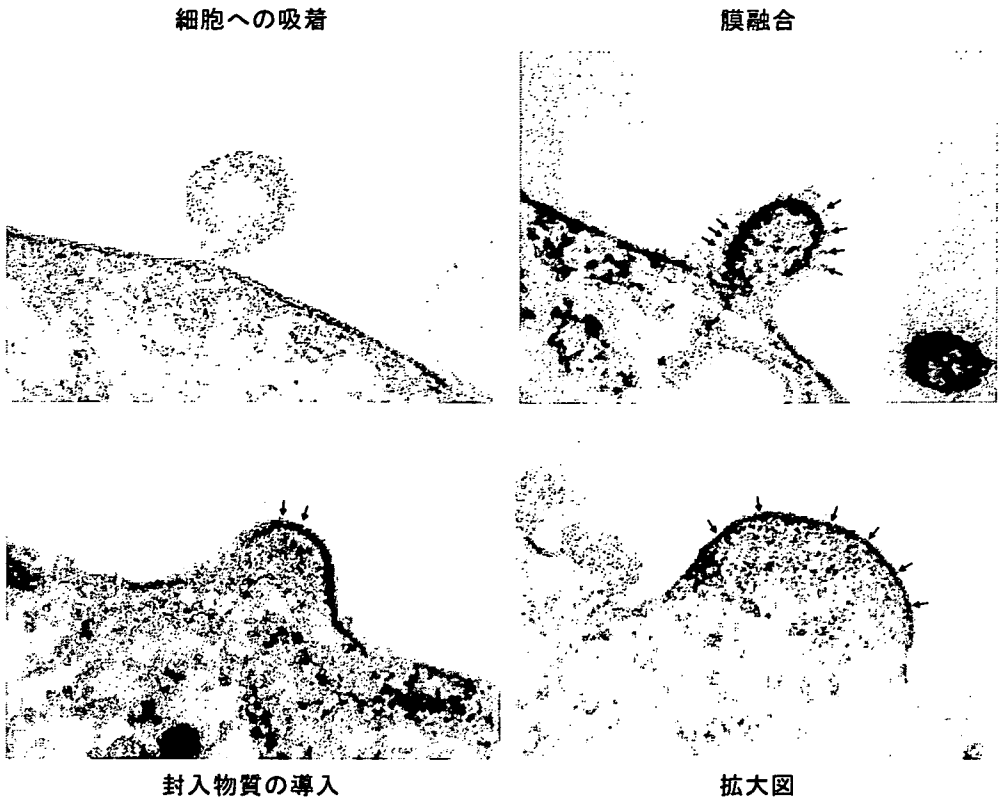
A. 膜融合による導入の電子顕微鏡写真



B. リポフェクションとの比較

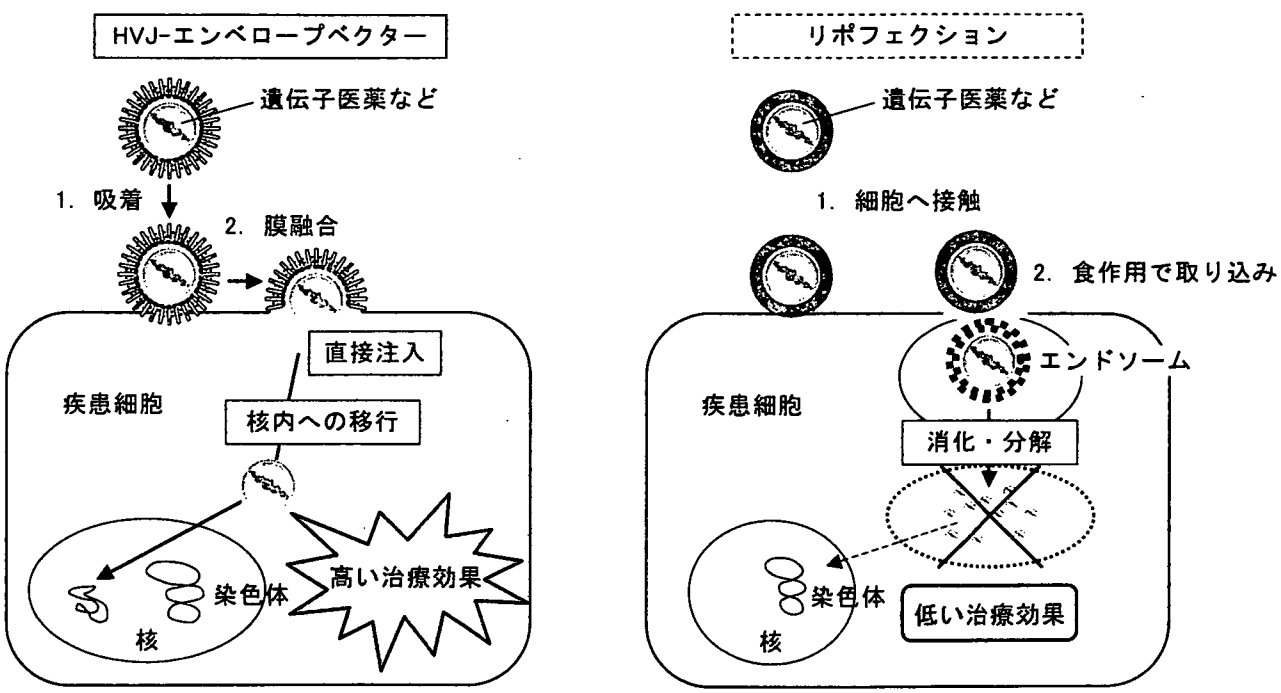


図5　膜融合による(HVJ-エンベロープベクター)導入の優位性

いながら，そのウイルスとしての性質については不活性化を行い，外膜部分のみをナノカプセルとして利用するため，位置付けとしては非ウイルス性の高性能ベクター(ドラッグデリバリーシステム)になると考えている。

2.4 HVJ-エンベロープベクターの特徴

HVJ-エンベロープベクターは，このように国内で開発が進められているユニークなデリバリーシステムである。そこで，その特徴について簡単に述べることにする。

第一の特徴は，ユニークな導入のメカニズムである。HVJ-エンベロープベクターにおいても膜融合により封入医薬品が直接標的細胞の細胞質に導入されるため，効率の高いデリバリーが可能である。その理由は，上記のようなエンドサイトーシスを介さないためであり，例えば核酸医薬を用いた検討では，正電荷リポソームで導入した場合には70％の核酸医薬の分解が認められたのに対して，HVJ-エンベロープベクターでは15％しか分解が認められなかったことが報告されている[18](図5B)。遺伝子医薬は製造原価が高額であることから，デリバリーされるまでに分解される比率を低減することは，医薬品として開発した場合のコストを考慮する上で重要な要因である。

第二の特徴は，広範な生体臓器や組織に対して低侵襲で高効率に導入できる点である。これまでに，動物試験においては脳神経組織，癌組織，筋組織，子宮，肝臓，心臓，肺，眼，脾臓，関節組織などに対して遺伝子医薬の導入が可能であることが明らかとなっている[12]。そして，HGF遺伝子をHVJ-エンベロープベクターに封入してラットの脳脊髄液内に注入することにより，延髄や小脳の神経細胞とグリア細胞，内耳の神経節細胞まで遺伝子導入が認められ，脳梗塞や難聴の予防と治療が可能であることが示唆されている[19),20]。また，Rad51に対する核酸医薬siRNAをHVJ-エンベロープベクターに封入して皮下に形成された癌組織に導入することにより，約50％の効率で細胞内導入することができ，シスプラチンの腫瘍抑制効果を10倍以上増強することも明らかになっている[21]。さらに，抗原遺伝子とサイトカインを産生する遺伝子をHVJ-エンベロープベクターに封入して，DNAワクチンとして筋肉内に導入することにより，感染症に対するワクチン効果と治療効果が認められることも明らかとなっている[22),23]。このように，動物の疾患モデルにおいて，実際に治療効果が認められるレベルの高い導入効率を持つことが明らかとなっている。さらに，導入効率が高いにも関わらず侵襲性が低いことも特徴である。例えば，心筋細胞に対して正電荷リポソームとHVJ-エンベロープベクターのそれぞれを用いて遺伝子を導入してLDHの放出を指標にして評価を行った実験では，正電荷リポソームではLDHの濃度が増加するのに対して，HVJ-エンベロープベクターの場合にはコントロールである無処理群と同レベルの濃度しか認められていない[24]。そのため，治療薬として連続投与を行う場合に問題となる投与部位の炎症反応も低レベルであり，マウスを用いた実験では連続投与が可能であることが明らかとなっている[25]。

第三の特徴は，治療に有効な免疫系を誘導することができる点である。詳細なメカニズムの解析については現在解析中であるが，癌細胞に対する免疫や，ウイルスや結核感染症の予防と治療に有効なTh1タイプの免疫を誘導できること，そして成立した免疫が長期間持続することが明らかとなっている。このような性質は，癌や感染症に対する特異的な免疫の活性化に有効であり，免疫療法剤としての開発が有望であることを示唆している。

2.5 HVJ-エンベロープベクターの臨床応用

HVJ-エンベロープベクターを医薬品として開発するためには，1) その特徴を活かした臨床プ

壊死，尾の痂皮形成あるいは尾の表皮剥離などが認められ，2,970 HAU 以下の群では，一般状態および剖検に異常はなかった。血液生化学検査では，9,450 および 7,830 HAU 群の雄で，LDH の高値およびA/G 比の低値がみられた。

9,450 HAU 群の生存例の病理組織学的検査では，投与部位皮下組織の浮腫，炎症，壊死，筋線維の再生，腎臓の尿細管好塩基性変化，細胞円柱，硝子円柱，メサンギウム細胞の増加，肺の血管周囲性単核細胞浸潤，限局性肺胞出血および肺胞単核細胞浸潤がみられた。1,350 HAU 以上の群の投与部位，腎臓あるいは肺でも同様の所見が認められたが，頻度は投与量の減少に伴い低下した。

3.3 単回，鼻腔内投与，マウス

HVJ-エンベロープベクターを 0(対照)，913，1,825 および 3,650 HAU/匹の用量で雌雄各群 5 匹のマウスに単回鼻腔内投与した。

死亡はいずれの群でもみられず，一般状態，体重および剖検も異常は認められなかった。病理組織学的検査では，肺の血管周囲性リンパ球浸潤が 913 HAU/匹以上の群で認められた。

3.4 反復，皮内投与，マウス

HVJ-エンベロープベクターの 0(生理食塩液，対照)，10，100，1,000，10,000，100,000 HAU/匹を各群 5 匹の雄 8 週齢のマウスに週 1 回計 2 回皮内投与し，その 14 日後に剖検，病理組織学的検査を実施した。

一般状態，体重に異常はなく，2 回目投与でアナフィラキシー症状はみられなかった。剖検では投与部位の皮膚に炎症性病変が認められ，病理組織学的には 10,000 HAU 以上では真皮の壊死，膿瘍形成，潰瘍形成および皮下膿瘍がみられたが，1,000 HAU まではそのような所見はなく，組織障害は軽度であった。

3.5 単回，静脈内投与，サル

16匹の雄カニクイザル(3～5 才，体重 2.8～4.6 kg)を用いて，単回，静脈内投与により HVJ-エンベロープベクターの安全性を検討した。試験には対照群(10 mL の生理食塩液投与，n＝4)，溶媒対照群(10 mL の HVJ-エンベロープリポソーム懸濁液投与，50 μM 脂質と HVJ-エンベロープベクター 66,666 HAU，n＝5)ならびに HVJ-リポソーム群(10 mL の DNA 非存在の HVJ-リポソーム懸濁液投与，50 μM 脂質と 666 μg の pcDNA3.1(＋)，HVJ-エンベロープベクター 66,666 HAU，n＝7)を設けた。

一般状態，体重，血液検査，血液生化学検査，尿検査は投与前，投与後 1，14，21，28 日目に測定あるいは実施し，観察期間終了後に剖検し，病理組織学的に検査した。その結果，一般状態，体重，血液学的検査，血液生化学検査および尿検査で異常は認められなかった。また，病理組織学的にも異常は認められなかった。pcDNA3.1(＋)が PCR 分析の結果，1 日目の全てのサルの肺，肝臓，脾臓，2 匹中 1 匹の腎臓，2 匹中 1 匹の心臓から検出された。しかし，DNA は 14，21，28 日の検査からは検出されなかった。ウイルスゲノム DNA も検出されなかった。

3.6 反復，筋肉内投与，サル

4 匹の雌雄カニクイザル(雄 2 匹，雌 2 匹)を用いて，反復，筋肉内投与による HVJ-エンベロープベクターの安全性を検討した[14]。2 mL の溶媒投与対照群〔HVJ-エンベロープリポソーム懸濁液(30 μM の脂質，40,000 HAU)〕，pUC-SR α/HGF を封入した HVJ-リポソーム群(30 μM 脂質，400 μgpUC-SR α/HGF，40,000 HAU)を設けた。

一般状態，血液検査，血液生化学検査および血清 HGF 検査は投与前，投与後 7，21，28，29 日目に実施したが，試験期間中，死亡はなく一般状

態も変化はなかった。血液検査でも顕著な変化はなかった。その他，毒性学的に意義のある変化は認められなかった。pUC-SRα/HGFを投与したサルにHGFの上昇が認められた。解剖および病理組織学的検査で異常所見は認められなかった。

3.7 非臨床安全性試験のまとめ

一般的にリポソームは，単球や顆粒球などの網内系細胞内に取り込まれやすいことが知られており[30),31)] そこで高濃度に分布し，その消失は穏やかであると考えられる。したがって，全身投与された場合，細網内皮系臓器への影響については注意しておく必要がある。また，薬物動態についても非ウイルスベクターは特定の臓器に取り込まれやすいと考えられるため，毒性との関連で十分検討しておく必要があると思われる。

しかし，今まで述べてきた安全性試験から，HVJ-エンベロープベクターの細網内皮系臓器への障害を示唆するような所見は得られていない。

以上，筆者らが実施した薬効モデル動物を使用した試験から得られた薬効量の約10倍量でも重篤な毒性が認められていないことからヒトでも十分な治療域が確保できると考えられた。これらのことから現在，膀胱癌を適応症として臨床試験を準備している。さらに今後，固形がん，感染症領域にも適応を拡大すべく検討している。

4. HVJ-エンベロープベクターの製造技術

4.1 製造技術の概要

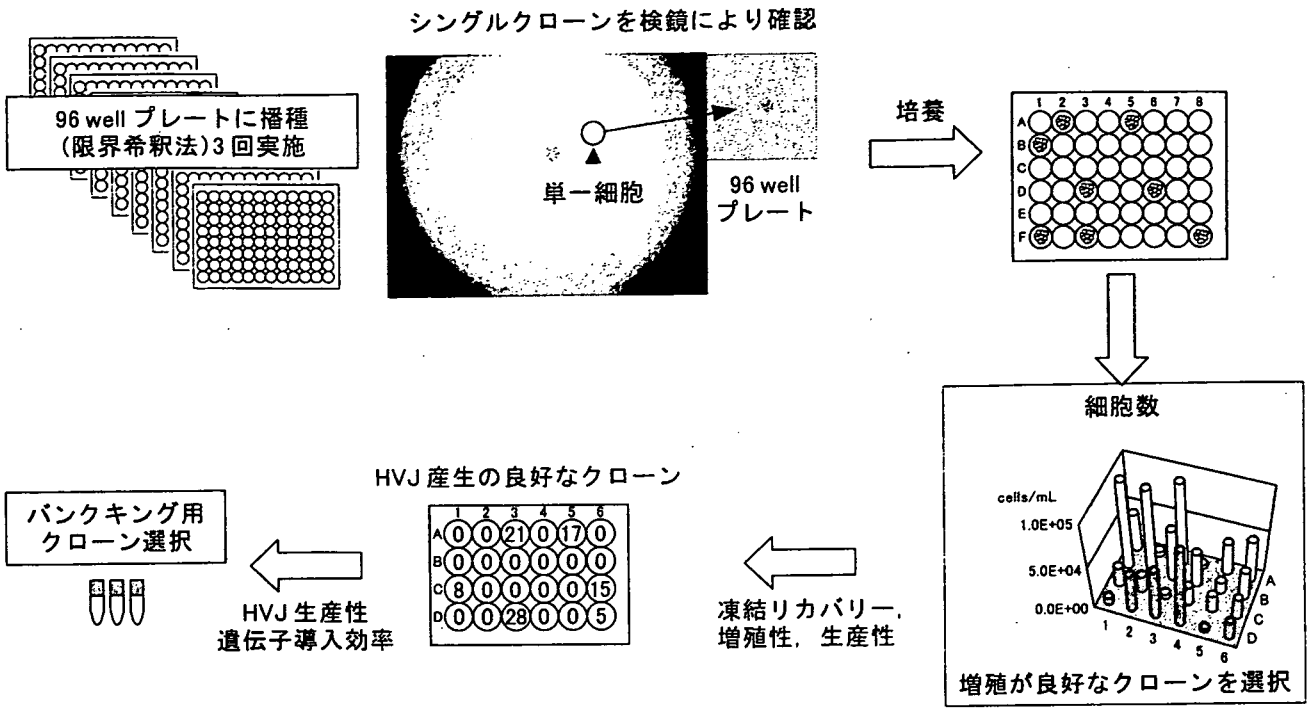
HVJ-エンベロープベクターは，ヒト培養細胞により産生されるバイオ医薬となるため，3項で述べたような確認申請が必要になると考えられる。ここでは，確認申請に必要とされる項目のうち，1) 製造方法の概要(原材料に関するデータも含む製造工程)，2) 品質管理，3) 安定性，4) 細胞・組織利用医薬品または医療用具の製造施設および設備に関連するデータについて記述する。

4.2 製造用材料(マスターバンク)の整備

現在HVJ-エンベロープベクターは，ヒト培養細胞を用いて製造を行っている。したがって，確認申請のためには，製造に使用する細胞の履歴に関する書類を整備した上でマスターセルバンクの作成を行い，ガイドラインに規定されている項目について検査を行う必要がある。筆者らは，製造に用いる予定の細胞とHVJについては，ガイドラインに従ってマスターバンク(マスターセルバンク，マスターウイルスバンク)の整備を行い，確認申請のために準備を行っている。そこで，整備過程も含めて，その概要について記述する。

HVJ-エンベロープベクターを構成する膜成分は基本的に産生細胞の細胞膜となるため，抗原性などの問題を低減することを目的として，ヒト培養細胞株を選択してマスターセルバンクの作成を実施した。バンクを作成するために，細胞のクローニングを実施(顕微鏡下で確実に単一細胞であることを確認)した後に，必要なスケールまで培養を行った。そして，購入からクローニングの過程における原材料の使用履歴，細胞増殖性，継代数，細胞数倍化レベル(PDL)など，必要な履歴についてはデータとして保存した。細胞培養に関しては，動物由来原料を含有しない無血清培養を行って，BSEなど混入を防止するようにした。そして，HVJの産生効率や細胞の増殖速度を指標にしてクローンの選択を行った後に(図6A)，必要なスケールまで拡大培養を行った時点でシードセルストックとして一旦凍結保存して，ウイルスなどの混入や無菌性についてGLP施設で検査を行って品質を確認した。さらに，シードセルストックからGMP準拠の自社施設のクリーンルーム内でマスターセルバンクの作成を行った。作成

A. マスターセルバンクの整備



B. 整備したマスターバンクの保存

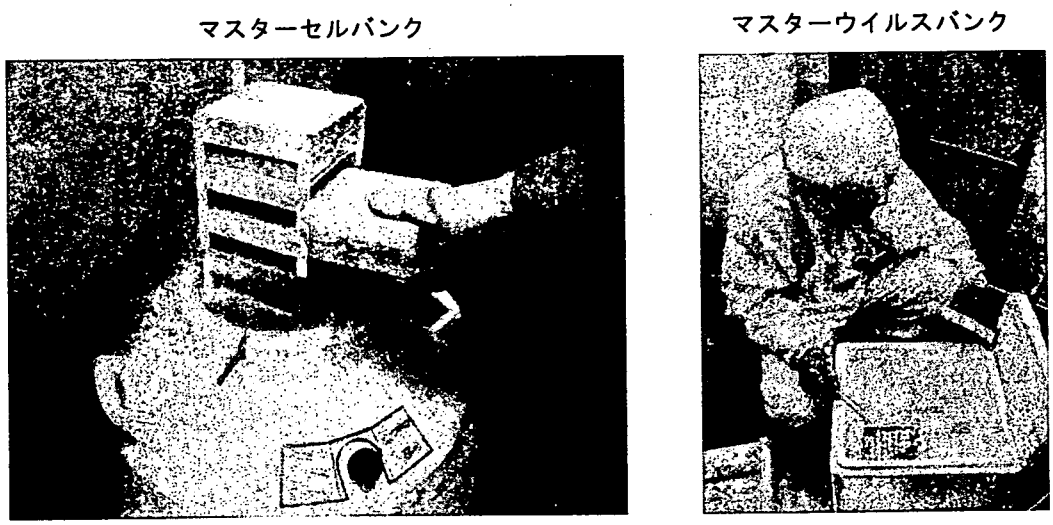


図 6　確認申請に向けたマスターバンクの整備

したマスターセルバンクの品質検査は，ガイドラインに従って検査項目を設定した上，GLP 施設を持つ外部委託機関により無菌性，マイコプラズマ混入否定試験，ウイルス混入否定試験，細胞の純度試験を実施して，それぞれの項目について陰性の結果を得た。作成したマスターセルバンクについては，リスク管理のために自社の製造施設と，国外の保管管理会社の両施設に分割して保管・管理を行っている(図 6B)。現在，バンクから起眠後製造に使用できる期間の設定を行った上で，CAL 試験(Cell At the Limit)を実施している。

HVJ についても，ATCC より購入後にクローニングを実施して履歴の整備を行った後に，産生効率を指標にしてシードストックを作成した。そ

して，上記のようにして整備を行ったマスターセルバンクを使用して，シードストックからGMP準拠の自社施設内でマスターウイルスバンクの作成を行った。さらに，マスターセルバンクと同様にガイドラインに従って必要な検査項目の設定を行った上で，GLP検査機関に委託して品質検査を行った。現在，自社の製造施設において作成したマスターウイルスバンクの保存管理を行っている(図6B)。

以上のように，バイオ医薬製造に必要なマスターバンク(細胞とHVJ)については自社において整備を行った上で，第三者機関である外部試験委託機関においてGLP品質検査試験を行って必要なデータを取得した上，製造用バンクとして保管管理を行っている。

4.3 バイオリアクターシステムによる製造

従来HVJ-エンベロープベクターは，発育鶏卵を用いて原料の製造を行っていた。この方法は，インターフェロン製造用原料として使用するHVJを産生する場合に採用されている方法である。また，ワクチン製造用のウイルスを産生する場合にも，同様の方法が採用されてきた。しかし，鶏卵の場合には品質的に季節変動があること，緊急時にニワトリを大量に飼育して迅速に対応することは困難であること，SPFレベルの発育鶏卵を大量に準備することが困難であること，無菌的に粒子を回収することが困難であることが課題であった。そこで，近年ワクチン製造用としては，発育鶏卵ではなく培養細胞を使用したバイオリアクターシステムによる無菌製造への切換えが進められている[32]。また，HVJ-エンベロープベクターの場合は，産生細胞の細胞膜成分を含むことから，発育鶏卵を製造に使用するとニワトリ由来成分の混入が起こるため，治療薬の製造には適当でないと判断された。そこで，ヒト培養細胞を用いたバイオリアクターシステムによる製造技術の開発を行った(図7)。

従来，実験室レベルでは培養細胞を用いたHVJの産生には付着細胞が用いられていたが，産生効率が非常に低レベルであることや，ビーズなどを使用した培養の場合には工業的なスケールアップを行うための工程が複雑になることから，使用する付着細胞を浮遊培養に馴化させた上，高密度培養を行うための条件検討を行った。さらに，種々の培養条件の検討を実施することで発育鶏卵により，製造した場合と同レベル以上の産生効率を得ることに成功している。さらに，BSE混入の問題を回避するために，動物由来成分を含まない無血清培地中での培養による製造技術を開発している。

バイオリアクターシステムに関しても，製造機器メーカーと共同で開発を行い，現在までにスケールアップが可能な無血清培地中で，浮遊培養条件下でバイオリアクターシステムによる製造を行っており，上記のように整備したバンクを使用して品質的にも安定したHVJ-エンベロープベクターを供給できる状況である。このように，従来は困難であったヒト培養細胞を用いてスケールアップ可能で医用材料レベルの製造に適合した技術を開発した上で，生産効率的にも従来法である発育鶏卵と同レベル以上の数値を達成している。今後は，治験薬製造に向けて工程のバリデーションを実施していく予定である。

4.4 カラムクロマトグラフィー法による精製

従来，ウイルスベクターやワクチン用ウイルスの製造においては，密度勾配遠心法による精製が一般的であった。しかし，筆者らは工業的なスケールアップに対応することが容易であること，治験薬GMPグレードの工程開発が可能であること，精製効率の高いこと，欧米での実績などを考慮して，カラムクロマトグラフィー法による精製技術を開発した(図8)。従来は，エンベロープウイルス粒子は物理的に弱いために精製工程で破壊

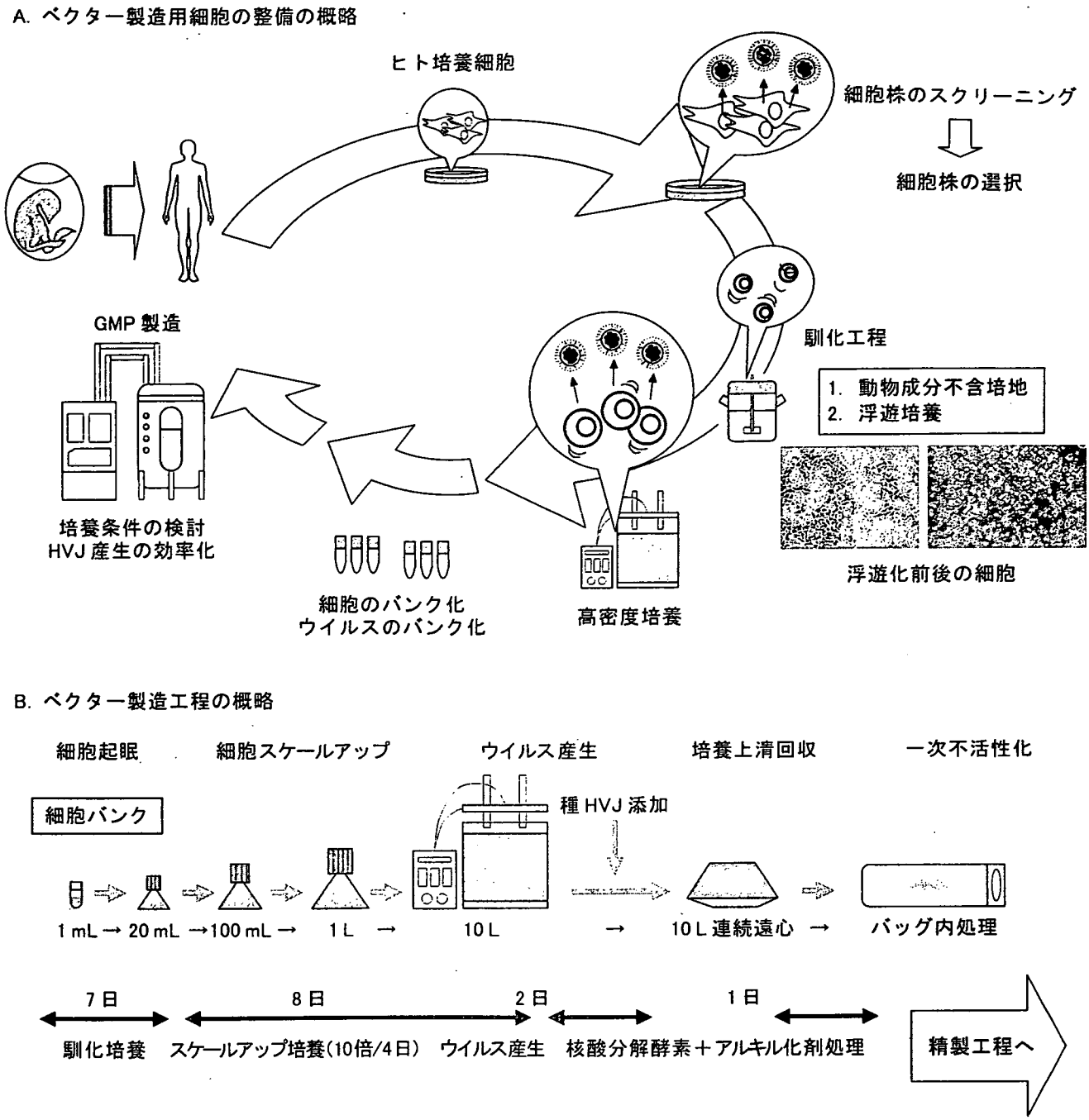
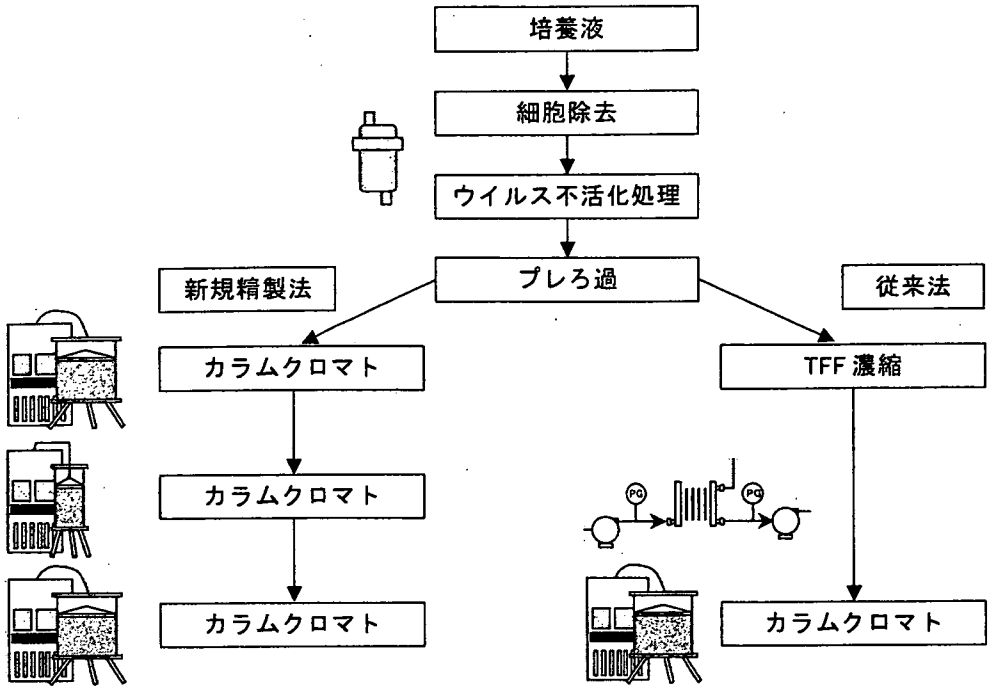


図 7　バイオリアクターシステムによるベクターの製造

されてしまうために回収率が悪いと考えられていた上，数百ナノメートルの粒子の場合にはカラムに充填した樹脂のボイド部分を素通りしてしまうために，官能基によるトラップ効率が悪くなることが予測されていたため，カラムクロマトでの精製は困難であると考えられていた。HVJ-エンベロープの膜部分も基本的に細胞膜と同様であり，非常にマイルドな条件で精製工程を構築する必要があるため，特にせん断力がかかる濃縮工程を省くことを目的として，種々の条件を設定して試行錯誤を行った。その結果，現在までに 3 段のカラムを組み合わせることで膜濃縮工程を含まない精製工程を構築して，原薬となる HVJ-エンベロープベクターを製造することに成功している。収率の面でも，初期に予測された 10 %程度という数値を大きく上回る数値を得ており，工業レベルで

A. ベクター精製法の改良

3種類のカラムクロマトグラフィー法の組み合わせにより，従来法で実施していた濃縮工程を除外した。



B. ベクター精製工程の概略

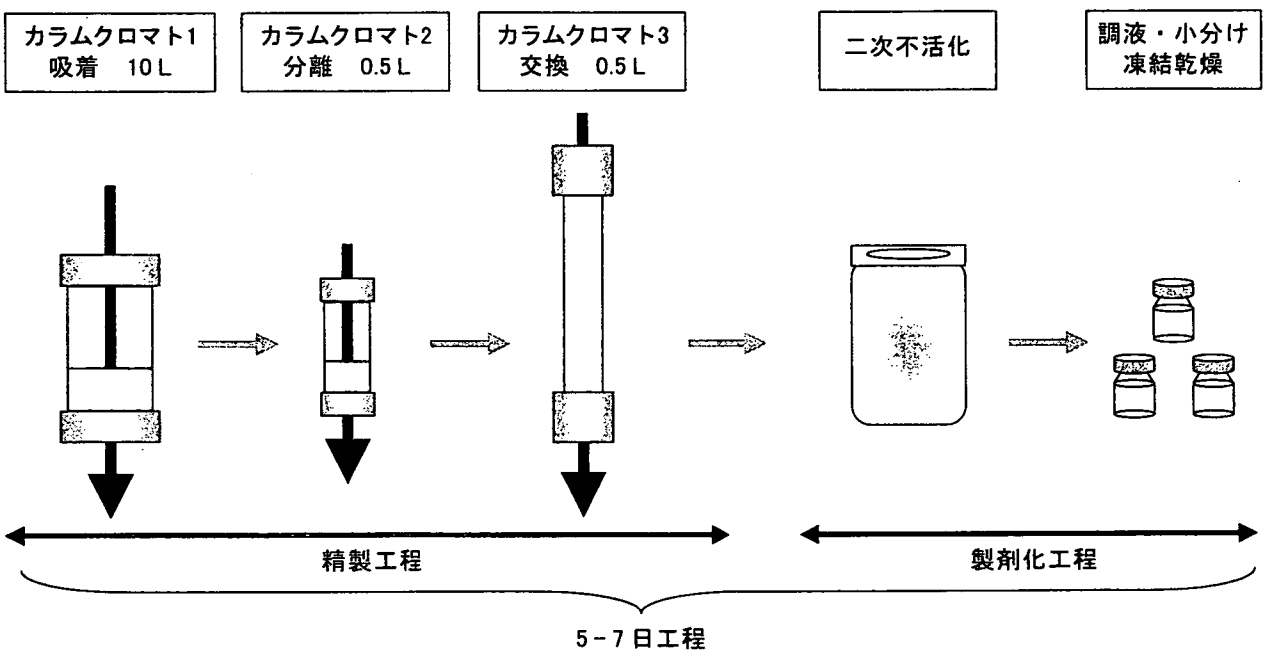


図8　カラムクロマトグラフィー法によるベクターの精製

も充分に通用する工程を構築している。今後，封入する遺伝子医薬の特性を考慮した上で，精製工程の改良とバリデーションの実施を進めていく予定である。

4.5　凍結乾燥法による製剤化

前項でも述べたように，従来エンベロープウイルスやHVJ-エンベロープベクターのように，物理的に弱い脂質二重膜を含む粒子は，限外濾過法やタンジェンシャルフロー濾過法による濃縮や，凍結乾燥は困難であると考えられていた。しかし，当時米国の遺伝子治療企業であるジェネティックセラピー社(Genetic Therapy Inc：GTI)で遺伝子治療用ベクターの製造技術を開発していた小谷らは，レトロウイルスベクターを凍結乾燥法により失活させることなく濃縮・製剤化できることを報告した[34]。この技術開発は，脂質二重膜により構成される粒子のように脆弱な構造を持つものであっても，凍結乾燥法による製剤化が可能であることを示す画期的な出来事であった。筆者らの研究所においても，HVJ-エンベロープベクターの最終製剤化には凍結乾燥法を想定して，添加剤の種類や濃度，温度条件など種々の条件検討を行っている。現在までの検討により，凍結乾燥により遺伝子導入活性を保持した状態で製剤化できることが明らかとなっている(図9)。今後はさらに封

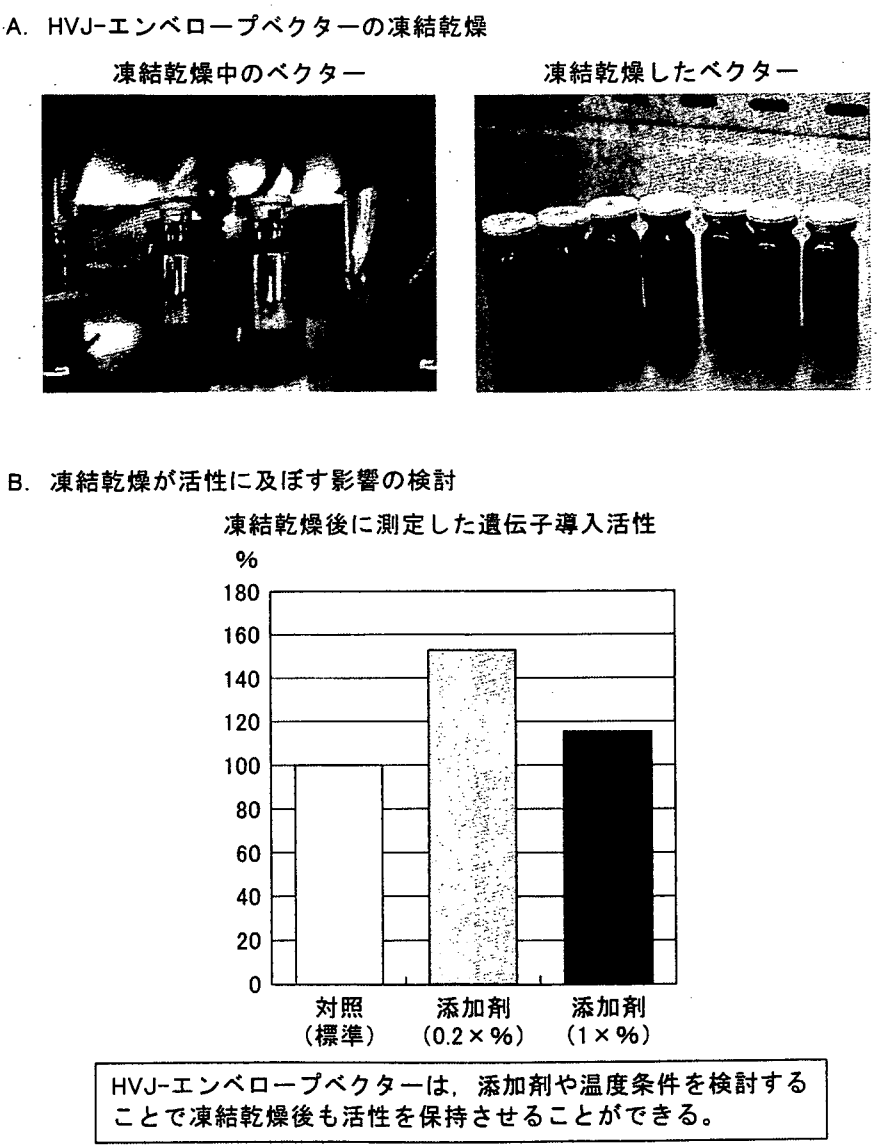


図9　凍結乾燥法による濃縮と製剤化検討

入する遺伝子医薬に応じて凍結乾燥条件の調整を行って，最終製剤化条件を設定した後に，工程のバリデーションと安定性試験を実施する予定である。また，品質管理項目や規格値については，現在暫定的に設定してデータの取得を行って，設定が適切であるかを検討している状況である。今後は，規制当局との相談を通じて最終的な設定を進めていく予定である。

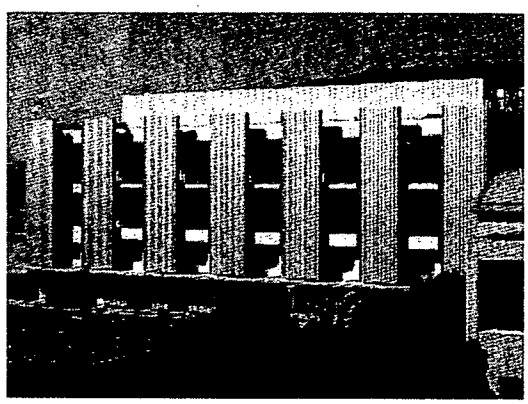
A. 製造用設備

バイオリアクターシステム

1L　　10L　　リアクターシステム全体　　精製システム

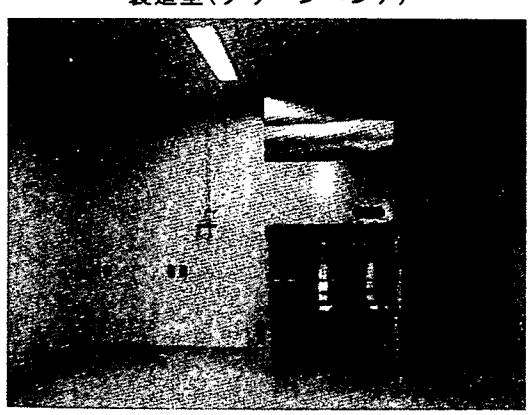
B. HVJ-エンベロープベクター製造用施設

外　観



製造室(滅菌水，スチーム)

製造室(クリーンベンチ)



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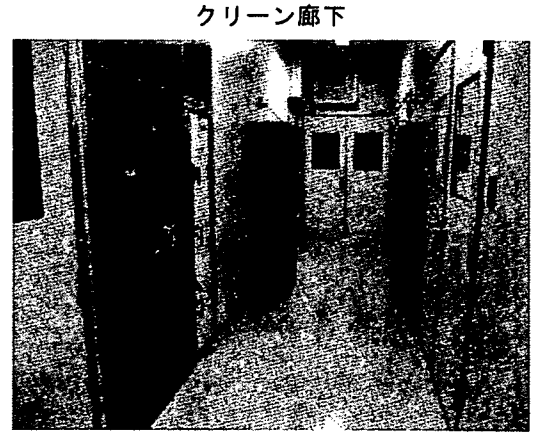

図10　HVJ-エンベロープベクター製造用施設・設備の整備

4.6 HVJ-エンベロープベクターの製造設備

4.1項の確認申請に関する項目の7)に記述したように，HVJ-エンベロープベクターの確認申請で承認を得るためには，ベクター製造用施設・設備の整備が重要である。筆者らは，産業技術総合研究所・関西センターと共同で，規制当局のガイドラインに準拠したバイオ医薬品候補のための治験薬製造施設の整備を行い，製造用機器の設置前に施設バリデーションデータを取得した。その後，製造用機器を設置しており，上記のように製造工程のバリデーションを進めるのと並行して，施設についても稼動状態でのモニタリングを進めて，申請に必要なバリデーションデータを取得する予定である(図10)。

5. 今後の課題

将来，HVJ-エンベロープベクターを遺伝子医薬用のデリバリーシステムとして汎用性の高いデリバリーシステムとしていくためには，標的化ベクターを開発していくことが重要であると考えられる。現在までに，表面電荷の調整，化学的な修飾，エンベロープタンパク質のキメラ化(単鎖抗体，リガンドなど)，突然変異体の単離など種々のアプローチで標的化に最適な技術の開発を行っている。特に癌細胞への標的化は，治療の有効性を向上する上で重要であるため，今後重点的に開発を進めていきたい。

謝　辞

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(中島俊洋／長澤鉄二／和田 博)

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Identification of an HLA-A*0201-Restricted T-Cell Epitope on the MPT51 Protein, a Major Secreted Protein Derived from *Mycobacterium tuberculosis*, by MPT51 Overlapping Peptide Screening[▽]

Taiki Aoshi,[1] Toshi Nagata,[2]* Mina Suzuki,[1] Masato Uchijima,[1] Dai Hashimoto,[2] Alireza Rafiei,[1] AQ: A Takafumi Suda,[2] Kingo Chida,[2] and Yukio Koide[1]

Department of Infectious Diseases,[1] Department of Health Science,[2] and Department of Internal Medicine, Second Division,[3] Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan

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CD8$^+$ T cells play a pivotal role in protection against *Mycobacterium tuberculosis* infection. We identified a novel HLA-A*0201-restricted CD8$^+$ T-cell epitope on a dominant secreted antigen of *M. tuberculosis*, MPT51, in HLA-A*0201 transgenic HHD mice. HHD mice were immunized with plasmid DNA encoding MPT51 with gene gun bombardment, and gamma interferon (IFN-γ) production by the immune splenocytes was analyzed. In response to overlapping synthetic peptides covering the mature MPT51 sequence, the splenocytes were stimulated to produce IFN-γ by only one peptide, p51-70. Three-color flow cytometric analysis of intracellular IFN-γ and cell surface CD4 and CD8 staining revealed that the MPT51 p51-70 peptide contains an immunodominant CD8$^+$ T-cell epitope. Further analysis using computer algorithms permitted identification of a bona fide T-cell epitope, p53-62. A major histocompatibility complex class I stabilization assay using T2 cells confirmed that this epitope binds to HLA-A*0201. The T cells were capable of lysing MPT51 p53-62 peptide-pulsed T2 cells. In addition, MPT51 p53-62-specific memory CD8$^+$ T cells were found in tuberculin skin test-positive HLA-A*0201$^+$ healthy individuals. Use of this HLA-A*0201-restricted CD8$^+$ T-cell epitope for analysis of the role of MPT51-specific T cells in *M. tuberculosis* infection and for design of vaccines against AQ: B **tuberculosis is feasible.**

Fn1 Tuberculosis (TB) is still a major cause of death due to infectious disease worldwide. There were an estimated 8.8 million new cases in 2005, and 1.6 million people died of TB (37). The problem of TB is increasing worldwide due to several factors, including the prevalence of multi-drug-resistant strains and coinfection with human immunodeficiency virus (23). The only TB vaccine currently available is the attenuated *Mycobacterium bovis* strain bacillus Calmette-Guérin (BCG), yet its efficacy against pulmonary TB in adults has been controversial (32). Therefore, there is an urgent need for an improved vaccine for TB (16).

Cell-mediated immunity plays a pivotal role in the control of *Mycobacterium tuberculosis* infection. There is mounting evidence that CD4$^+$ type 1 helper T lymphocytes (Th1) are involved in the development of resistance to this disease, primarily through the production of macrophage-activating cytokines, such as gamma interferon (IFN-γ) and tumor necrosis factor alpha. In addition, CD8$^+$ cytotoxic T lymphocytes (CTL) contribute to disease resistance since susceptibility to *M. tuberculosis* is increased in mice with a deficiency in CD8$^+$ T cells (17, 18, 31).

To design a new generation of vaccines, information on the antigenic make-up of *M. tuberculosis* must be obtained in order to identify immunodominant proteins and epitopes. Secreted and surface-exposed cell wall proteins seem to play a pivotal role in the induction of protective cellular immunity against TB (2, 4). The mouse model of TB infection revealed that memory cells from immune mice produced substantial amounts of IFN-γ in response to two fractions of culture filtrate of *M. tuberculosis*, represented by 6- to 10-kDa proteins and the antigen 85 (Ag85) complex, a 30- to 32-kDa protein family (3).

The Ag85 complex (Ag85A, Ag85B, and Ag85C), which has mycolyltransferase activity in cell wall synthesis and in the biogenesis of cord factor (5) and the ability to bind to fibronectin (1), has been shown to be a major fraction of the secreted proteins of *M. tuberculosis* (35). Another major secreted protein, MPT51, was demonstrated to cross-react with the three components of the Ag85 complex at antibody levels and to exhibit primary protein structure similarity (37 to 43% at the amino acid level) with these components (22, 36). Using a DNA vaccine encoding MPT51, we found that MPT51 can induce specific cellular immune responses and protective immunity against challenge with *M. tuberculosis* (20), and we identified murine T-cell epitopes using C57BL/6 and BALB/c mouse strains (34).

Here, we identified an HLA-A*0201-restricted CD8$^+$ T-cell epitope on MPT51 by using a strategy that included HLA-A*0201 transgenic mice, gene gun immunization with expression plasmid DNA encoding MPT51, overlapping synthetic peptides spanning the entire mature MPT51 amino acid sequence, and computer algorithms.

* Corresponding author. Mailing address: Department of Health Science, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan. Phone and fax: 81-53-435-2332. E-mail: tnagata@hama-med.ac.jp.

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MATERIALS AND METHODS

Mice. HLA-A*0201 transgenic mice (HHD mice) (25) were kindly donated by F. A. Lemonnier (Pasteur Institute, France). HHD mice express a monochain in which the C terminus of human β2-microglobulin is covalently linked to the N terminus of the HLA-A2.1 heavy chain in a chimeric configuration (α3 domain of mouse origin) (25). In HHD mice, the HLA-A*0201 monochain is the only type of major histocompatibility complex (MHC) class I molecule expressed. The mice were kept under specific-pathogen-free conditions and fed autoclaved food and water ad libitum at the Institute for Experimental Animals of the Hamamatsu University School of Medicine. Two- to 3-month-old female mice were used in all experiments. Animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine. We confirmed that HLA-A*0201 was expressed on spleen cells of the HHD mice that we used (data not shown).

Human subjects. HLA-A*0201$^+$ healthy donors who had previously been vaccinated with *M. bovis* BCG were recruited from the Hamamatsu University School of Medicine. Blood samples were taken after written permission was obtained from the individuals participating in this study.

Peptides. Peptides spanning the entire mature MPT51 amino acid sequence of *M. tuberculosis* (266 amino acid residues) were synthesized as 20-mers overlapping by 10 residues; the only exception was the carboxyl-terminal 12-mer from amino acid 255 to amino acid 266, which was described previously (34). Briefly, lyophilized peptides were purchased from Invitrogen Corporation (Carlsbad, CA), and the purity was confirmed by mass spectrometry. To identify the potential HLA-A*0201-restricted CD8$^+$ T-cell epitopes in a 20-mer peptide, computer-based T-cell epitope prediction algorithm programs were used, which were accessed through the websites of the National Institutes of Health BioInformatics and Molecular Analysis Section (BIMAS) HLA Peptide Binding Predictions (http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform) (24) and SYFPEITHI Epitope Prediction (http://www.syfpeithi.de/) (27). All peptides were dissolved in distilled water to obtain a concentration of 1 mM and stored at −80°C until use.

Immunization of mice. Mice were immunized with pCI-MPT51, a plasmid DNA vaccine encoding the mature MPT51 molecule (34), employing a gene gun bombardment system. For DNA immunization with the Helios gene gun system (Bio-Rad Laboratories, Hercules, CA), cartridges of DNA-coated gold particles were prepared according to the manufacturer's instructions. To immunize mice, the shaved abdominal skin was wiped with 70% ethanol. Mice were inoculated with 2 μg of the plasmid DNA four times at 1-week intervals.

Cell lines. The human transporter associated with peptide loading (TAP)-deficient T2 cell line (29) was kindly donated by Peter Creswell (Yale University School of Medicine). The cells were cultured in RPMI 1640 medium (Sigma-Aldrich, Inc., St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (Thermo Electron, Melbourne, Australia) (RPMI/10FCS) in an incubator with a humidified atmosphere containing 5% CO_2.

Preparation of splenocyte culture supernatants and measurement of IFN-γ amounts. Spleen cells were harvested from MPT51 DNA-immune mice. Recovered cells were plated in 96-well plates at a concentration of 1×10^6 cells per well in the presence or absence of 5 μM of each MPT51 peptide at 37°C with an atmosphere containing 5% CO_2. Supernatants were harvested 24 h later and stored at −20°C until they were assayed. The concentration of IFN-γ in the culture supernatants was determined by a sandwich enzyme-linked immunosorbent assay (ELISA). The ELISA was carried out as described previously (34), with some modifications. The following method was used. The 96-well ELISA plates (EIA/RIA A/2; Costar, Cambridge, MA) were coated with 2 μg ml^{-1} of capture antibody (anti-murine IFN-γ monoclonal antibody [MAb] R4-6A2; BD Biosciences, San Jose, CA) at 4°C overnight, washed with phosphate-buffered saline supplemented with 0.05% Tween 20 (PBS-Tween), and blocked with Block One blocking solution (Nakalai Tesque, Kyoto, Japan) at room temperature for 45 min. After washing with PBS-Tween, the culture supernatants were added to the plates and the plates were incubated at 4°C overnight. After washing with PBS-Tween, 0.5 μg ml^{-1} of biotin-labeled anti-murine IFN-γ MAb XMG1.2 (BD Biosciences) was added to the plates, and the plates were incubated for 1 h at room temperature. After washing with PBS-Tween, horseradish peroxidase-conjugated streptavidin (eBioscience, San Diego, CA) was added, and the preparations were incubated for 30 min at room temperature. After washing, the 3,3′,5,5′-tetramethylbenzidine one-component horseradish peroxidase microwell substrate (BioFX Laboratories, Owings Mills, MD) was added to the plates to detect bound horseradish peroxidase-conjugated streptavidin. After 5 min, the enzyme reaction was stopped by adding 2 M H_2SO_4, and then the absorbance at 450 nm was measured using an EZS-ABS microplate reader (Asahi Techno Glass, Tokyo, Japan).

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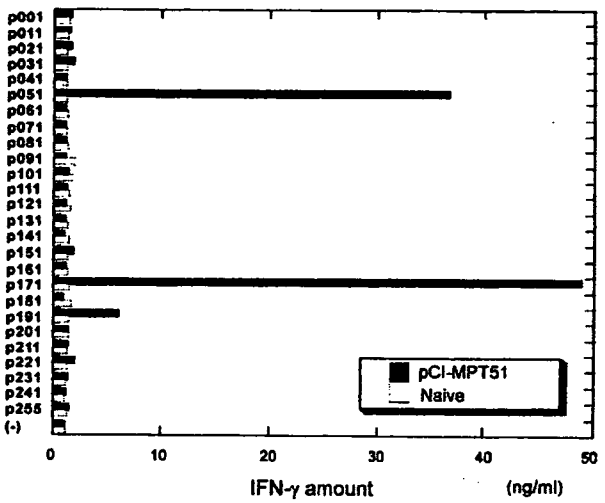

FIG. 1. IFN-γ production by spleen cells from HHD mice immunized with pCI-MPT51. The IFN-γ production by splenocytes from HHD mice immunized with the pCI-MPT51 plasmid in response to 1 of 26 overlapping peptides (5 μM) covering the MPT51 molecule or medium alone (−) was evaluated. Splenocytes from naïve HHD mice were also examined as a control. The data are representative of the results of three independent experiments.

MHC stabilization assay. The abilities of peptides to bind to HLA-A*0201 were measured by determining the stabilization of class I molecules on the surface of T2 cells (33). T2 cells (1×10^6 cells ml^{-1}) were cultured at 26°C overnight and then incubated for 1 h in the presence or absence of peptides (50 or 250 μM). Cells were then incubated at 37°C for 2 h and washed with FACS buffer [phosphate-buffered saline supplemented with 1% fetal calf serum], and the cell surface expression of HLA-A*0201 molecules was detected by flow cytometry (EPICS XL; Beckman Coulter, Fullerton, CA) using a mouse MAb specific for HLA class I molecules (34-1-2S; Cedarlane, Ontario, Canada), followed by treatment with fluorescein isothiocyanate (FITC)-labeled anti-mouse immunoglobulin antibodies (Rockland, Gilbertsville, PA). The results were expressed as the mean fluorescence intensity (MFI) ratio, determined as follows: [(MFI observed in the presence of peptide at 37°C/MFI observed in the absence of peptide at 26°C) − (MFI observed in the absence of peptide at 37°C/MFI observed in the absence of peptide at 26°C)] × 100.

Intracellular IFN-γ staining. An antigen-specific T-cell subset was also identified by simultaneous flow cytometric assessment of the T-cell phenotype and intracellular IFN-γ synthesis.

The methods used for cell surface staining of CD4 and CD8 and intracellular IFN-γ staining have been described previously (34). Intracellular IFN-γ staining was performed using a Cytofix/Cytoperm Plus (with GolgiStop) kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions.

Cytotoxicity assay. One week after the last immunization, immune spleen cells (2×10^7 cells) were cocultured for 5 days with 2×10^7 syngeneic splenocytes treated with 100 μg ml^{-1} of mitomycin C (Kyowa Hakko, Tokyo, Japan) and pulsed with peptide for 2 h at 37°C. Each well also received 10 U ml^{-1} of human recombinant interleukin-2 (Hoffmann-La Roche, Nutley, NJ). Cell-mediated cytotoxicity was measured by using a conventional ^{51}Cr release assay as described previously (34). Briefly, the target cells used in this study were T2 cells pulsed with peptide at a concentration of 1 μM for 15 h at 37°C. Target cells (1×10^4 cells/well) were incubated for 5 h in triplicate at 37°C with serial dilutions of effector cells, and the level of specific lysis of the target cells was determined by using the following equation: percentage of specific lysis = [(experimental counts per minute − spontaneous counts per minute)/(total counts per minute − spontaneous counts per minute)] × 100.

Tetramer staining. A phycoerythrin (PE)-labeled HLA-A*0201/MPT51 p53-62 tetramer complex was kindly supplied by the NIH Tetramer Facility. After 10 days of in vitro stimulation with the MPT51 p53-62 peptide, spleen cells of immune HHD mice were treated with ammonium chloride and potassium chloride lysis buffer for 5 min at room temperature to remove erythrocytes,

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TABLE 1. Candidate HLA-A*0201-restricted T-cell epitopes in the p51-70 peptide of the MPT51 molecule

Peptide[a]	Amino acid sequence[b]	Estimated scores for restriction molecules	
		BIMAS	SYFPEITHI
p51-70	MNTLAGKGISVVAPAG GAYS		
Nonamers			
p53-61	**TLAGKGISV**	69.552	27
p54-62	LAGKGISVV	1.549	22
Decamers			
p53-62	**TLAGKGISVV**	65.588	28
p50-59	AMNTLAGKGI	7.535	19
p52-61	NTLAGKGISV	3.574	18

[a] Data for peptides ranked in the top 20 in the BIMAS or SYFPEITHI algorithms are shown.
[b] Boldface type indicates peptide sequences that were synthesized and used for experiments. Underlining indicates anchor residues. The G residues in p53-62 are residues that are associated with good binding to A*0201, as suggested by Ruppert et al. (28).

washed twice with RPMI 1640 medium, and resuspended in RPMI/10FCS. For some experiments, peripheral blood mononuclear cells (PBMCs) from purified protein derivative (PPD)-reactive HLA-A*0201$^+$ human healthy subjects were prepared by LeucoSep (Greiner Bio-One, Frickenhausen, Germany) according to the manufacturer's instructions. These cells (1×10^6 cells) were washed twice with FACS buffer and stained with the PE-labeled HLA-A*0201/MPT51 p53-62 tetramer and FITC-labeled anti-mouse or -human CD8 MAb for 30 min at 4°C. The cells were then washed with FACS buffer twice and analyzed with a digital flow cytometer (EPICS XL; Beckman Coulter).

RESULTS

IFN-γ production in response to overlapping synthetic peptides from MPT51 in HHD mice. Splenocytes from HHD mice immunized with a DNA vaccine encoding mature MPT51 (pCI-MPT51) were stimulated with the overlapping MPT51 peptides for 24 h, and the IFN-γ concentrations in culture supernatants were determined by ELISA. As shown in Fig. 1, robust IFN-γ production was observed in splenocytes from MPT51 DNA-vaccinated HHD mice after stimulation with peptide 51 (p51) (amino acid residues 51 to 70) and peptide 171 (p171) (amino acid residues 171 to 190). In addition, weak IFN-γ production was observed in the splenocytes in the presence of peptide 191 (p191) (amino acid residues 191 to 210). Since the HHD mice that we used in this study had a C57BL/6 background (25) and we observed that only CD4$^+$ T cells produced IFN-γ in response to p171 and p191, we concluded that CD4$^+$ T cells responded to these peptides presented on H2-A^b molecules and produced IFN-γ (34). As expected, spleen cells from naïve HHD mice showed no significant IFN-γ production in response to any MPT51 peptide.

Identification of a 10-mer CD8$^+$ T-cell epitope in peptide p51-70 of MPT51. Since CD8$^+$ T-cell epitopes presented by MHC class I molecules comprise 8 to 10 amino acids and are generally 9 amino acids long, we pursued a line of inquiry to identify the fine HLA-A*0201-restricted CD8$^+$ T-cell epitope. We predicted candidate peptides in the 20-mer peptide by using the computer-based programs BIMAS HLA Peptide Binding Predictions and SYFPEITHI Epitope Prediction. Using the BIMAS program, we found that a 9-mer peptide, p53-61 (TLAGKGISV), and a 10-mer peptide, p53-62 (TLAGKGISVV), showed high scores for binding to the HLA-A*0201 molecule in the region containing amino acid residues 51 to 70 (the binding scores were 69.552 for p53-61 and 65.588 for p53-62) (Table 1). In addition, the SYFPEITHI program also produced high scores for these peptides (27 for p53-61 and 28 for p53-62) (Table 1). Therefore, we synthesized p53-61 (TLAGKGISV) and p53-62 (TLAGKGISVV). In addition, we synthesized the p21-29 peptide (FLAGGPHAV) since this peptide had the highest HLA-A*0201 binding scores with the BIMAS and SYFPEITHI programs (319.939 and 29, respectively). Three-color flow cytometric analysis showed that the number of IFN-γ-producing CD8$^+$ T cells increased in the

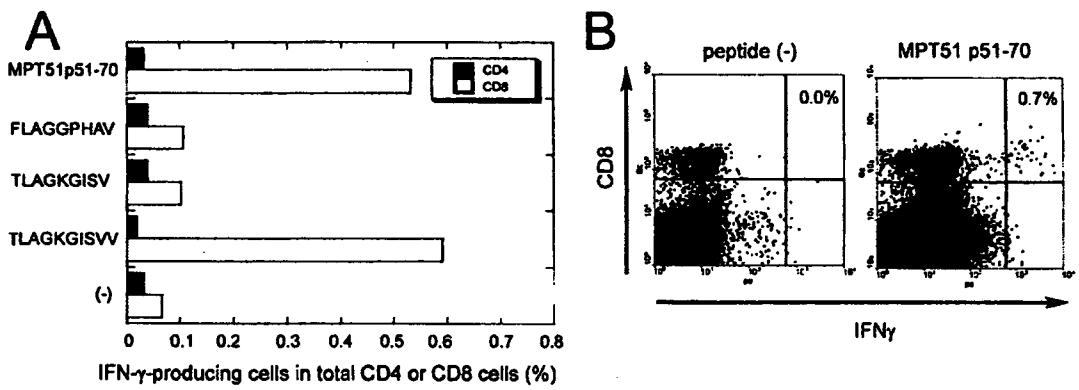

FIG. 2. Identification of a T-cell epitope in the MPT51 p53-62 peptide and the T-cell subset recognizing the epitope in HHD mice. (A) Levels of IFN-γ-producing T-cell subsets in spleens of HHD mice immunized with the pCI-MPT51 plasmid. Three-color flow cytometric analysis was performed for detection of intracellular IFN-γ and cell surface CD4 and CD8 molecules after immune splenocytes were cultured in the presence of the MPT51-derived peptides p51-70 (20-mer peptide), p21-29 (FLAGGPHAV), p53-61 (TLAGKGISV), and p53-62 (TLAGKGISVV). The data are the percentages of IFN-γ-producing CD4$^+$ or CD8$^+$ cells in the total CD4$^+$ or CD8$^+$ cells after 4 h of stimulation with peptides. The results of a representative experiment are shown. (B) Representative flow cytometry data for intracellular IFN-γ and cell surface CD8 staining of spleen cells of HHD mice immunized with the pCI-MPT51 plasmid after 4 h of stimulation with the MPT51 p51-70 peptide. The percentages of IFN-γ-producing cells in the total CD8$^+$ cells are shown.

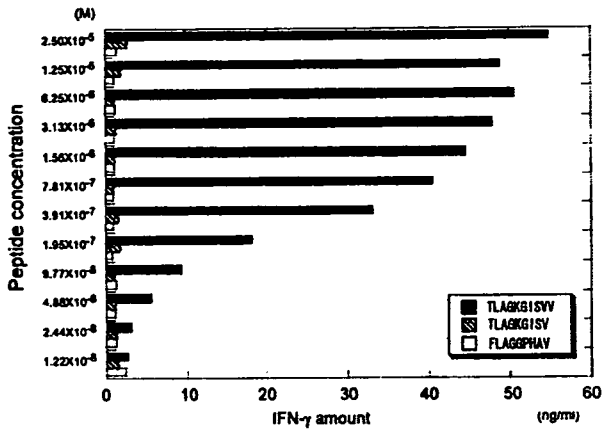

FIG. 3. MPT51 p53-62 is a dominant T-cell epitope in HHD mice. The IFN-γ production by splenocytes from HHD mice immunized with the pCI-MPT51 plasmid in response to twofold serially diluted doses of candidate peptides MPT51 p53-62 (TLAGKGISVV), p53-61 (TLAGKGISV), and p21-29 (FLAGGPHAV) was evaluated. The data are representative of the results of three independent experiments.

presence of p53-62 (TLAGKGISVV) but not in the presence of p53-61 (TLAGKGISV) or p21-29 (FLAGGPHAV) (Fig. 2). The MPT51 p53-62 peptide was confirmed to stimulate splenocytes derived from MPT51 DNA-immune HHD mice in a dose-dependent manner. The minimum concentration of this peptide for inducing IFN-γ production by the splenocytes was approximately 5×10^{-8} M (50 nM) (Fig. 3).

Binding affinity of the p53-62 peptide to the HLA-A*0201 molecule. We then examined the binding affinity of the MPT51 p53-62 peptide to the HLA-A*0201 molecule by measuring the binding stability with T2 cells, and we compared this peptide with several other *M. tuberculosis*-derived epitopes in terms of binding stability. T2 cells are defective for endogenous class I presentation due to the TAP deficiency, but peptide loading on MHC molecules stabilizes the expression of MHC on the cell surface (33). The MHC molecules stabilized with the appropriate peptides could be detected by flow cytometry with an MAb to the HLA-A*0201 molecule. As shown in Fig. 4A, MPT51 p21-29 (FLAGGPHAV) and MPT51 p53-62 (TLAGKGISVV) were strongly bound to the HLA-A*0201 molecule on T2 cells, whereas MPT51 p53-61 (TLAGKGISV), a known *M. tuberculosis* Ag85A-derived HLA-A*0201-binding peptide (KLIANNTRV) (30), and an *M. tuberculosis* ESAT6-derived HLA-A*0201-binding peptide (LLDEGKQSL) (19) were relatively weakly bound to the HLA-A*0201 molecule.

To obtain insight into T-cell recognition of the MPT51 p53-62/HLA-A*0201 complex on T2 cells, we examined the cytotoxic T-cell response of immune mice to the peptide-MHC complex. As shown in Fig. 4B, immune splenocytes of MPT51 DNA-immune HHD mice after in vitro stimulation with MPT51 p53-62 peptide-pulsed autologous splenocytes lysed the peptide-pulsed T2 cells substantially. However, the immune splenocytes did not lyse MPT51 p21-29 peptide-pulsed T2 cells after in vitro stimulation with the peptide-pulsed autologous splenocytes (Fig. 4A), although the peptide bound relatively strongly to HLA-A*0201 on T2 cells (data not shown).

Detection of MPT51 p53-62-specific $CD8^+$ T cells in PBMCs of HLA-A*0201$^+$ PPD-reactive healthy subjects. Finally, we examined whether HLA-A*0201$^+$ PPD-reactive healthy subjects do have MPT51 p53-62-specific memory T cells. We screened PBMCs of HLA-A*0201$^+$ individuals for the presence of the memory T cells. HLA-A*0201$^+$ PPD-reactive PBMCs were subjected to MPT51 p53-62/HLA-A*0201 tetramer staining after in vitro stimulation with mitomycin C-treated MPT51 p53-62-pulsed autologous PBMCs for 10 days. As shown in Fig. 5A, PBMCs from some HLA-A*0201-positive PPD-reactive individuals showed larger amounts of MPT51 p53-62/HLA-A*0201 tetramer-positive $CD8^+$ T cells by flow cytometric analysis than PBMCs from HLA-A*0201-negative individuals. The PBMCs of two of five HLA-A*0201-positive individuals were tetramer positive. In parallel, the

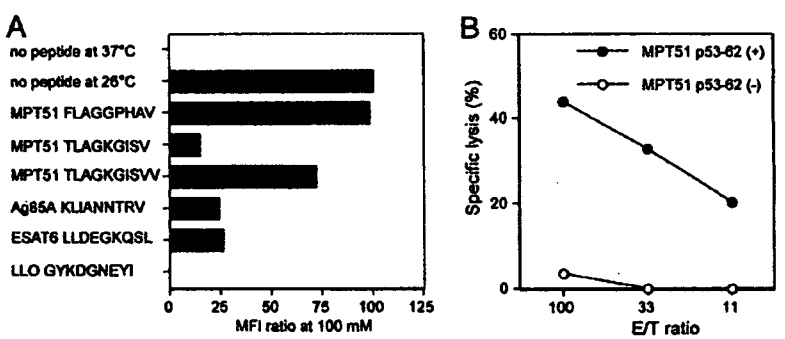

FIG. 4. MPT51 p53-62 peptide binds to cell surface HLA-A*0201 molecules and can be recognized by immune T cells in the context of HLA-A*0201. (A) HLA binding assay with T2 cells showing that MPT51 p21-29 (FLAGGPHAV) and MPT51 p53-62 (TLAGKGISVV) bound to HLA-A*0201 strongly, whereas MPT51 p53-61 (TLAGKGISV), the Ag85A-derived peptide KLIANNTRV, and the ESAT6-derived peptide LLDEGKQSL bound to HLA-A*0201 relatively weakly. The MFI ratios in the presence of the indicated peptides at a concentration of 100 μM are shown. The listeriolysin O (LLO)-derived peptide GYKDGNEYI was used as a negative control. The expression of HLA-A*0201 on T2 cells cultured in the absence of any peptide at 37 or 26°C is also shown. Representative data from three independent experiments are shown. (B) Lysis of MPT51 p53-62 peptide-pulsed T2 cells by splenocytes from MPT51 DNA-immune HHD mice. Immune splenocytes (effectors) were incubated with target cells using the effector/target cell ratios (E/T ratio) indicated on the *x* axis. Representative data from three independent experiments are shown.

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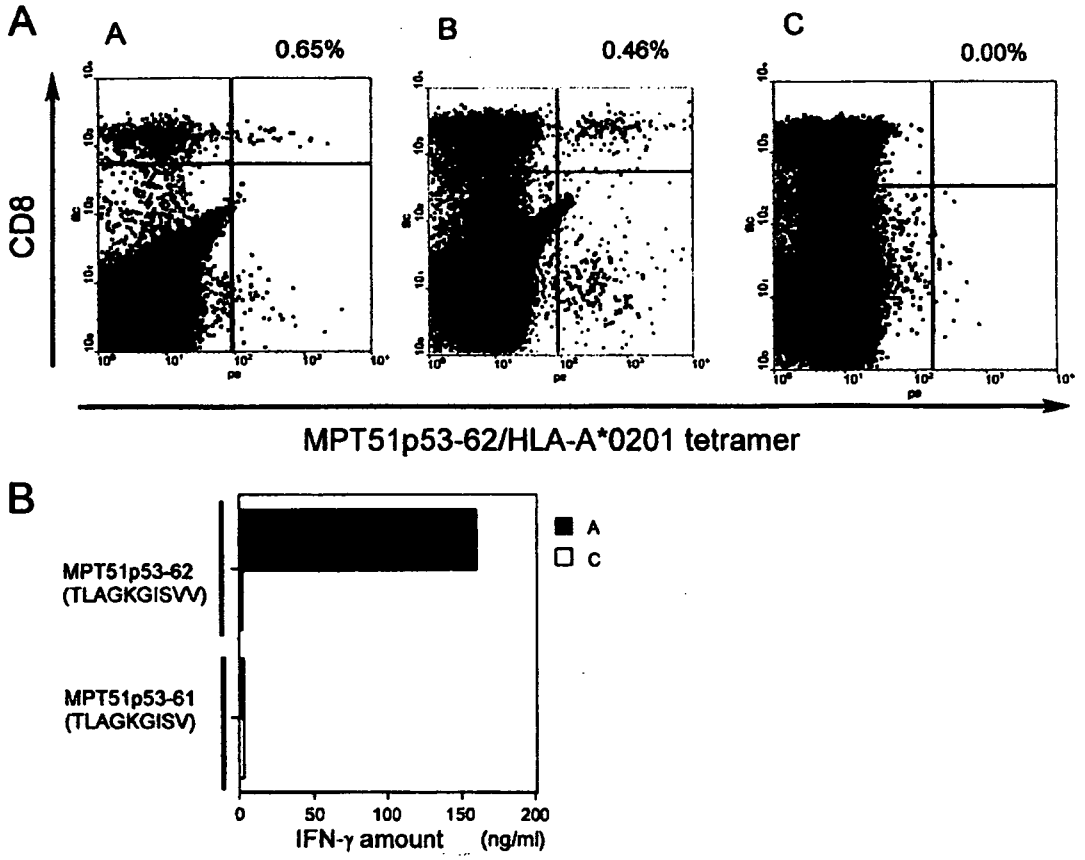

FIG. 5. Detection of MPT51 p53-62-specific memory T cells in PBMCs of HLA-A*0201$^+$ PPD-reactive healthy subjects. (A) Flow cytometric analyses to detect MPT51 p53-62-specific memory T cells in PBMCs of HLA-A*0201$^+$ PPD-reactive healthy subjects using MPT51 p53-62/HLA-A*0201 tetramer. PBMCs of the healthy subjects were prepared and cultured for 10 days together with mitomycin C-treated, MPT51 p53-62-pulsed autologous PBMCs and then subjected to flow cytometric analysis after treatment with PE-conjugated MPT51 p53-62/HLA-A*0201 tetramer and FITC-conjugated anti-human CD8 MAb staining (graphs A and B). HLA-unmatched PBMCs were used as a negative control (graph C). Representative flow cytometry patterns are shown. The percentages of tetramer-positive cells in the total CD8$^+$ cells are indicated. (B) IFN-γ production by PBMCs of HLA-A*0201$^+$ PPD-reactive healthy subjects stimulated with MPT51 p53-62 (TLAGKGISVV)- or p53-61 (TLAGKGISV)-pulsed autologous PBMCs for 10 days as evaluated by an IFN-γ ELISA. Samples A and C correspond to graphs A and C in panel A.

tetramer-positive PBMCs produced large amounts of IFN-γ after in vitro stimulation (Fig. 5B).

DISCUSSION

Here we identified induction of an MPT51 p53-62/HLA-A*0201-specific T-cell population by using HLA-A*0201 transgenic mice (HHD mice) and the MPT51 expression plasmid pCI-MPT51. From the data described above, we were able to draw the following conclusions about a T-cell epitope on the mature MPT51 molecule of *M. tuberculosis*: (i) MPT51 p53-62 peptide is a bona fide HLA-A*0201-restricted CD8$^+$ T-cell epitope and (ii) epitope-specific memory T cells were detected in PBMCs of HLA-A*0201-positive PPD-reactive healthy subjects.

A greater understanding of the nature of protective immunity to *M. tuberculosis* would facilitate development of a vaccine. The cellular arm of the immune response mediated by CD4$^+$ Th1 and CD8$^+$ CTL has been determined to be a pivotal component of protective immunity against *M. tuberculosis* (17). IFN-γ secretion, cytotoxic ability, and direct killing of *M. tuberculosis* by CD8$^+$ T cells have been speculated to be involved in protection (18). We report here that an MPT51 p53-62 peptide/HLA-A*0201 complex can be recognized by CD8$^+$ T cells producing IFN-γ and exhibiting CTL activity.

Reports concerning the involvement of CD8$^+$ T cells in containing *M. tuberculosis* infection in human have been accumulating, and intense efforts have been made to identify *M. tuberculosis*-derived CD8$^+$ T-cell epitopes that can be presented by HLA class I molecules. *M. tuberculosis*-derived HLA-A*0201-restricted T-cell epitopes have been identified, including epitopes in Ag85A (30), ESAT-6 (19), Ag85B (14), heat shock protein 65 (7), the 16-kDa protein (6), the 28-kDa protein (8), the 38-kDa protein (8), superoxide dismutase (9), alanine dehydrogenase (9), glutamine synthetase (9), the 19-kDa protein (21), and Rv0341 (12).

MPT51 is a dominant *M. tuberculosis*-derived secreted molecule which is related to the Ag85 family molecules Ag85A,

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Ag85B, and Ag85C. Such molecules have been found in a variety of mycobacteria (22). Functionally, these molecules have been implicated in fibronectin binding, like Ag85 family molecules (1). However, MPT51 appears not to have mycolyltransferase activity, which Ag85 family molecules have, since MPT51 does not have the catalytic triad (Ser-His-Glu) in its amino acid sequence (36). Therefore, MPT51 seems to have a function that remains to be clarified. Importantly, MPT51 has been reported to be a potential marker for the diagnosis of TB, especially in AIDS patients. Ramalingam and colleagues (26) reported that early immune responses against 38- and 27-kDa (MPT51) proteins were detected in pulmonary TB patients, accompanied by human immunodeficiency virus coinfection. In addition, we demonstrated that MPT51 DNA vaccination using an attenuated *Listeria* carrier vaccination system induced protection against *M. tuberculosis* infection in mice (20).

HLA transgenic mice have been widely used for detection of HLA-restricted T-cell epitopes. In this study we used HHD mice. In HHD mice, the HLA-A*0201 monochain is the only type of MHC class I molecule expressed (25). Firat and colleagues (11) reported that not only the size but also the diversity of the $CD8^+$ T-cell receptor repertoire is substantially larger in HHD mice than in A^*0201/K^b transgenic mice, which still express mouse $H2^b$ class I molecules. In addition, we used the computer algorithm programs BIMAS and SYFPEITHI for epitope prediction. These programs were helpful for narrowing down the amino acid region of the bona fide T-cell epitope.

HLA-A*0201-restricted $CD8^+$ T-cell epitopes have been identified in a variety of antigens, including antigens derived from cancers, viruses, bacteria, and protozoans. The main anchor amino acid positions are position 2 (Leu) and position 9 (Val), which were conserved in MPT51 p53-62 (TLAGKGIS VV). Most HLA-A*0201-restricted T-cell epitopes were nonamer peptides (10, 24), but some epitopes were decamer peptides, such as influenza virus matrix protein p59-68 (15). It is shown here that the MPT51 p53-62 decamer peptide was capable of binding to HLA-A*0201 and stimulating $CD8^+$ T cells of immune HHD mice, but the MPT51 p53-61 nonamer was not able to do these things. The conformational and electrostatic differences between the nonamer and the decamer should affect their binding affinity to the HLA-A*0201 molecule and subsequent T-cell responses. Ruppert and colleagues (28) studied in detail the roles of different amino acid residues at each position of nonamer or decamer peptides for binding to the HLA-A*0201 molecule. They suggested that the nonamer and decamer peptides have different preferences for amino acid residues for binding to the HLA-A*0201 molecule. For example, they showed that Tyr, Phe, and Trp residues at positions 1, 3, and 5 in nonamer peptides and Gly residues at positions 4 and 6 in decamer peptides are preferred for binding to HLA-A*0201. According to the speculation of these workers, the MPT51 p53-62 peptide seems to have better A*0201 binding features than the MPT51 p53-61 peptide (Gly residues at positions 4 and 6 in the MPT51 p53-62 peptide are suggested to be associated with good A*0201 binding) (Table 1). Interestingly, the MPT51 p21-29 peptide (FLAGGPHAV) was not immunogenic in terms of IFN-γ production and CTL ability, although this peptide showed high affinity to HLA-A*0201 (Fig. 4A), as predicted by MHC binding algorithms. Previous reports showed that there is a strong association between immunodominance and HLA binding affinity (13). But the results described here suggest that binding of peptides to the restricted MHC molecules is a prerequisite for T-cell epitopes; however, not all the peptides which show high-affinity binding for MHC molecules are necessarily immunodominant epitopes.

When we examined HLA-A*0201$^+$ PPD-reactive PBMCs for the response against MPT51 p53-62, we observed the specific $CD8^+$ T-cell response in some individuals. However, we could not detect $CD8^+$ T-cell responses in HLA-matched subjects without in vitro stimulation with the peptide. Therefore, we cannot rule out the possibility that these T cells were primed in vitro during stimulation with the peptide. The frequency of the memory T cells and the kinetics after *M. tuberculosis* infection are important issues to be clarified in the future.

In conclusion, we identified one HLA-A*0201-restricted $CD8^+$ CTL epitope on MPT51 in HHD mice, which may play a pivotal role in protection against *M. tuberculosis* infection. The identification of T-cell epitopes should be very useful for further elucidating the role of MPT51-specific T cells in protective immunity using tetramer staining or intracellular cytokine staining and also for future vaccine design.

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