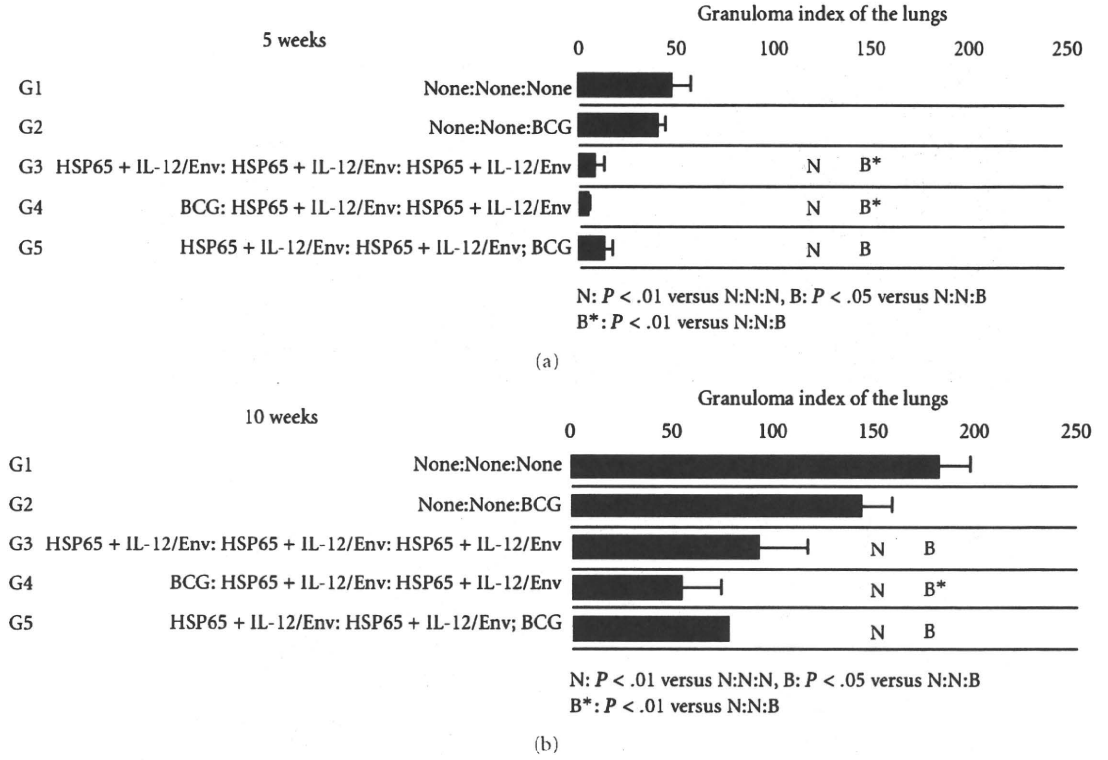

FIGURE 7: Granuloma index of the G1, G2, G3, G4, and G5 (DNA/DNA/BCG) groups in the lungs 5 weeks and 10 weeks after *M. tuberculosis* challenge. Results are expressed as the mean ± S.D. of triplicates of 5 mice per group. The statistical significance of differences between the groups was determined by Dunnett test, $P < .01$ as compared with the naive (N) group or the BCG alone (B) group. $P < .05$ as compared with the BCG alone (B*) group. The statistical significance of differences ($P < .05$) of granuloma index of 5 weeks G3 group compared to the G4 group.

both *in vitro* and *in vivo* [40, 43, 44]. Therefore, HVJ-E was used as an efficient and safe vector for DNA vaccine against TB in the present study.

Mycobacterial heat shock protein 65 (HSP65) is a potential target for protective immunity and has been studied extensively [19]. Several studies have reported that *hsp65* DNA vaccines can strongly induce protective immune responses in mice against virulent *M. tuberculosis* infections [20–22]. Protection is attributed to the establishment of a cellular immune response dominated by HSP65-specific T cells which produce IFN-γ and are cytotoxic towards infected cells. Furthermore, Lowrie and colleagues have reported that this vaccine reduces bacterial loads in mice infected with *M. tuberculosis* when given therapeutically after infection [32].

One of the major roles of IL-12 is the induction of IFN-γ-mediated immune responses to microbial pathogens. Cooper and colleagues have demonstrated the importance of IL-12 in generation of the protective response to tuberculosis [45]. Coadministration of the *IL-12* gene, which induces an IFN-γ-mediated immune response to microbial pathogens, with various tuberculosis DNA vaccines including *hsp65* DNA [46], and 35 K MW DNA [47], may boost the efficacy of these DNA vaccines to the levels achieved with BCG in the mouse model, although an inhibitory effect rather than a synergistic effect on immunotherapy was observed in mice coadministered *hsp65* DNA vaccine plus the *IL-12* gene [32].

In conclusion, we have shown efficacy of a novel HVJ-E DNA vaccine encapsulating HSP65 DNA with IL-12 DNA in the mouse model of TB. These results suggest that HSP65 + IL-12/HVJ could be a promising candidate for a new tuberculosis vaccine superior to BCG. To this aim, protective efficacy and immune responses were further studied in nonhuman primates before proceeding to human clinical trials.

In Japan and other countries, BCG is inoculated into human infants up to 6 months after birth. Therefore, BCG prime in infants and HSP65 + hIL-12/HVJ boost in adults (including junior high school students, high school students, and the elderly) may be required for significant improvement of clinical protective efficacy against TB. Thus, our results with the HSP65 + hIL-12/HVJ vaccine in a murine prophylactic model and cynomolgus monkey prophylactic model provide a significant rationale for moving this vaccine into clinical trials. Indeed, multiple animal models are available to accumulate essential data on the HVJ-E DNA vaccine in anticipation of a phase I clinical trial.

4. Conclusions

Vaccination by BCG prime with a novel vaccine (IgHSP65 + mIL-12/HVJ-E) boost resulted in significant protective efficacy (10,000-fold greater than BCG alone) against TB

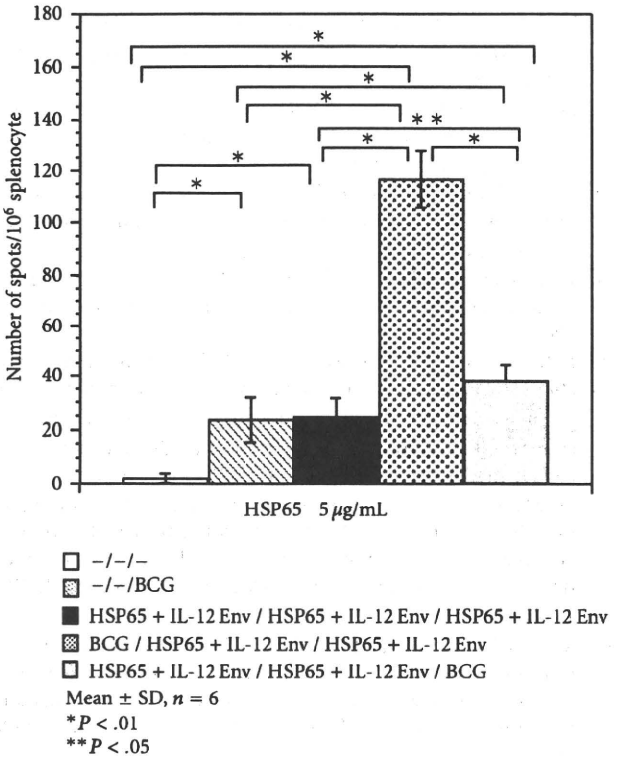

FIGURE 8: ELISPOT assay for IFN-γ antigen-specific responses in the spleens of vaccinated mice following stimulation with rHSP65 protein. Spleen cell cultures were stimulated with rHSP65 protein for 20 h. The numbers of IFN-γ-secreting cells specific for rHSP65 protein per million cells were determined individually by ELISPOT assay. Results are expressed as the mean ± S.D. of 6 wells of 3 mice per group. The statistical significance of differences between individual groups in the number of IFN-γ-secreting cells was determined by Dunnett test. The statistical significance of differences ($P < .01$) of the G1 (naive) group compared to the G2 (BCG alone group), G3 (DNA/DNA/DNA), G4 (BCG/DNA/DNA), or G5 (DNA/DNA/BCG). The statistical significance of the G2 group difference ($P < .01$) compared to the G4 or the G5. The statistical significance of the G3 group differences ($P < .01$) compared to the G4. $P < .01$; the G4 group compared to the G5. The statistical significance of the G3 group differences ($P < .05$) compared to the G5.

infection in the lungs of mice. In addition to bacterial loads, significant protective immunity was demonstrated by histopathological analysis of the lungs. This vaccine showed extremely significant protection against TB, suggesting that further development for eventual testing in clinical trials may be warranted.

Acknowledgments

This paper was supported by Health and Labor Science Research Grants from MHLW, international collaborative study Grants from the Human Science foundation, and Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science, and Technology (Japan), Research on Publicly Essential Drugs and Medical Devices from Japan Health Sciences Foundation, and a Grant from the Osaka Tuberculosis Foundation.

References

[1] J. L. Flynn and J. Chan, "Immunology of tuberculosis," *Annual Review of Immunology*, vol. 19, pp. 93–129, 2001.

[2] J. Hess, U. Schaible, B. Raupach, and S. H. E. Kaufmann, "Exploiting the immune system: toward new vaccines against intracellular bacteria," *Advances in Immunology*, vol. 75, pp. 1–88, 2000.

[3] A. Geluk, K. E. Van Meijgaarden, K. L. M. C. Franken et al., "Identification of major epitopes of *Mycobacterium tuberculosis* AG85B that are recognized by HLA-A*0201-restricted $CD8^+$ T cells in HLA-transgenic mice and humans," *Journal of Immunology*, vol. 165, no. 11, pp. 6463–6471, 2000.

[4] A. Lalvani, R. Brookes, R. J. Wilkinson et al., "Human cytolytic and interferon γ-secreting CD8+ T lymphocytes specific for *Mycobacterium tuberculosis*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 1, pp. 270–275, 1998.

[5] P. Wong and E. G. Pamer, "CD8 T cell responses to infectious pathogens," *Annual Review of Immunology*, vol. 21, pp. 29–70, 2003.

[6] H. McShane, S. Behboudi, N. Goonetilleke, R. Brookes, and A. V. S. Hill, "Protective immunity against *Mycobacterium tuberculosis* induced by dendritic cells pulsed with both CD8(+)- and CD1(+)-T-cell epitopes from antigen 85A," *Infection and Immunity*, vol. 70, no. 3, pp. 1623–1626, 2002.

[7] I. K. Srivastava and M. A. Liu, "Gene vaccines," *Annals of Internal Medicine*, vol. 138, no. 7, pp. 550–148, 2003.

[8] M. Okada and T. Kishimoto, "The potential application and limitation of cytokine/growth factor manipulation in cancer therapy," in *Cell proliferation in Cancer: Regulatory Mechanisms of Neoplastic Cell Growth*, L. Pusztai, C. Lewis, and E. Yap, Eds., pp. 218–244, Oxford University Press, New York, NY, USA, 1996.

[9] M. J. Roy, M. S. Wu, L. J. Barr et al., "Induction of antigen-specific $CD8^+$ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine," *Vaccine*, vol. 19, no. 7-8, pp. 764–778, 2000.

[10] T. P. Le, K. M. Coonan, R. C. Hedstrom et al., "Safety, tolerability and humoral immune responses after intramuscular administration of a malaria DNA vaccine to healthy adult volunteers," *Vaccine*, vol. 18, no. 18, pp. 1893–1901, 2000.

[11] A. C. Moore and A. V. S. Hill, "Progress in DNA-based heterologous prime-boost immunization strategies for malaria," *Immunological Reviews*, vol. 199, pp. 126–143, 2004.

[12] S. Yoshida, S. I. Kashiwamura, Y. Hosoya et al., "Direct immunization of malaria DNA vaccine into the liver by gene gun protects against lethal challenge of Plasmodium berghei sporozoite," *Biochemical and Biophysical Research Communications*, vol. 271, no. 1, pp. 107–115, 2000.

[13] J. D. Boyer, M. A. Chattergoon, K. E. Ugen et al., "Enhancement of cellular immune response in HIV-1 seropositive individuals: a DNA-based trial," *Clinical Immunology*, vol. 90, no. 1, pp. 100–107, 1999.

[14] K. Huygen, "DNA vaccines: application to tuberculosis," *International Journal of Tuberculosis and Lung Disease*, vol. 2, no. 12, pp. 971–978, 1998.