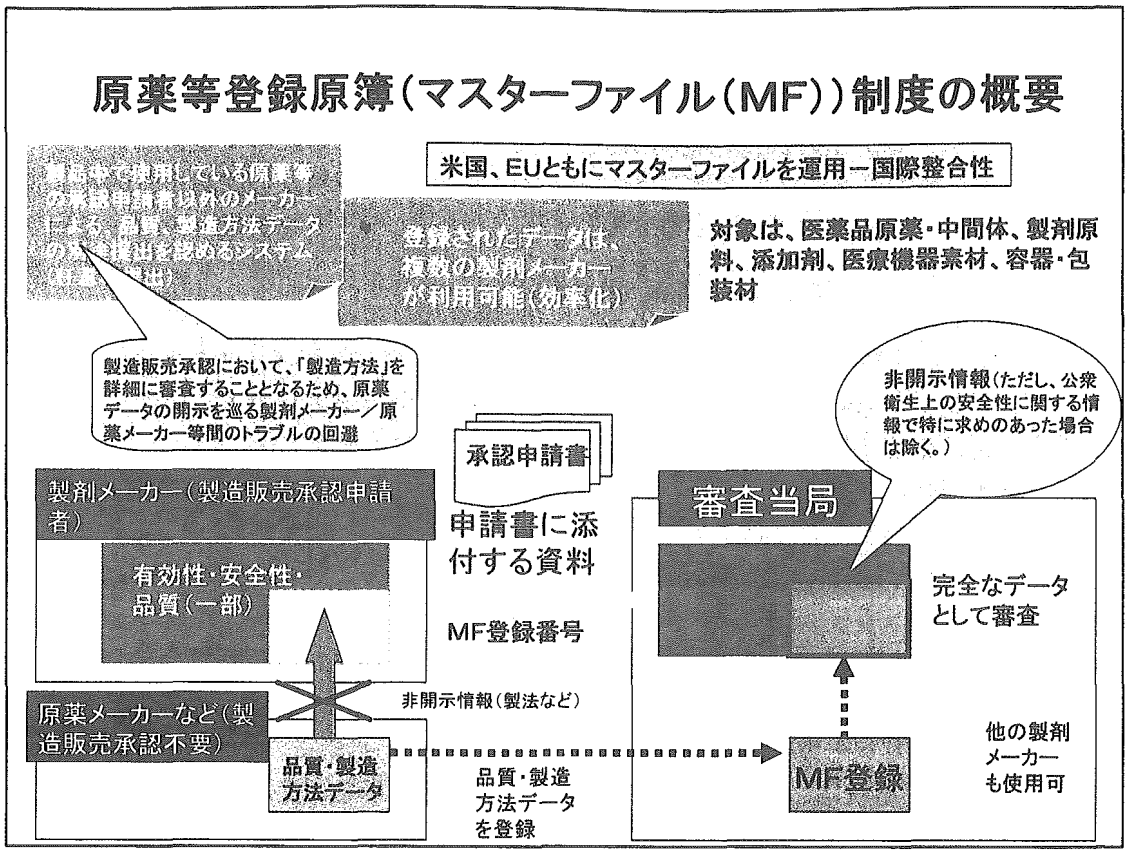
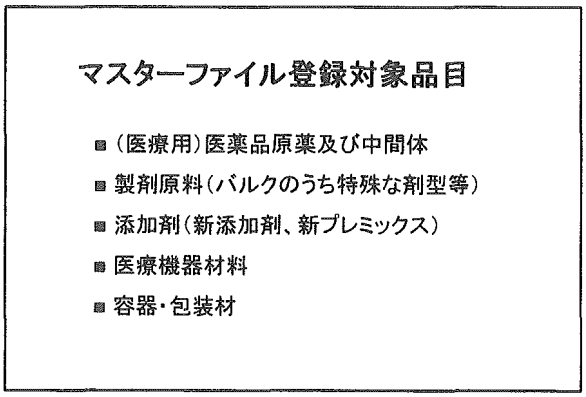
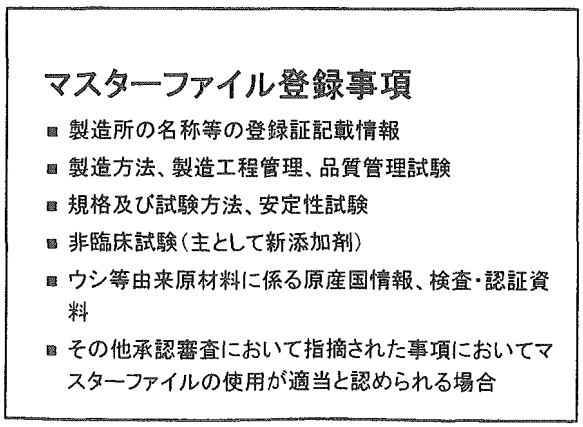
スライド36



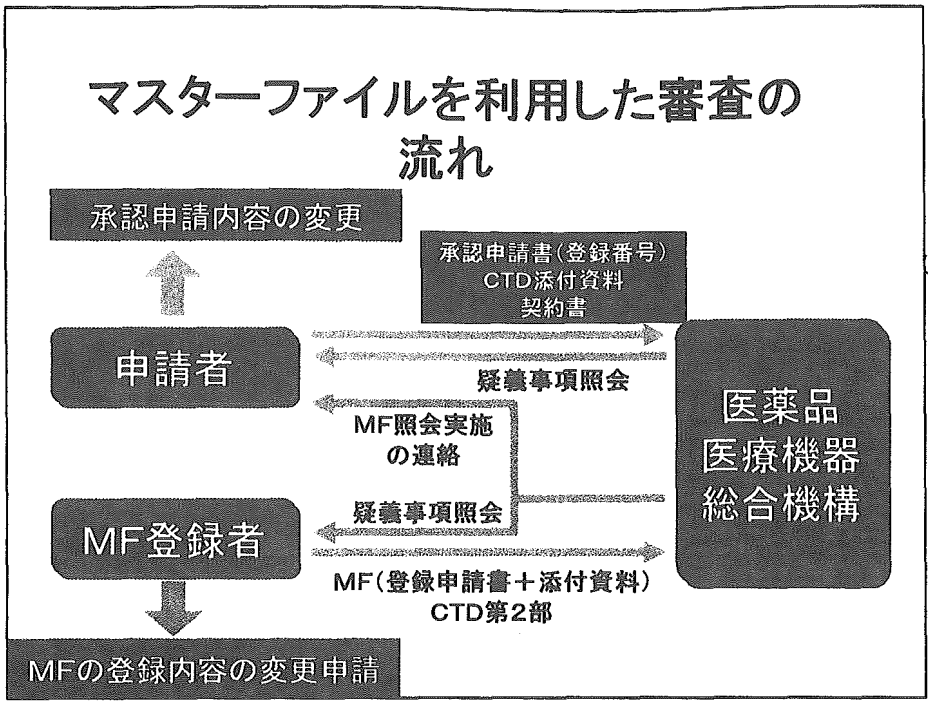
スライド37



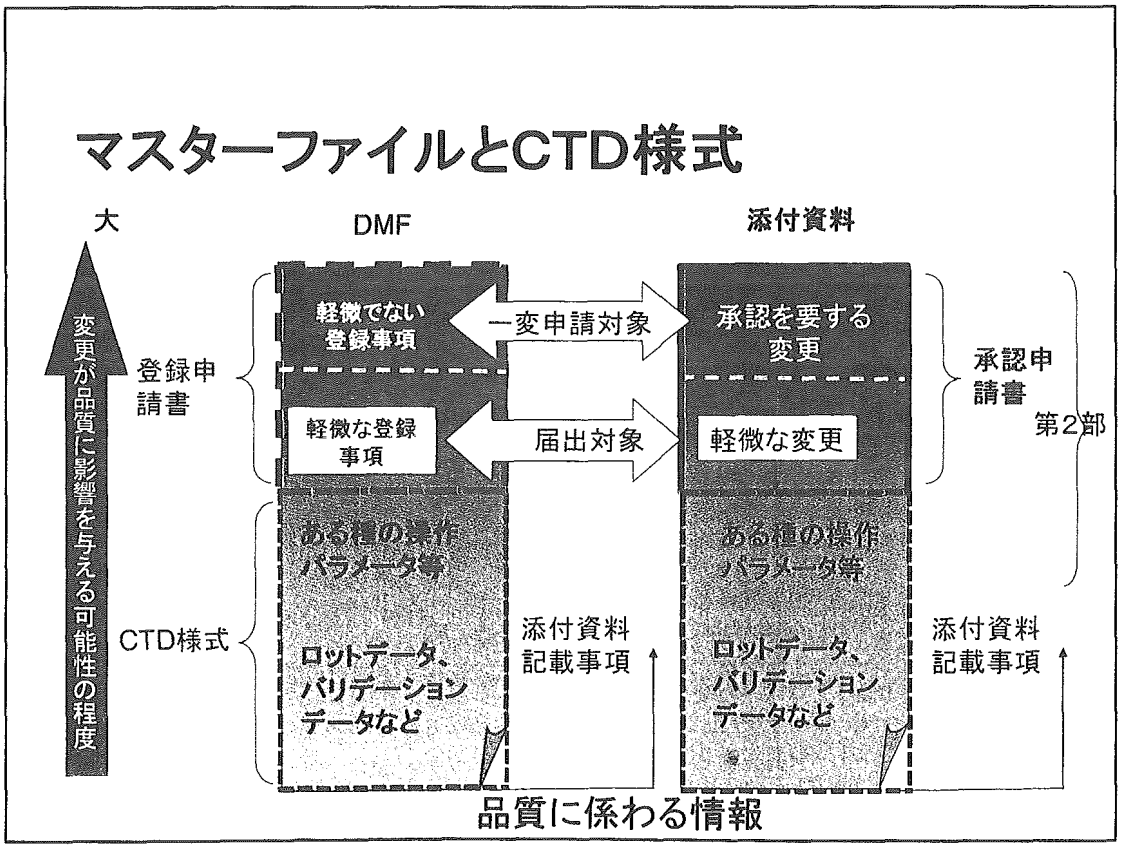
スライド38



スライド39



スライド40



ターファイルを使う場合は、マスターファイルのデータの部分があって、登録事項がある。この登録事項というのはその登録申請書です。

登録申請書に登録したい事項が書かれるのですが、それも一変対象になる部分、即ち軽微でない登録事項と軽微な登録事項がある。ここの関係は承認申請書と全く一致しますので、誤解しないようにして下さい。

スライド41をご覧下さい。

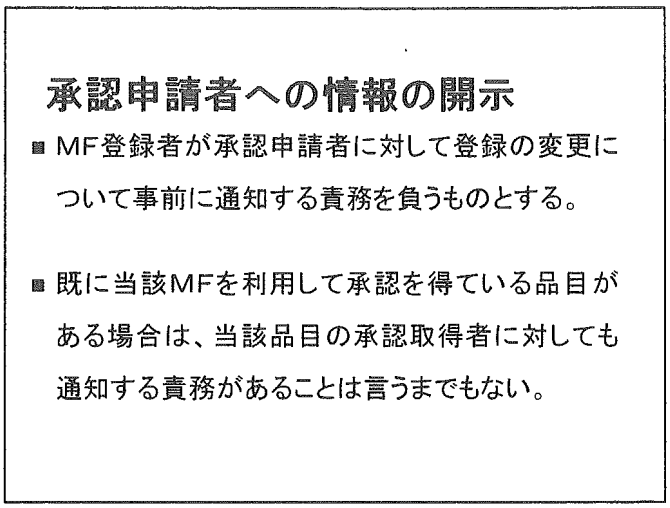
ただ、マスターファイルの登録者が製剤メーカーにデータを開示しないで規制当局にデータを渡すことができると言いましても、やはり最低限必要なデータは原薬メーカーから製剤メーカーに渡らなければいけないということです。そのために承認申請者への情報開示ということが義務づけられています。

それから、変更するときには事前に通知するということは極めて大事です。

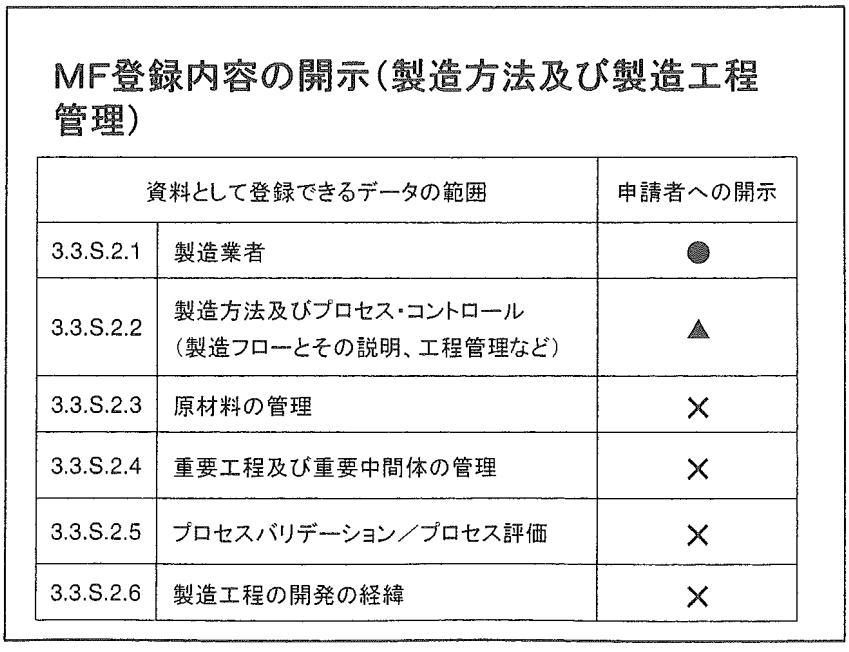
スライド42をご覧下さい。

どのようなことを開示しなければいけないか

スライド41

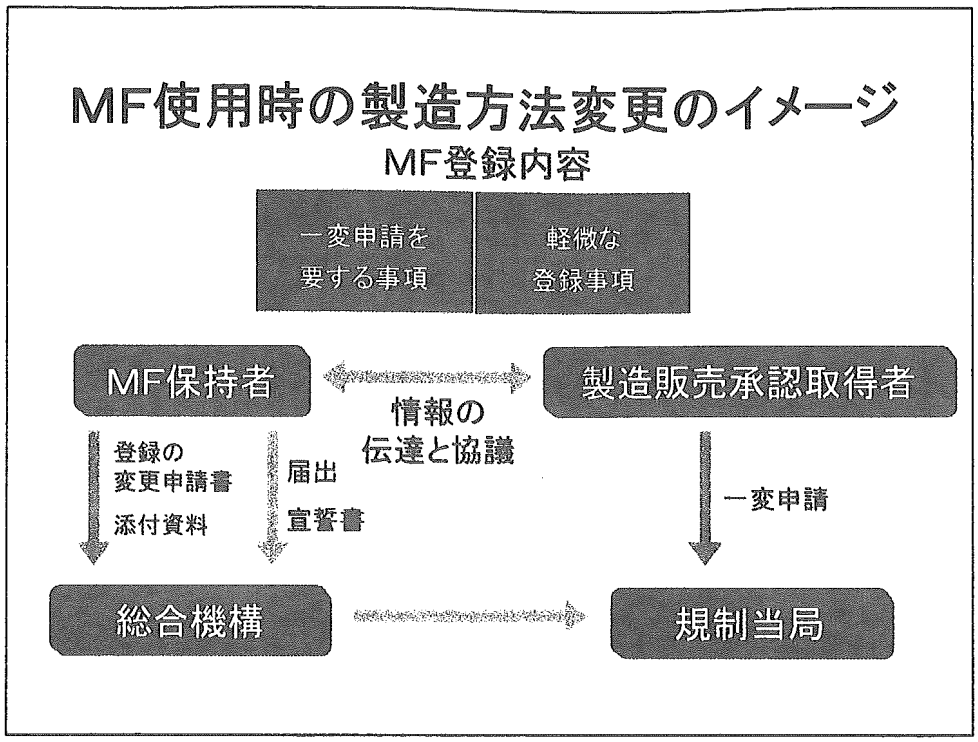


スライド42



資料として登録できるデータの範囲		申請者への開示
3.3.S.2.1	製造業者	●
3.3.S.2.2	製造方法及びプロセス・コントロール（製造フローとその説明、工程管理など）	▲
3.3.S.2.3	原材料の管理	×
3.3.S.2.4	重要工程及び重要中間体の管理	×
3.3.S.2.5	プロセスバリデーション／プロセス評価	×
3.3.S.2.6	製造工程の開発の経緯	×

スライド43



という例がマスターファイルの通知に出ていますけれども、規格及び試験方法は開示を必要とする部分ですが、製造工程については重要工程とかプロセスバリデーションとかそういったことは開示の必要はありません。ただし、製造方法の概略のプロセスについては「▲」になっているというのは、これは部分的に開示するというようなことであります。

スライド43をご覧下さい。

これはマスターファイルを利用しているときの製造方法変更手続きのイメージです。マスターファイル保持者が何か変えようというときに「一変を要する事項か、軽微な事項か」で手法が異なります。いずれにせよ、「変える」ということについては、マスターファイル保持者は、実際にそれを使って製剤をつくっている業者に対して情報をきちっと伝達することが必要です。それが一変の対象であれば、製造販売取得業者は規制当局に一変申請をする。申請が認められて初めてマスターファイル保持者の登録変更が認められるということになります。届出の場合は、この届出の内容が製造販売承認書に書かれていない、単にマスターファイル記載事項であれば、これはマスターファイルの届出だけでいいということになりますが、その場合についてもやはりまずここのところで情報がきちんと製造業者に伝達され、変更されたということが製造業者が分かるということが極めて大事です。

原薬の製法の影響というのは、本来、製剤の品質にどう影響するかということで最終的には判断されるべきことであります。ここのところは十分な注意をして頂きたいというように思います。

以上で私の説明を終わりますけれども、去年の７月と６月に大阪と京都でお話をさせて頂く機会があって、そのときにもいろいろな質問をお受けしました。「まだこれから考える部分がいっぱいあります」というようにお答えした部分もあるのですが、それから半年間研究班の活動をしてきて、やはり完全に詰まったわけではありません。これからいろいろな活動をまだやって、相談活動の支援も含めていろいろな活動をやっていくつもりではおります。今後ともよろしくお願いいたします。

——研修会プロシィーディング——

医薬品研究
IYAKUHIN KENKYU
34 (8) 508～512(2003)

品質に関わるトピックの動向***

—Q5E：バイオ医薬品のコンパラビリティー—

川西　徹*，松木　滋**

1. 東京会議までの経過

1.1 ブリュッセル会議（2002年2月）

ブリュッセル会議で作成されたコンセプト・ペーパーの概略をTable 1に示します．

1．製造工程に変更を加えたバイオテクノロジー応用医薬品/生物起源由来医薬品のコンパラビリティーに関するガイドラインを作成する．2．適用範囲はQ6B（バイオテクノロジー応用医薬品/生物起源由来医薬品の規格及び試験方法の設定について）[1]と同じ．3．製造工程変更後の製品の申請資料として，製造工程変更前の製品について得られたデータを用いる場合を対象とする．4．コンパラビリティーを確立するために必要な，科学的な考え方に焦点を絞って議論する．5．製品の品質と製造工程の両方の観点から議論する．6．まず品質分野のエキスパートが一次案を作成し，必要であれば安全性分野及び有効性分野のグループの参加を求め，非臨床，臨床分野の問題を議論する．7．FDAは内部事情によりドラフト作成作業に入れないので，当面ワシントン会議に向けてPhRMAが調整役，JPMAが事務局としてプレドラフト作成を行うこととされました．

1.2 ワシントン会議（2002年9月）

前回ワシントン会議の内容をTable 2にまとめました．後発品の取り扱いについて議論しましたが，運営委員会により後発品を適用範囲に含まないことが確認されました．ガイドラインに盛り込むアイテムを挙げたPhRMA原案のアウトラインの各項目についてJPMAのposition paper等を参考にして議論しました．また，極によってはコンセプト・ペーパーの作成に関わったメンバーの交代があったこともあり，共通認識を深める目的で非公式のワークショップも開催されました．

また，EWGが正式に発足しPhRMAとJPMA両者がラポーターとしてドラフト作成を進めることになりました．

1.3 前回ワシントン会議以降

ワシントン会議以降の経過をTable 3に示します．まずガイドライン案の作成を本格的に進めるために，基本になるドラフトはラポーターが作成することになって，各極のガイドラインやposition paperの比較表，更にガイドラインのたたき台としてDraft 0を2002年11月に示しました．続いて三極6団体からコメントを収集し，そのコメントを踏まえて，2003年2月の東京会議で用いるガイドライン案としてDraft 1をラポーターが提示しました．

2. 東京会議の内容

東京会議での検討内容をTable 4にまとめました．まず，三極6団体とCanadaからそれぞれの考え方が表明されました．その後に東京会議の前に提示されたガイドライン案のDraft 1について詳細に検討しました．この検討の各段階で議論された項目の中で，今後調和に向けて議論すべき主な検討項目を特定化しました．そして，次回ブリュッセル会議に向けてDraft作成の日程について検討しました．

2.1 三極から考え方の表明（Table 5）

三極いずれの団体からもDraft 1の内容はDraft 0に対して大きく改善されたことが表明されました．

日本からはコンパラビリティーのガイドラインに

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*** 当協会主催の第8回ICH即時報告会（平成15年3月6日：東京，3月11日：大阪）における講演による．

Table 1　2002年2月コンセプト・ペーパー概略

1. 製造工程に変更を加えたバイオテクノロジー応用医薬品/生物起源由来医薬品のコンパラビリティーに関するガイドラインを作成する.
2. 適用範囲はQ6Bと同じ.
3. 製造工程変更後の製品の申請資料として，変更前の製品のデータを用いる場合が対象.
4. 科学的な考え方に焦点を絞って議論する.
5. 製品の品質と製造工程の観点から議論する.
6. Qualityのエキスパートが一次案を作成する.
7. ワシントン会議に向けPhRMAを調整役，JPMAを事務局としてプレドラフト作成を行う.

Table 2　2002年9月ワシントン会議討議内容

1. 運営委員会で後発品は適用範囲に含まない事が確認された.
2. PhRMA原案のOutlineを基に，JPMA position paper等を参考にして，用語の定義，ガイドラインに入れるべき項目等について討議
3. PhRMA主催非公開ワークショップ（トピック：(1)製造方法の変更に伴う製品の抗原性評価に関する考察；(2)製薬企業によるコンパラビリティー評価例の報告)
4. EWGが正式に発足し，PhRMAとJPMAがラポーターとなることとされた.

Table 3　前回ワシントン会議以降の経過

1. ラポーターが各極のガイドライン，あるいはPosition Paperの比較表を作成，さらに，ガイドラインのたたき台として“Draft 0”（各国の主張をほぼすべて取り入れたバージョン）を提示（2002年11月）
2. “Draft 0”について三極からコメント収集（2002年12月～2003年1月）
3. 東京会議に用いる“Draft 1”をラポーターが提示.（2003年1月）

Table 4　東京会議の内容のまとめ

1. 三極から考え方の表明
2. ガイドライン案(Draft 1)の検討
3. 調和に向け今後の主要な検討項目の特定化
4. ブリュッセル会議への日程の検討

Table 5　三極から考え方の表明

日本:	Draft 1は大きく改善した．製品の品質によるコンパラビリティー評価を重視すべき．詳細な製造工程関連の記述は現行審査システムでは適用が難しい.
欧州:	Draft 1は大きく改善した．製造工程の記述，非臨床，臨床の取り扱いの検討を要請.
米国:	Draft 1は大きく改善した．リスク評価，コンパラビリティー評価手順，開発段階のコンパラビリティーについて検討を要請．品質の改善を目的とした場合についても盛り込むべき.

おいては基本的に製品の品質の比較による評価を重視すべきであること，更に日本の現行の審査システムにおいては詳細な製造工程関連の記述は難しいことが主張されました.

欧州からは反対に製造プロセスを詳細に盛り込むべきであると共に，非臨床及び臨床分野の取り扱いについての記述が要請されました.

米国からは製造工程を変更したときのリスク評価やコンパラビリティー評価を行う際の手順及び開発段階でのコンパラビリティー評価の検討が要請されました．更に品質の改善を目的とした製造工程の変更についても考え方を整理すべきであると要請されました.

2.2　ガイドライン案（Draft 1）の検討

(Table 6)

今回の東京会議では多くの時間をかけてガイドライン案であるDraft 1の内容について詳細に検討しました．その結果，ガイドラインを作成する上で調和に向けて障害になるような大きな問題はないことを確認しました.

討議の結果については，現段階でガイドラインに盛り込むべきとされた項目について説明します．ただし，まだ案の段階であり，今後調和の過程において大きな変更も考えられますので，不確定なものであることをご承知おき下さい.

1番のIntroductionではそれぞれ目的，背景，適用範囲，基本原則が述べられています.

2番のGuidelinesからコンパラビリティーの検討作業にあたっての具体的な指針が述べられています．その最初にコンパラビリティーを検討するため

Table 6 ガイドラインTOC案
ICH Q5E: Comparability of Biotechnological/Biological Products Subject to Changes in their Manufacturing Process

1	Introduction
1.1	Objectives
1.2	Background
1.3	Scope of the Guideline
1.4	General Principles
2	Guidelines
2.1	Considerations for Establishing Comparability
2.2	Key Quality Criteria
2.2.1	Analytical Techniques
2.2.2	Characterisation
2.2.3	Specifications
2.2.4	Stability
2.3	The Manufacturing Process
2.3.1	Drug Substance and Drug Product
2.3.2	Description of the Manufacturing process and controls
2.3.3	Assessing Impact of Manufacturing Changes
2.3.4	Process Evaluation / Validation: Consistency of Batches before and after manufacturing change
2.4	The Comparability exercise (Study)
2.4.1	Overall Strategy
2.4.2	Post marketing
2.4.3	Drug Substance
2.4.4	Drug Product
2.5	Development
2.6	Nonclinical and clinical data
3	Glossary

に考慮すべきポイントについての総論，次に品質関係でコンパラビリティーを検討する上での要素を述べます．これには分析法の適格性，物理的化学的，生物学的あるいは免疫学的な特性解析，製品規格，製品の安定性に関する比較が含まれます．

次に製造工程について順番に述べます．最初に原薬と製剤の製造工程，製造工程と工程管理の記述，製造工程変更によるインパクトの評価，製造工程の変更前後のロットの恒常性を確認するための工程評価/検証と続きます．後ほど述べますが，この製造工程の内容については，どのような順番・構成で記述するか今後大きな議論になると考えています．

続いてコンパラビリティー評価研究の具体的な流れについての説明です．まず全体の方策，市販後の場合，原薬・製剤の場合，開発段階でのコンパラビリティー，そして非臨床・臨床のデータという流れになり，最後に用語集となる予定です．

2.3 今後の検討課題 (Table 7)

今回の東京会議ではガイドライン案であるDraft 1を詳細に検討し，その結果，調和ガイドラインの作成をめざす上で，調和を阻む大きな問題点はないことを確認しました．しかし，今後調整が必要と考えられる主要な検討課題がいくつか浮かび上がりました．

まず製造工程の記述です．これは欧州が強く主張している点ですが，このガイドラインに製造工程関係の要素を詳細に記述し，CPMPのガイドラインのような工程管理の規格値及び適否の判定基準やインプロセスコントロールもコンパラビリティー評価の重要な要素として記述しようという主張です．これには日米が反対していますから相当な議論になる

Table 7 今後の検討課題

1. 製造工程の記述
2. コンパラビリティーを評価する手順
3. 開発段階のコンパラビリティー評価
4. 試験方法の分析能力の検討
5. 非臨床及び臨床研究について

と思います．

次にコンパラビリティー評価の手順です．コンパラビリティーを成立させるための評価は幾つかのステップを踏んで段階的に進めますが，その順番はケースにより異なります．そこで，基本的な考え方をどのようにガイドラインに記述するかという課題があります．

開発段階では製造工程を改良するために様々な変更が行われますが，多種多様なケースをどのようにまとめ，ガイドラインに盛り込むべきかが問題です．またコンパラビリティーを成立させるために用いる試験方法の分析能力についてどのように評価して使用すれば良いのかといった課題もあります．

そして，これも大きな問題ですが，非臨床及び臨床研究についてはどのような内容を盛り込むべきかという課題があります．非臨床及び臨床研究関連の記述は，最初に述べたようにコンセプト・ペーパー（Table 1）では，まず品質分野のエキスパートが一次案を作成し，必要であれば安全性分野及び有効性分野のグループの参加を求めて前臨床及び臨床分野の問題を議論することになっていました．

今回の東京会議において，非臨床及び臨床研究の具体的な手順についてEWGの考え方をまとめました（Table 8）．その結果，まず品質分野のエキスパートが集まってガイドラインを作成し，Step 2とします．それから非臨床及び臨床分野の専門家の意見を聞き，Step 4の前にその内容を品質分野のエキスパートが検討することとしました．

2.4　ブリュッセル会議までの日程（Table 9）

EWGでは2003年11月の大阪でのICH6でStep 2にしようと議論を進めていますが，正式にEWGメンバーが直接顔を合わせて討論できる機会は7月に予定されているブリュッセル会議しかありません．先ほど述べた問題点を解決するには，EWG会議が大阪ICH 6までに一回では，Step 2に進むのは困難ということがEWG内での共通認識でした．そこで，ブリュッセル会議までの5月上旬に臨時EWG会議の開催を提案することとしました．

Table 8　非臨床及び臨床研究

1. 品質分野の専門家が討議してガイドラインを作成し，Step 2とする．
2. 非臨床及び臨床分野の専門家にはその後に意見を聞く．
3. Step 4に先立ち，非臨床及び臨床分野の専門家からのコメントについて，品質分野の専門家が検討する．

Table 9　ブリュッセル会議迄の日程

1. Draft 2(ラポーターが作成): 3月上旬
2. 各極からのコメントの収集: 4月中旬
3. 臨時EWG会議: 5月上旬 製造工程の記述等について検討する予定．
4. Draft 3作成及びコメント収集: 6月

2.5　臨時EWG会議（Table 10）

臨時EWG会議では，先ほど述べた問題点の中で最も大きな問題点と思われる製造工程の記述について検討する予定です．先ほど述べたように三極の考え方が大きく異なっています．特に欧州から詳細に盛り込むべきであるとの意見が出されていますので，その記述内容については，かなり突っ込んだ話し合いをすることになりそうです．

その他の課題も検討する予定ですが，このトピックがQ5シリーズとQ6Bガイドラインを踏まえて，その応用によって成立するコンパラビリティー評価法についての調和を目指したガイドラインであることから，各課題について相当詳細かつ念入りな議論を行うことが調和のための必須要件であると思っています．

2.6　臨時EWG会議（Table 11）

今回の東京会議で予想以上に検討が進みましたことから，11月の大阪ICH6でStep 2に到達できる可能性が出て来ました．大阪でStep 2に達するためには5月の臨時会議と7月のブリュッセル会議で十分な検討を行い，確実にドラフト作成を進めるこ

Table 10　臨時EWG会議 —製造工程の記述—

1. 製造工程の記述について，考え方が三極間において大きく異なっている．
2. 欧州から詳細に盛り込むべきとの意見．
3. 製造工程の記述内容について検討する．

Table 11　今後の予定

2003年5月	臨時EWG
2003年7月	ブリュッセルEWG
2003年11月	ICH 6 (Step 2?)

とが必要であると思っています．

文　献

1) 厚生労働省医薬局審査管理課長：生物薬品（バイオテクノロジー応用医薬品/生物起源由来医薬品）の規格及び試験方法の設定について，医薬審発第571号，平成13年5月1日．

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Detection of Replication-Competent Adenoviruses Spiked into Recombinant Adenovirus Vector Products by Infectivity PCR

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The presence of replication-competent adenovirus (RCA) in clinical lots of adenovirus vectors raises a variety of safety concerns. To detect RCA in adenovirus vector products, the cell culture/cytopathic effect (CPE) method has generally been preferred. However, it is difficult to evaluate the amount of RCA clearly and quantitatively by this method. In addition, the cell culture/CPE method requires large-scale cell culturing and a substantial amount of time. For the purpose of establishing a method to detect RCA more sensitively and rapidly, we developed the infectivity PCR, a hybrid method that combines the infectivity assay and quantitative PCR. This method allows RCA to be quantified by real-time quantitative PCR using primers and a probe designed for E1 DNA. By infectivity PCR, 1 pfu of RCA spiked into 10^9 particles of adenovirus vectors could be detected. In contrast, CPE was observed in the cells infected with 10^4 pfu of RCA spiked into 10^9 particles of adenovirus vectors. The glass-beads method was suitable for extracting DNA rapidly from the RCA-infected cells. These results showed that infectivity PCR combined with the glass-beads-based DNA extraction method was useful for the detection of RCA in adenovirus vector products.

Key Words: replication-competent adenovirus, adenovirus vector, infectivity PCR

Introduction

Recombinant adenovirus vector is one of the most promising vectors available for human gene therapy. In fact, adenovirus vector-based gene therapies now account for 26.9% of all clinical gene-therapy protocols [1]. Considerable efforts have been made to improve the potency of adenovirus vectors to make them more useful for gene therapy, e.g., regulation of target-cell specificity by modifying fiber protein [2–7], application of cassettes that enable the regulation of gene expression [8,9], and reduction of immunogenicity by deleting all viral genome sequences [10]. However, there have been few studies investigating the establishment of a system to ensure the safety of these vectors.

The vast majority of adenovirus vectors are constructed by inserting the therapeutic genes in place of the essential viral E1 sequence in the adenovirus. The generation of E1-deleted adenovirus vectors relies on the complementation functions present in HEK293 cells into whose genome E1 DNA has been inserted. However, HEK293 cells are prone to the generation of replication-competent adenovirus (RCA) as a result of recombination events between the vector DNA and the integrated adenovirus sequences present in the cells [11]. The presence of RCA in adenovirus vector products raises the possibilities of adenovirus infection, unintended vector replication due to the presence of wild-type helper function, and exacerbation of host inflammation response [12]. Because it is extremely difficult to avoid completely the emergence of RCA in adenovirus vector products by means of the current production technique, examining the level of RCA in each lot of adenovirus vector products is important [12]. In addition, examining for the presence of RCA in the patients who have been administrated adenovirus vectors

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is important to test for viral shedding during the clinical study.

As a method to detect RCA in adenovirus vector products, the cell culture/cytopathic effect (CPE) assay has generally been used [13,14]. In the cell culture/CPE assay, the vector products are infected into cells, the RCA are amplified, and the CPE induced by the RCA is observed. By this method, the presence of RCA is judged by microscopic observation, and thus the results may not always be accurate and quantitative. In addition, this method requires large-scale cell culturing and a substantial amount of time. As a sensitive method to detect viral DNA, PCR is thought to be useful [11,15]. However, because the infectivity of the viral DNA cannot be measured by PCR, the cell culture/CPE assay is still recommended by the FDA [12]. For all of the above reasons, there is need for a more sensitive, quantitative, and rapid method for the detection of RCA.

In the present study, we established an infectivity PCR method for detecting RCA. Infectivity PCR is a hybrid method that combines the best features of the infectivity assay and PCR. By this method, the virus is allowed to replicate in the cell culture, as in the traditional cell culture/CPE assay, and the amount of virus replicated in the cells is determined by quantitative PCR rather than by observing CPE. For efficient extraction of the DNA from RCA-infected cells, a novel glass-bead method was developed. Our results demonstrated that the infectivity PCR method combined with glass-beads-based DNA extraction was superior to the classical cell culture/CPE method for detecting RCA.

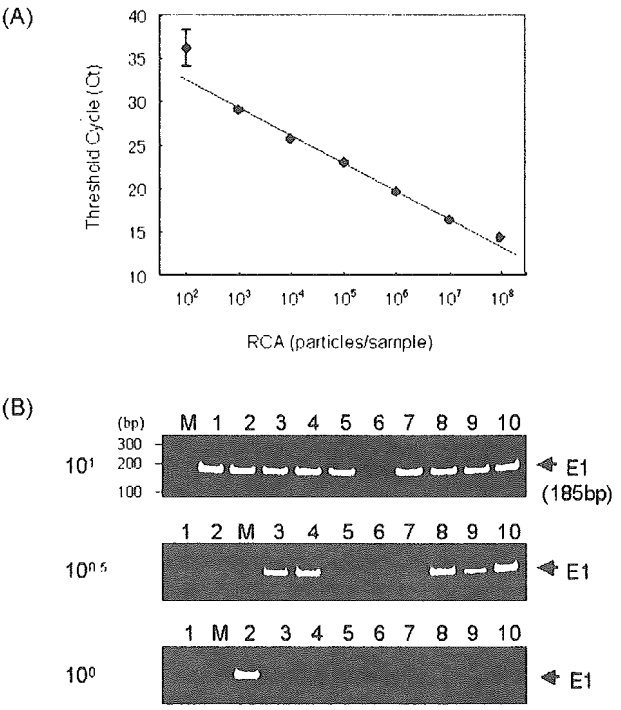


FIG. 1. Detection of RCA by PCR. Viral genome DNA was extracted from serial log dilutions of RCA and amplification of each sample was performed by (A) real-time quantitative PCR or (B) nested PCR. (A) Standard curve for the determination of RCA quantity generated from the amplification plot of real-time quantitative PCR. Data are the means ± SD of triplicate amplifications. (B) Detection of a low copy number of RCA by nested PCR ($n = 10$). The particle numbers in the samples were 10^1, $10^{0.5}$, and 10^0. M, molecular weight marker.

Results

Quantification of RCA by Real-Time Quantitative PCR

As a first step in establishing an infectivity PCR method, we developed a real-time quantitative PCR that can quantify the copy number of the RCA genome. We designed four pairs of primers and probes for the detection of E1 DNA, which is included in the RCA genome but not in the adenovirus vectors. Among them, the Ad5dE1-1035F and Ad5dE1-1105R primers and the Ad5dE1-1058TM probe worked well for quantifying the RCA genome. Fig. 1A shows the standard curve with the starting quantity of RCA on the *x* axis and the threshold cycle (Ct denotes the PCR cycle at which the threshold line intercepts the amplification curve) on the *y* axis. The threshold cycle and the log-transformed concentration showed a high, inverse correlation in a linear fusion from 10^8 to 10^3 particles.

Detection of RCA by Nested PCR

Since the nested PCR method is known to be suitable for detecting low concentrations of DNA, we applied it here to detect the DNA extracted from 10^1, $10^{0.5}$, or 10^0 particle(s)/tube of RCA. When nested PCR was performed in 10 tubes, the amplification succeeded in the ratios of 9/10 for 10^1 particles, 5/10 for $10^{0.5}$ particles, and 1/10 for 10^0 particles (Fig. 1B). Although DNA concentration cannot be quantitatively determined by nested PCR, it can be estimated from the hit rate of the amplification. This estimation assumes that, when low concentrations of samples are transferred from a stock tube to PCR tubes, viral particles will not necessarily be present in all the reaction tubes. If the PCR is optimized, then as little as one copy of DNA can be amplified, and the relationship between the hit rate in the PCR and the average copy number in the PCR tubes can be calculated as follows: 1/10 for 0.105, 2/10 for 0.233, 3/10 for 0.357, 4/10 for 0.511, 5/10 for 0.693, 6/10 for 0.916, 7/10 for 1.20, 8/10 for 1.61, and 9/10 for 2.30 [16,17]. In our experiments, half of the extracted DNA was subjected to the nested PCR; therefore, the hit rate of the PCR and the RCA copy number were 1/10 for 0.5, 5/10 for 1.58, and 9/10 for 5. Although the hit rate in this experiment seemed to be slightly lower than the theoretical values, this does not

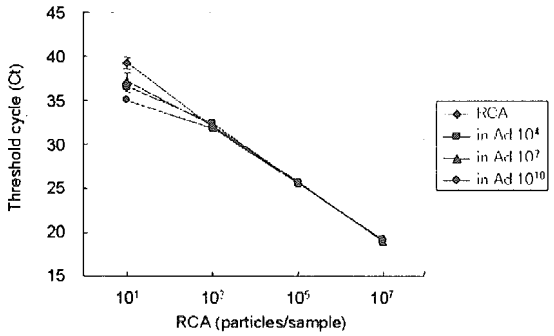


FIG. 2. Detection of RCA spiked into adenovirus vectors by real-time quantitative PCR. 10^1, 10^3, 10^5, or 10^7 particles of RCA were spiked into 0, 10^4, 10^7, or 10^{10} particles of adenovirus vectors (AdHM4LacZ). Viral DNA was extracted from each sample, and E1 DNA was detected by real-time quantitative PCR. Data are the means ± SD of triplicate amplifications. The background Ct (Ad without any RCA) was 39.1 for 10^4 particles of adenovirus vectors, 39.2 ± 1.6 for 10^7 particles, and 35.2 ± 0.04 for 10^{10} particles.

necessarily invalidate the results, since DNA may have been lost during the DNA extraction, and the detection limit may have been higher than one copy. These results demonstrated that the nested PCR could be used for the detection of lower concentrations of RCA and that the detection limit might be fewer than 10^1 particles.

Measurement of RCA in Adenovirus Vector Products

Because, in practice, it is often necessary to detect very slight amounts of RCA in high concentrations of adenovirus vectors, we next tried to quantitate the amount of RCA spiked into adenovirus vectors. We extracted viral genome DNA from 10^1, 10^3, 10^5, or 10^7 particles of RCA spiked into 10^4, 10^7, or 10^{10} particles of adenovirus vectors and then measured the amount of RCA by real-time quantitative PCR. As shown in Fig. 2, when the RCA concentration was higher than 10^3 particles/sample, the amount of RCA could be measured without major interference by coexisting adenovirus vectors. However, when the spiked RCA concentration was 10^1 particles, the Ct value seemed to be lowered by the presence of adenovirus vectors. From these results, although RCA was thought to be detectable even in the presence of adenovirus vectors, the adenovirus vectors used in this experiment might have contained a certain amount of E1 DNA. We therefore examined several lots of adenovirus vectors for the presence of E1 DNA by nested PCR. When 10^{10} particles of adenovirus vectors were subjected to the nested PCR, E1 DNA was detected in all seven lots tested. When 10^9 particles of adenovirus vectors were tested, E1 DNA was detected in four of the seven lots tested (data not shown).

Because we could not distinguish whether the E1 DNA detected in adenovirus vectors was derived from RCA or HEK293 cells, we tested for the presence of HEK293 cell-derived DNA in the vector products by PCR. For this purpose, we used primers designed for the sequence of the pregnancy-specific glycoprotein (PSG) gene, in which the adenovirus E1 gene is inserted into the HEK293 cell genome [18]. In all lots examined, PSG DNA was detected in 10^{10} particles of adenovirus vectors, meaning that the adenovirus vector products contained HEK293 cell-derived DNA as impurities (Fig. 3A). E1 DNA was barely detected in 10^{10} particles of adenovirus vectors (Fig. 3B). These results showed that adenovirus vectors can be subjected to the direct measurement of RCA by PCR, although the results are complicated by the presence of residual HEK293 cell DNA. To separate RCA from HEK293 cell-derived DNA, we attempted to establish an infectivity PCR method for the measurement of RCA in adenovirus vectors. In the infectivity PCR assay, RCAs are expected to be selectively amplified in the cells that support their growth.

Comparison of the Amount of RCA in Cells and Cultured Medium from RCA-Infected Cell Culture

To determine a suitable source for the PCR template in infectivity PCR, we compared the amounts of E1 DNA in the cells and cultured medium from the RCA-infected cell culture. We infected HeLa cells with serially diluted RCA and cultured them. We extracted DNA from the cells and cultured media and then subjected it to real-time quantitative PCR. In this experiment, we used one-third of the cells or one-hundredth of the supernatant from each dish for DNA extraction. A 100-fold higher amount of RCA was

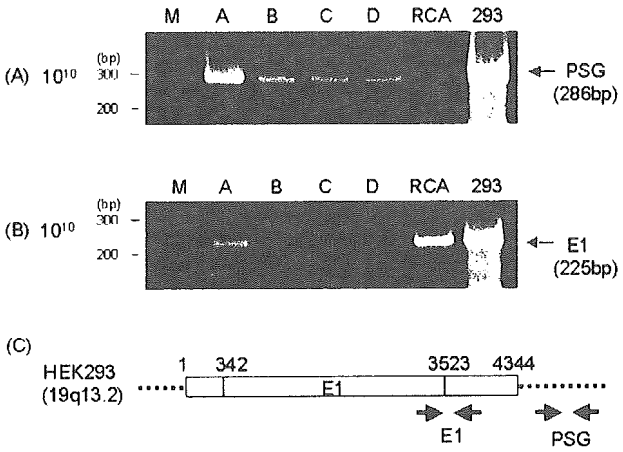
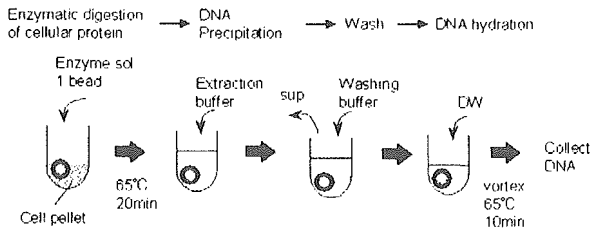


FIG. 3. Detection of pregnancy-specific glycoprotein (PSG) or E1 DNA in adenovirus vectors by PCR. Viral DNA was extracted from 10^{10} particles of adenovirus vectors and then (A) 293 cell-derived pregnancy-specific glycoprotein DNA or (B) E1 DNA was detected by PCR. DNA from HEK293 cells was used as a positive control. Lane A, AdHM4LacZ (Lot 0516); lane B, AdHM10LacZ-3 (Lot 0529); lane C, AdHM10LacZ-4 (Lot 0529); lane D, AdHM10LacZ-5 (Lot 0529), lane RCA, replication-competent adenoviruses; lane 293, genomic DNA extracted from HEK293 cells; lane M, molecular weight marker. (C) E1 DNA inserted into the chromosome of HEK293 cells and the positions of the PCR primers used are shown.

(A)



(B)

Method	EX R&D	Glass Beads
Raw data	8.9×10^8	4.5×10^8
	3.4×10^8	4.9×10^8
Average	6.15×10^8	4.7×10^8

FIG. 4. DNA extraction using glass beads. (A) Procedure of DNA extraction from cell pellets using glass beads. (B) Comparison of the efficiency of DNA extraction. DNA was extracted from RCA-infected HeLa cell lysate by the EX-R&D reagent or glass-beads method. The copy number of E1 DNA in each sample was determined by real-time quantitative PCR.

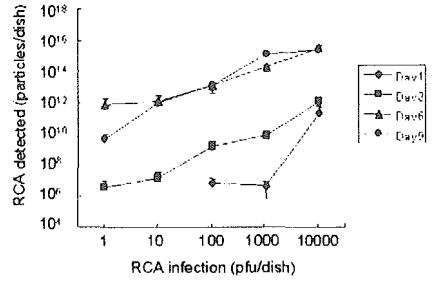
detected in the DNA extracted from the cells compared with that from the cultured medium (data not shown). Therefore, we considered the cells to be a suitable source for further examination of the propagation of RCA.

In the preparation of DNA from RCA-infected cells, cellular genomic DNA caused high viscosity and disturbed the subsequent PCR. However, cellular genomic DNA could be digested by nuclease after freezing and thawing without damaging the viral DNA, because the viral DNA is protected by capsid proteins. By this procedure, we could extract viral DNA from more than 1×10^6 cells and use it as a template for the PCR, although this procedure was laborious. We considered that glass beads, which were initially developed for extracting cellular DNA, might be used to simplify this step, and therefore we examined a glass-beads-based DNA extraction method (Fig. 4A). The protocol for the glass-beads method is simple and less time-consuming than extracting DNA after freezing and thawing followed by nuclease treatment. For comparing the efficiency of DNA extraction using glass beads to that using the SMI TEST EX-R&D, we extracted DNA from cells that contain the same amount of RCA. We used the SMI TEST EX-R&D as an example of a method that can extract DNA with high efficiency [19]. As shown in Fig. 4B, we detected equal amounts of RCA in DNA extracted by these two methods, meaning that glass beads are useful for extracting DNA rapidly, with an efficiency similar to that of the previous method.

Infectivity PCR for the Detection of RCA

For examining the infectivity PCR, we used RCA in the absence of adenovirus vectors as a preliminary experiment. HeLa cells were infected with serial log-diluted RCA (0, 0.1, 1, 10, 100, 1000, 10,000 pfu) in triplicate and cultured. After culturing them for 1, 3, 6, or 9 days, we harvested the cells and extracted the DNA using glass beads. We then measured the amount of RCA in each sample by real-time quantitative PCR. As shown in Figs. 5A and 5B, we detected RCA in all of the triplicate samples that had been infected with more than 1 pfu of RCA. RCA was not detected in the samples that had been infected with 0.1 pfu of RCA. Therefore, we concluded that at least 1 pfu of RCA was detectable by this assay. In parallel with this infectivity PCR assay, we observed the cells by microscopy and tested for CPE (Fig. 5C). On day 6, we observed weak signs of CPE in the cells that had been infected with 1000 pfu of RCA, and we clearly observed CPE in the cells that had been infected with 10,000 pfu of RCA. On day 9, we observed slight CPE in the cells that had been infected with 100 pfu of RCA, and we clearly observed CPE in the cells that had been infected with 1000 or 10,000 pfu of RCA. These results showed that the sensitivity of infectivity PCR was 100 or 1000 times higher than that of the CPE assay. In addition, by the infectivity PCR method, RCA could be detected at an earlier time point than by the CPE assay. Since A549 cells are often used for CPE assay, we also examined infectivity PCR using A549 cells. The viability of the cells decreased later than day 6, and it was difficult to test the CPE. The amount of RCA amplified in A549 cells tended to be lower than that in HeLa cells(data not shown). Therefore, we used HeLa cells for further examination.

(A)



(B) Infectivity PCR

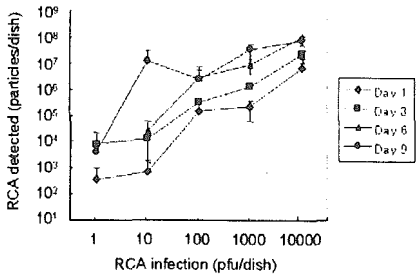
pfu/dish	Day1	Day3	Day6	Day9
10000	3/3	3/3	3/3	3/3
1000	3/3	3/3	3/3	3/3
100	3/3	3/3	3/3	3/3
10	0/3	2/3	3/3	3/3
1	0/3	1/3	2/3	2/3
0.1	0/3	0/3	0/3	0/3
0	0/3	0/3	0/3	0/3

(C) CPE method

pfu/dish	Day1	Day3	Day6	Day9
10000	0/3	0/3	3/3	3/3
1000	0/3	0/3	3/3	3/3
100	0/3	0/3	0/3	3/3
10	0/3	0/3	0/3	0/3
1	0/3	0/3	0/3	0/3
0.1	0/3	0/3	0/3	0/3
0	0/3	0/3	0/3	0/3

FIG. 5. Comparison of RCA detection by CPE assay and infectivity PCR. HeLa cells were infected with serial dilutions of RCA in medium. CPE was observed and cells were harvested on days 1, 3, 6, and 9. The viral DNA was extracted by glass beads and then the amount of RCA was determined by real-time quantitative PCR. (A) RCA growth curve in HeLa cells. Data are the means ± SD ($n = 3$). (B) The ratio of E1 DNA-positive samples is indicated ($n = 3$). (C) The number of CPE-positive samples is indicated ($n = 3$).

(A)



(B) Infectivity PCR (TaqMan PCR)

pfu/dish	Day1	Day3	Day6	Day9
10000	3/3	3/3	3/3	3/3
1000	3/3	3/3	3/3	3/3
100	3/3	3/3	3/3	3/3
10	1/3	2/3	3/3	3/3
1	1/3	1/3	0/3	2/3
0.1	0/3	0/3	0/3	0/3
0	0/3	0/3	0/3	0/3

(C) Infectivity PCR (Nested PCR)

pfu/dish	Day1	Day3	Day6	Day9
1	2/3	2/3	1/3	2/3

(D) CPE method

pfu/dish	Day1	Day3	Day6	Day9
10000	0/3	0/3	0/3	1/3
1000	0/3	0/3	0/3	0/3
100	0/3	0/3	0/3	0/3
10	0/3	0/3	0/3	0/3
1	0/3	0/3	0/3	0/3
0.1	0/3	0/3	0/3	0/3
0	0/3	0/3	0/3	0/3

FIG. 6. Comparison of the detection of RCA spiked into adenovirus vectors by CPE assay and infectivity PCR. HeLa cells were infected with serial dilutions of RCA spiked into 10^9 particles of adenovirus vectors. CPE was observed and cells were harvested on days 1, 3, 6, and 9. The viral DNA was extracted by glass beads, and then the amount of RCA was determined by real-time quantitative PCR or nested PCR. (A) RCA growth curve in HeLa cells. Data are the means ± SD (n = 3). (B and C) The ratio of E1 DNA-positive samples is indicated (n = 3). (D) The number of CPE-positive samples is indicated (n = 3).

Infectivity PCR for the Detection of RCA Spiked into Adenovirus Vector Products

Finally, we applied the infectivity PCR method for detecting RCA spiked into adenovirus vectors (Fig. 6). We infected 1.5×10^6 HeLa cells with 0, 0.1, 1, 10, 100, 1000, or 10,000 pfu of RCA spiked into 10^9 particles of adenovirus vectors (AdHM10LacZ-3). Because 10^9 particles of the adenovirus vector were equivalent to 5×10^7 infectious units, the multiplicity of infection (m.o.i.) was about 33 in this assay. We harvested the cells on days 1, 3, 6, and 9, and then we extracted the DNA using glass beads. By real-time quantitative PCR, we detected RCA in the samples that had been infected with more than 1 pfu of RCA (Figs. 6A and 6B). We detected no RCA in the samples infected with 0.1 pfu of RCA. Therefore we concluded the detection limit of this assay to be 1 pfu. This result was the same as that in the assay performed in the absence of adenovirus vectors. The absolute value of the amplified RCA was lower than in the assay without adenovirus vectors. When we also examined the presence of RCA by nested PCR (Fig. 6C), we detected E1 DNA in the samples infected with 1 pfu of RCA, but we did not detect it in those infected with 0.1 pfu of RCA, meaning that the detection limit was still 1 pfu. We observed a slight sign of CPE on day 9 in only one of the three dishes that had been infected with 10,000 pfu of RCA (Fig. 6D). CPE appeared to be suppressed by the presence of adenovirus vectors. From these results, when we measured RCA spiked into adenovirus vectors, the sensitivity of the assay was almost 10,000 times higher by infectivity PCR than by CPE assay. Thus the RCA contamination in adenovirus vectors could be detected with high sensitivity and short-term cell culture by the infectivity PCR method reported here.

Discussion

The infectivity PCR assay was developed for detecting RCA in adenovirus vectors (Fig. 7). The cell culture/CPE method in common use involves an infection of HeLa or A549 cells, harvesting, blind passage on fresh cells, and the search for CPE (Fig. 7A). It usually takes more than 4 weeks. In the method we reported here, HeLa cells are infected with adenovirus vector products and cultured for 3 or more days. The cells are harvested and viral DNA is extracted using glass beads, and then the amount of E1 DNA is measured by real-time quantitative PCR. At least 1 pfu of RCA spiked into adenovirus vectors can be detected by this method. The infectivity PCR method thus provides a more sensitive, rapid, and simple means of testing the quality of adenovirus vectors than the traditional CPE assay.

As a sensitive method for the detection of viral genome, PCR has been widely used. Real-time quantitative PCR is sensitive and highly reproducible over a wide dynamic range in addition to having high-throughput capacity. In our study, 10^3 to 10^8 particles of RCA could be detected quantitatively by real-time quantitative PCR. Nested PCR is known to be suitable for the detection of smaller amounts of DNA. By nested PCR, fewer than 10^1 particles of RCA could be detected. While the PCR method has advantages in terms of sensitivity, it has a disadvantage in that not only infectious virus but also uninfectious viral particles or DNA fragments are detected. In our experiments, we used primers and a probe for the E1 sequence that is present in RCA but not in adenovirus vectors. However, in addition to RCA, HEK293 cells in which the adenovirus vectors had been propagated had the E1 sequence in their genome. Although the adenovirus vectors had been purified by CsCl ultracentrifugation after treating the HEK293 cell lysate with nuclease, HEK293 cell-derived E1 DNA was detected in adenovirus vector products, and thus if the E1 DNA was detected in adenovirus vector products by PCR, it is not clear whether the origin is RCA or HEK293 cells. The presence of cellular E1 DNA in adenovirus vector products might not be limited to our sample, because purification by CsCl ultracentrifugation is the method used for the adenovirus vectors for clinical studies [20], and cellular DNA has been detected in adenovirus reference materials [21]. On the other hand, the fact that the ratio of infectious titer to particle concentration was 1:8.6 (see Materials and Methods) means that not all of the RCA particles

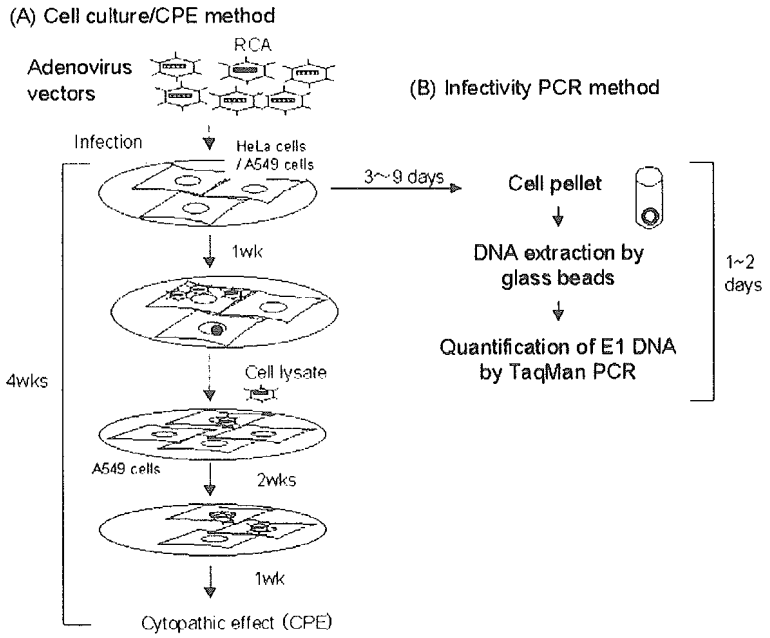


FIG. 7. (A) The cell culture/CPE method in common use involves an infection of HeLa or A549 cells, harvesting, blind passage on fresh cells, and the search for CPE. It usually takes more than four weeks. (B) In infectivity PCR method, HeLa cell are infected with adenovirus vector products, and cultured for three or more days. The cells are harvested and viral DNA is extracted using glass beads, and then the amount of E1 DNA is measured by real-time quantitative PCR.

were infectious. Therefore, infection of the adenovirus vectors into cells was thought to be indispensable for the selective amplification of the infectious RCA particles.

In the infectivity PCR assay, RCA could be detected with a detection limit of 1 pfu irrespective of the presence of adenovirus vectors. On the other hand, CPE was observed in the cells that had been infected with 10^2 to 10^4 pfu of RCA without adenovirus vectors or 10^4 pfu of RCA spiked into adenovirus vectors. Therefore, for the detection of RCA spiked into adenovirus vectors, the infectivity PCR was shown to be almost 10,000 times more sensitive than the CPE assay.

Since nested PCR was able to detect smaller amounts of DNA than real-time quantitative PCR, nested PCR was expected to contribute to an increase in the sensitivity of the infectivity PCR assay. However, the detection limit of the assay was still 1 pfu even when nested PCR was used. This might have been because, at lower concentrations of RCA, there was a threshold in the steps of infection or amplification in the cells, and thus the RCA was not linearly amplified.

The infectivity PCR was also shown to have the advantage of requiring fewer cells than the CPE assay. It is known that too high an input m.o.i. may lead to suppression of RCA outgrowth by the vector [12,22]. Because adenovirus vectors are prepared as high-titer stocks, a large-scale cell culturing, e.g., roller-bottle culture, is required to test the presence of RCA at a low m.o.i. Although the presence of adenovirus vectors seemed to interfere with the CPE, RCA could be detected by real-time quantitative PCR with the same detection limit of 1 pfu even in the presence of adenovirus vectors. This means that infectivity PCR can be performed at a higher m.o.i. than the CPE assay and therefore may contribute to a decrease in the number of the cells required for the assay.

The FDA currently recommends that adenovirus vector preparations contain <1 RCA in 3×10^{10} particles. Since, in our method, 10^9 particles of adenovirus vectors can be tested using one 10-cm-diameter plate, 3×10^{10} particles of adenovirus vectors can be tested using 30 plates. If 3×10^{10} particles are tested in the standard culture method with blind passage and come out positive, the only possible conclusion would be that there was >1 pfu in 3×10^{10} particles. Our method has the potential to be much more effective and quantitative than the standard culture method, because each plate can be tested separately, and then the actual level of RCA contamination can be estimated using the Poisson distribution.

With a goal of ensuring the safety of gene therapy, official guidelines have been published for the testing of replication-competent retroviruses (RCR) [23]. These guidelines, entitled *Supplemental Guidance on Testing for Replication-Competent Retrovirus in Retroviral Vector-Based Gene Therapy Products and During Follow-Up of Patients in Clinical Trials Using Retroviral Vectors*, provide information on RCR testing during manufacture (including timing), amount of material to be tested, and general testing methods. However, with respect to RCA in adenovirus vectors, the only available guidelines are the descriptions included within the *Guidance for Human Somatic Cell Therapy and Gene Therapy* [12]. In these guidelines, the cell culture/

CPE method is recommended for testing the presence of RCA. Although it is recommended that the assay sensitivity should be validated by spiking the test inocula with increasingly smaller numbers of wild-type adenovirus particles, there are currently no detailed guidelines for the RCA test. With regard to the safety concerns of adenovirus vector-based gene therapy, most research has focused on the immune response against capsid proteins [24]. In addition, preclinical safety studies are inherently limited in their assessment of RCA-related risks, since there are no animal models that support extensive replication of human wild-type adenovirus. This fact may have limited the attention paid to establishing an RCA detection method. Our method reported here could be used to test the RCA contamination in clinical lots of adenovirus vectors or to detect RCA in patients undergoing adenovirus vector-based gene therapy. Together with the recent efforts to develop cell lines that can propagate adenovirus vectors without emergence of RCA [25–27], this method might improve the safety of adenovirus vector-based gene therapy.

Materials and Methods

Cells. HeLa cells were obtained from the Japanese Cancer Research Resource Bank (Tokyo, Japan) and maintained in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS). HEK293 cells were obtained from Clontech (Palo Alto, CA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS.

Replication-competent adenovirus. The seed RCA (adenovirus type 5) was purchased from ATCC (Manassas, VA). For the amplification of the RCA, HeLa cells were infected with the RCA and harvested when CPE was observed. The cell lysate was prepared by four cycles of freezing and thawing and then was added to another dish of HeLa cells. After the amplification was repeated, the cell lysate from five 150-mm-diameter dishes was subjected to RCA purification by CsCl ultracentrifugation. The particle concentration and infectious titer of RCA were measured according to the standard operating procedure for adenovirus reference material (ATCC VR1516). For determining the particle concentration, RCA was diluted with the excipient solution (20 mM Tris, 25 mM NaCl, 2.5% glycerol (w/v), pH 8.0). After incubation for 15 min at room temperature, the absorbance value at 260 nm was measured. The particle concentration was determined as 5.826×10^{11} particles/ml. For determining the infectious titer, HEK293 cells were seeded in 96-well plates at 4×10^4 cells/well. After culturing for 1 day, medium was replaced with 200 μl of medium containing 5×10^7 to 1.28×10^{10} times diluted RCA stock. The plates were incubated for 60 min, and then the medium was replaced with 200 μl of fresh medium. On day 10, each well was examined for signs of CPE using a light microscope. The infectious titer was calculated as the normalized adjusted standard titer [28] and determined to be 6.767×10^{10} NIU/ml. The ratio of infectious titer to particle concentration was 1:8.6.

After we had prepared our in-house RCA standard, the Adenovirus Reference Material (ATCC VR-1516) developed under the guidance of the Adenovirus Reference Material Working Group and the U.S. Food and Drug Administration was distributed [21]. The ratio of infectious titer to particle concentration of the reference material is 1:8.3. The ratio of our in-house RCA was 1:8.9 (5.8×10^{11} particles/ml vs 6.5×10^{10} IU/ml) when these values were measured using the Adenovirus Reference Material as a reference. Since the ratios for our in-house standard and the reference material were similar, our in-house RCA standard was considered to have qualities similar to those of the reference material.

Adenovirus vector. Adenovirus vectors were prepared as described previously [29]. In brief, the plasmid harboring β-galactosidase in the E1-deleted region of the adenovirus, pAdHM10LacZ, was digested with *Pac*I. The linearized plasmid was transfected into subconfluent HEK293 cells plated in a 60-mm dish using SuperFect (Qiagen, Valencia, CA) according to the manufacturer's protocol. Ten days later, cells were harvested and adenovirus vectors were released by four cycles of freezing and thawing. The vectors were amplified by further infecting into HEK293 cells and then purified by CsCl step gradient ultracentrifugation followed by CsCl linear gradient ultracentrifugation.

Real-time quantitative PCR. The ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) was used for detecting real-time quantitative PCR products. The DNA extracted from RCA standard or RCA-infected cells was dissolved in 20 μl of distilled H_2O. Ten microliters of the DNA sample was used as template in a subsequent real-time quantitative PCR with 0.5 μM each primer, 0.16 μM TaqMan probe, and 25 μl of TaqMan universal PCR master mix (Applied Biosystems). The PCR was initially denatured at 95°C for 10 min and then subjected to cycles of 95°C for 15 s and 60°C for 1 min. The reaction was carried out for 50 cycles. A calibration curve was generated for the DNA extracted from purified RCA and validated using linear regression analysis. The sequences of the primers and probe used were as follows: Ad5dE1-1035F, TCCGGTCCTTCTAACACACCTC; Ad5dE1-1105R, ACGGCAACTGGTTTAATGGG; and Ad5dE1-1058TM probe, FAM-TGAGATACACCCGGTGGTCCCGC-TAMRA. These sequences were designed using Primer Express software version 1.0 (Applied Biosystems), and it was confirmed that they amplified the products of desired molecular weight.

Nested PCR for E1 DNA. The primers used for the first PCR were Jzp5-Ad3473F, CGCTGAGTTTGGCTCTAGCGAT, and Jzp6-Ad3698R, CATCACATTCTGACGCACCC. The primers for the second PCR were Jzp5-2-Ad3483F, GGCTCTAGCGATGAAGATACAG, and Jzp6-2-Ad3668R, GGGCATGCGCGTTGTCAAAT. The amplification conditions for the PCR consisted of 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, followed by a final incubation at 72°C for 7 min. Two microliters of the 50-μl reaction mixture from the first PCR was subjected to a second PCR. For the second PCR, the number of amplifications was set to 25.

PCR for pregnancy-specific glycoprotein gene. To detect the DNA derived from HEK293 cells, a pregnancy-specific glycoprotein gene adjacent to the E1 gene in HEK293 cells was selected as a target. The primers used for the PCR were PSG3-293-5281F, CTCATGCCTGCCTCTTTCACT, and PSG4-293-5567R, AGAGCCATCCA CACAATGTGC.

Nucleic acid extraction using SMI TEST EX-R&D. Cells were harvested and centrifuged at 2000 rpm for 5 min and then suspended in PBS(−). After four cycles of freezing and thawing, the nucleic acids derived from the cells were digested by incubating with DNase I (0.2 mg/ml), RNase A (0.2 mg/ml), and $MgCl_2$ (10 mM) at 37°C for 30 min. Then DNA was extracted using SMI TEST EX-R&D (Genome Science Laboratories, Fukushima, Japan) according to the manufacturer's instructions. When DNA was extracted from purified RCA or adenovirus vectors, the samples were subjected directly to DNA extraction using SMI TEST EX-R&D reagents.

Nucleic acid extraction using glass beads. Glass beads (GSB 07) approximately 7 mm in diameter were obtained from Nippon Rikagaku Kikai Co. (Tokyo, Japan). The glass beads were treated with 30% hydrogen fluoride (HF) solution for 1 h and then extensively washed with distilled water. The HF-treated glass beads were used for the extraction of viral genome.

For nucleic acid extraction, cells were harvested in 10-ml tubes and centrifuged at 2000 rpm for 5 min. After the medium was removed, one glass bead and 250 μl of extraction solution (200 mM Tris–HCl (pH 8.0), 200 mM NaCl, 1% *N*-lauroylsarcosinate, 1% SDS, 2% 2-mercaptoethanol, and 1 mg/ml proteinase K) were added to each tube, and then the samples were stirred for 10 s. After incubation at 65°C for 20 min, 2 ml of 70% isopropyl alcohol was added. The samples were incubated at room temperature for 1 min with gentle stirring. The solution was removed, and the beads were washed with 2 ml of 75% ethanol twice. Then the beads were

dried at room temperature. DNA was eluted from each bead by adding 50 μl of distilled H_2O and incubating at 65°C for 5 min. Twenty microliters of the sample was assayed for E1 DNA.

Infectivity PCR using HeLa cells. HeLa cells (1.5×10^6) were seeded in 100-mm-diameter dishes. One day later (day 0), the cells were infected with 1 to 10^4 pfu of RCA spiked into 10^9 particles of adenovirus vectors by incubating at 37°C for 2 h in 1 ml of MEM containing 1% FCS. After infection, the medium was replaced with MEM containing 10% FCS. On days 1, 3, 6, and 9, the cells and supernatant were harvested. DNA was extracted, and the amount of E1 DNA was measured by real-time quantitative PCR. The experiments were done in triplicate.

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Improved sensitivity for insulin in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry by premixing α-cyano-4-hydroxycinnamic acid matrix with transferrin

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This report describes an enhancement of the signal intensities of proteins and peptides in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS). When α-cyano-4-hydroxycinnamic acid (CHCA) premixed with human transferrin (Tf) was used as a matrix, the signal intensity of insulin was amplified to more than ten times that of the respective control in CHCA without Tf. The detection limit of insulin was 0.39 fmol on-probe in the presence of Tf, while it was 6.3 fmol in the absence of Tf. The signal intensity of insulin was also enhanced when the CHCA matrix was premixed with proteins other than Tf (80 kDa), such as horse ferritin (20 kDa), bovine serum albumin (BSA, 66 kDa), or human immunoglobulin G (150 kDa). The optimum spectrum of insulin was obtained when the added amount of protein was in the range 0.26–0.62 pmol, regardless of the molecular weight of the added protein. Tf and BSA outperformed the other tested proteins, as determined by improvements in the resulting spectra. When the mass spectra of several peptides and proteins were recorded in the presence of Tf or BSA, the signal intensities of large peptides such as glucagon were enhanced, though those of smaller peptides were not enhanced. In addition, the signal enhancement achieved with Tf and BSA was more pronounced for the proteins, including cytochrome C, than for the large peptides. This enhancement effect could be applied to improve the sensitivity of MALDI-TOFMS to large peptides and proteins. Copyright © 2004 John Wiley & Sons, Ltd.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) and electrospray ionization mass spectrometry have been widely used in studies of protein chemistry, including proteomics studies aimed at sequence identification or quantitative analyses following enzymatic digestion by isotope-coded affinity tags and other tagging systems.[1–8] In particular, MALDI-TOFMS has been used for the qualitative and quantitative analysis of intact proteins.[9–11] When the MALDI technique was first introduced as an ionization method for proteins, a mixture of fine metal powder and glycerol, or nicotinic acid, was used as the matrix.[12,13] Progress has been made with other matrix materials such as sinapinic acid, 2,5-dihydroxybenzoic acid (DHB), and α-cyano 4-hydroxycinnamic acid (CHCA), which have some desirable properties such as less intense adduct peaks and a relative insensitivity to contamination.[14–16] With the MALDI approach, analyte proteins are dispersed on a surface in a thin layer of matrix. The energy of an incident pulse of laser photons is absorbed by the matrix to form a jet of matrix vapor that lifts the analyte proteins from the surface and transforms some of them into ions.[13]

However, the mechanisms by which laser light irradiation is able to generate macromolecular ions have not been fully verified to date. It has been reported that the ionization of macromolecules by the MALDI process is affected by several factors. For example, peptide signal intensity was increased by the use of acetone as the solvent for CHCA matrix instead of employing the commonly used solvent, a mixture of acetonitrile and aqueous 0.1% trifluoroacetic acid (TFA).[17] The signal-to-noise (S/N) ratios for macromolecules are low in DHB matrix, but the addition of suitable additives (fructose, glucose, fucose, or 2-hydroxy-5-methoxybenzoic acid) to the DHB matrix improved its performance in the high molecular mass range.[18–21] In the CHCA and sinapinic acid matrices, the detection of higher molecular weight proteins was improved by using polytetrafluoroethylene (Teflon) as sample support.[22,23]

Recently, we investigated a method of identifying and quantifying proteins in blood using mass spectrometry. During the present study, we discovered that the signal intensity of human insulin was augmented more than 10-fold when transferrin (Tf) was mixed with the CHCA matrix

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solution used for MALDI-TOFMS. This phenomenon was not specific to either insulin or Tf, which suggested that such enhancements could be used more generally to improve the sensitivity of protein analysis with MALDI-TOFMS.

EXPERIMENTAL

Materials

Human atrial natriuretic peptide (hANP), glucagon, insulin, insulin-like growth factor-1 (IGF-1), transferrin (Tf), bovine serum albumin (BSA), horse spleen ferritin (106 mg/mL in 0.15 M NaCl), and ProteoMass Peptide & Protein, were purchased from Sigma (St. Louis, MO, USA). Human immunoglobulin G (IgG, 11.3 mg/mL in 0.01 M sodium phosphate, 0.5 M NaCl, pH 7.6) was obtained from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Human insulin, IGF-1, glucagon, and hANP stock solutions were prepared at concentrations of 100 pmol/μL by dissolving them in 0.1% TFA. Tf and BSA stock solutions were prepared at concentrations of 10 mg/mL by dissolving the materials in Millipore deionized water. ProteoMass Peptide & Protein stock solutions, which include bradykinin fragment 1-7, human angiotensin II, synthetic peptide $P_{14}R$, human ACTH fragment 18-39, bovine insulin oxidized B chain, bovine insulin, equine cytochrome C, equine apomyoglobin, rabbit aldolase, and BSA, were prepared at concentrations of 100 pmol/μL each, according to the manufacturer's instructions.

Sample application and data acquisition

The Tf-mixed CHCA was a 5:1 mixture of the CHCA solution (10 mg/mL in 50% acetonitrile in 0.1% aqueous TFA) and Tf solution (0.10 μg/μL; the final concentration was approximately 8.3 ng/μL), corresponding to 0.21 pmol Tf on each well of the target plate, if not otherwise noted. The control CHCA was a mixture of the CHCA solution and deionized water (5:1). A portion of each sample solution was immediately mixed with an equal volume of the matrix solution with or without Tf, and an aliquot of 2 μL (corresponding to 1 μL of sample solution) was applied to a stainless steel target plate. Mass spectrometric analyses were performed using an AB4700 proteomics analyzer (Applied Biosystems, Foster, CA, USA). The operating conditions were as follows: Nd:YAG laser (355 nm), linear mode, and detection of positive ions. The spectra were generated by signal averaging 50 laser shots into a single spectrum. The signal intensity was obtained after performing background correction and noise reduction using the Data Processor software (Applied Biosystems). This software was also used to determine the detection limit.

To confirm whether or not the matrix solution was at an optimum composition, serially diluted CHCA, DHB, or sinapinic acid solutions (from 10 to 0.078 mg/mL in 50% acetonitrile, 50% 0.1% TFA) were added to the insulin solution (100 fmol/μL). The most intense signal was obtained when 10 mg/mL CHCA was added to the insulin solution.

RESULTS AND DISCUSSION

Human insulin solution (6.3 fmol/μL) was mixed with an equal volume of Tf-mixed CHCA or control CHCA. When

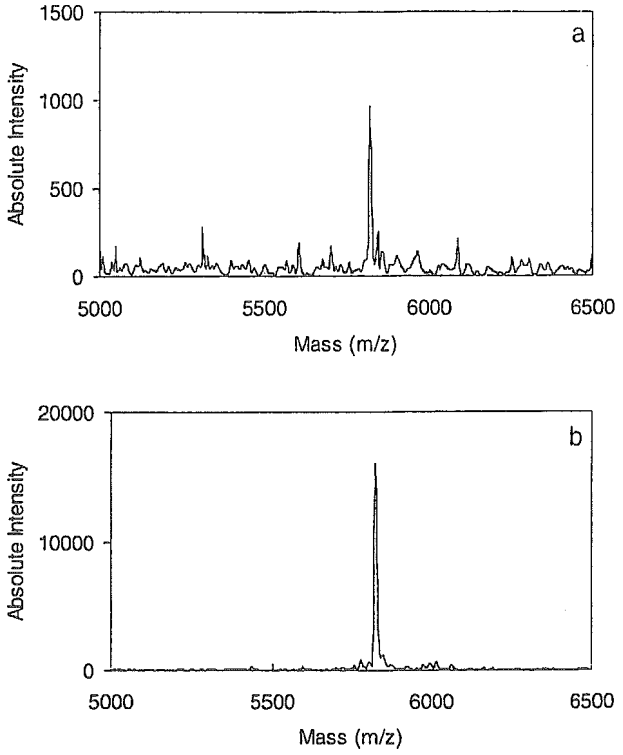

Figure 1. MALDI mass spectra of human insulin. The insulin solution (6.3 fmol/μL) and matrix solution were mixed together in equal volumes; 2 μL of the resulting mixture were applied to a target plate, allowed to dry, and analyzed by MALDI-TOFMS (see Experimental). The matrix solution was a 5:1 mixture of CHCA solution (10 mg/mL in 50% acetonitrile in 0.1% aqueous TFA) with deionized water or Tf solution (0.10 μg/μL). (a) Control CHCA used as matrix. (b) Tf-mixed CHCA used as matrix.

the Tf-mixed CHCA was used as matrix, the signal intensity of insulin in the MALDI-TOFMS detection system was amplified more than 10-fold relative to that achieved with the control CHCA (Fig. 1). To assess the sensitivity of insulin detection, the matrix solution was added to serially diluted insulin solutions (from 100 to 0.20 fmol/μL in deionized water), and samples were then spotted on a target plate. The detection limit of insulin was 0.39 fmol on the target plate in a Tf-mixed CHCA matrix under the present experimental conditions, whereas this limit was 6.3 fmol in the case of CHCA without Tf (Fig. 2).

To obtain the optimum concentration of Tf for the enhancement of insulin measurement sensitivity, the CHCA solution was mixed with serially diluted Tf solutions (from 1.0 μg/μL to 7.8 ng/μL) before addition to the insulin solution (100 fmol/μL). The signal intensity increased in a Tf-concentration-dependent manner (Fig. 3(a)). However, the S/N ratio decreased when the Tf concentration was more than 125 ng/μL (Fig. 3(b)), though it should be noted that the S/N value was still higher than the corresponding control value, i.e., 15 ± 7. A signal for 0.39 fmol/μL insulin was detected in the CHCA solution mixed with 0.1 μg/μL Tf (Fig. 2), whereas the signal for 1.6 fmol/μL insulin was not detected in the CHCA solution mixed with 1.0 μg/μL Tf (data not shown). These results suggest that the detection limit was also decreased in the presence of a high concentration of Tf.

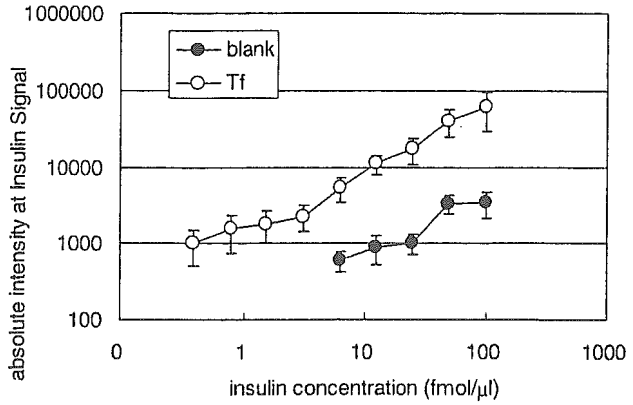

Figure 2. Dependence of insulin signals on insulin concentration. Sequentially diluted human insulin solution (100 to 0.20 fmol/μL in deionized water) and matrix solution were mixed in equal volumes. The matrix solution was a 5:1 mixture of the CHCA solution with either deionized water or Tf solution (0.10 μg/μL). The absolute intensity of the insulin signal obtained from Tf-mixed CHCA (open circles) is compared with that obtained for the control CHCA (closed circles). Each point represents the mean ± S.E. of four tests.

It is known that an excess amount of protein components can strongly influence the behavior of the MALDI process, resulting in partial or complete ion signal suppression.[24] In addition, the optimum mass ratio between the analyte and matrix for MALDI analysis has been demonstrated empirically.[15] When the CHCA was mixed with 1.0 μg/μL Tf, the excess amount of Tf might have suppressed the signal intensity of insulin as well. However, if that amount is appropriate, Tf appears somehow capable of enhancing the signal.

To determine whether or not the enhancement of the insulin MALDI-TOFMS signal intensity was specific to Tf, the CHCA solution was mixed with serially diluted solutions of several peptides and proteins before its addition to the insulin solution. The insulin signal intensity was also enhanced in the presence of ferritin (20 kDa), BSA (66 kDa), or IgG (150 kDa) (Fig. 4(a)). However, this was not found to occur in a simple concentration-dependent manner in the case of either ferritin or IgG; furthermore, when the CHCA solution was mixed with more than 2.0 μg/μL of these protein solutions, no insulin signal was detected. The enhancement of the insulin signal intensity was relatively small in the presence of peptides such as hANP (3.1 kDa) and glucagon (3.4 kDa). In addition, when the CHCA solution was mixed with more than 77 ng/μL of hANP or 87 ng/μL of glucagon, no insulin signal was detected. Among the tested peptides and proteins, the insulin signal intensity was enhanced most effectively in the presence of Tf (80 kDa) or BSA. Therefore, it is probable that this type of enhancement requires an added protein of moderate molecular weight, namely 66–80 kDa.

With regard to the results for the serial dilutions of the added peptides and proteins, the highest S/N values were obtained at 4.8 ng/μL hANP, 5.4 ng/μL glucagon, 66 ng/μL ferritin, 0.13 μg/μL BSA, 0.13 μg/μL Tf, or 0.57 μg/μL

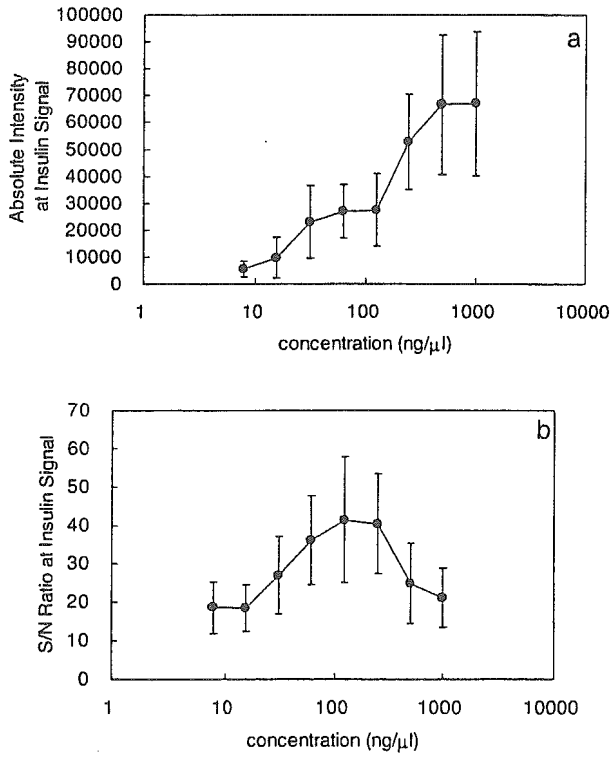

Figure 3. Dependence of insulin signal on Tf concentration. Serially diluted Tf solution was added to five volumes of the CHCA solution before mixing the resulting solution with an equal volume of human insulin (100 fmol/μL): (a) absolute intensity (arbitrary units) and (b) S/N ratio of the insulin signal in the MALDI analysis. Each point represents the mean ± S.E. of four tests.

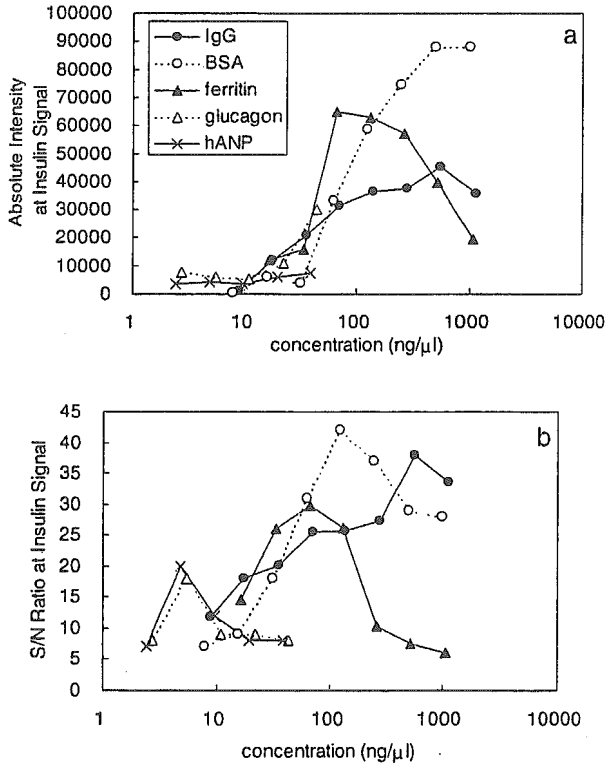

Figure 4. Dependence of insulin signal on concentrations of various added proteins. Serially diluted IgG, BSA, ferritin, glucagon, or hANP solution was added to the CHCA solution before the solution was mixed with the human insulin solution (100 fmol/μL): (a) absolute intensity (units) and (b) S/N ratio of the insulin signal. Each point represents the average of duplicate samples.