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別紙1

厚生労働科学研究研究費補助金

萌芽的先端医療技術推進研究事業

細胞結合配列を用いた易吸収性ペプチド製剤の設計

平成16年度　総括研究報告書

主任研究者　矢野　明

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別紙3

厚生労働科学研究費補助金（萌芽的先端医療技術推進研究事業）
総括研究報告書

細胞結合配列を用いた易吸収性ペプチド製剤の設計

主任研究者　矢野　明　　国立保健医療科学院　主任研究官

研究要旨　アルギニン-グリシン-アスパラギン酸（ＲＧＤ）を代表とする細胞結合配列をペプチドのN末端に付加することで、ペプチドの免疫原性が増強され、その水溶液を鼻腔粘膜へ滴下するだけで血清中に抗ペプチド抗体を誘導できることを確認した。オボアルブミンに対する抗体を誘導可能なモデルペプチドだけでなく、A型インフルエンザウイルス(H1N1)のHAタンパク質由来のHA1ペプチドや、M2タンパク質由来のM2ペプチドにおいても鼻腔免疫のみで血清抗体を誘導可能なペプチドを設計することができた。

A．研究目的

　　近年、生理活性を持った様々なペプチドが報告され、薬剤としての応用が期待されている。ワクチンの分野においても、ペプチドは必要最小限のエピトープに対する免疫を誘導でき、安全性が高いことから、新しいタイプの感染防御ワクチンやガン細胞のみを攻撃させるガンワクチンとしての開発が行われている。しかし、ワクチンとして用いるためには抗原性が弱いという致命的な欠陥がある。そこで、リポソームなどのベクターを用いてペプチドを免疫するというアプローチが考えられ、様々なベクターの研究開発が精力的に行われている。これに対し、本研究ではペプチド製剤そのものに生体組織への親和性、選択性を持たせ、ペプチドの免疫原性を高めることを目的としている。これに成功すればワクチン以外のペプチド製剤に関しても、その有効性を高め使用濃度を下げられる可能性がある。将来は鼻からペプチド溶液を滴下あるいは噴霧するだけで免疫誘導可能になり、手軽に感染予防やガン予防あるいは治療が可能になるかもしれない。

　　研究初年度は、以前発見した細胞結合配列の効果についてモデルペプチドワクチンを用い確証を得ること、具体的な疾患を含めた様々なペプチド配列に対しての一般性を確認すること、さらには細胞生物学的な実験を行い細胞への結合、取り込みが行われているか確認を行うことを目的とした。また、ペプチドの製造法にも適用可能な、タンパク質の生物学的生産方法に関して若干の検討も行う。

B．研究方法

　　モデルペプチドとして、以前から用いている"う蝕細菌"由来のSmU、オボアルブミン由来のOVApを含むペプチド（RGD-OMP-KK-SmU, RGD-OMP-KK-OVAp）に加え、A型インフルエンザウイルス(H1N1)由来のHAタンパク質の部分配列やM2タンパク質の部分配列を含むペプチド（RGD-Gag-KK-HA1, RGD-Gag-KK-M2）を設計し、AdvancedChemtech社のマルチペプチドシンセサイザーModel350を用いて合成した。逆相クロマトグラフィーにより>90%に精製し、これらペプチドをマウスの鼻腔に50μg、あるいは皮下に100μgを2週おきに3-5回免疫した。その後、血清抗体価、糞中への分泌抗体価をELISA法によって測定し、細胞結合配列の免疫原性増強効果の一般性を検証した。

　　細胞結合配列の効果に関して、細胞生物学的検証を行うため、マウスのマクロファージ様培養細胞、および、対照として単球様培養細胞を用いた。これら培養細胞に細胞結合性配列をもつペプチドを作用させ、ペプチドの細胞内外における動態の可視化に関して検討を行う。各種蛍光ラベルを付加したペプチドを設計し、培養細胞の密度、培養液の組成、ペプチドの濃度、標識法等を調整し、実験系の構築を進める。

　　また、ペプチドにも適用可能なタンパク質の生物学的な製造法に関して、植物を用いた生産系の検討を行う。

　　（倫理面への配慮）

　　動物愛護の観点に則り、倫理的に問題のないよう考慮したうえで実験を行う。また実験動物に苦痛を

ほとんど与えない経鼻免疫実験を主体とする。培養細胞を用いた実験系を積極的に取り入れる。研究に利用する培養細胞、アミノ酸配列情報などは公的機関が提供するものを用いる。

C．研究結果

本研究事業以前に確認していたRGD-OMP-KK-SmU ペプチド 50 µg をマウスBALB/c 系統に鼻腔免疫したときに、抗 SmU 抗体が誘導可能であること、また RGD-OMP-KK-OVAp 50 µg の BALB/c への鼻腔免疫によって抗 OVA 抗体が誘導可能であることを再確認できた。投与する濃度の検討も行ったが、RGD-OMP-KK-OVAp ペプチドの場合 10 µg で鼻腔免疫が可能であることがわかった。また、B10.D2 や B10.S など、他のマウス系統にも抗体誘導が確認でき、細胞結合配列を用いた免疫原性の増強効果の一般性が確認できた。一方、他のグループによって報告されていたインフルエンザウイルスの中和エピトープを持つペプチド、HA1, M2、に細胞結合配列を用いた設計を適用し、RGD-Gag-KK-HA1, RGD-Gag-KK-M2 というペプチドを作製し、これを B10.A 系統のマウスに鼻腔免疫したところ、抗 HA1 および抗 M2 ペプチド抗体が誘導できた。これらペプチドが誘導したのは、ほとんど C 末端側のペプチド SmU, OVAp, HA1, M2 に対する抗体のみであった。OMP、Gag は、ともに多種の MHC-II に認識されることがわかっているマルチアグレトープタイプのペプチドであり、様々な個体の T 細胞エピトープとして働くと考えられる。また、OMP、あるいは Gag ペプチドをフロインドの不完全アジュバントを用いて皮下免疫しても、ペプチドに交差する抗体が誘導できなかった。したがって両ペプチドは B 細胞エピトープとしては弱い性質を持つことがわかった。

RGD を持つペプチドにビオチンタグや FITC などの蛍光ラベルを入れ、マウス培養細胞におけるペプチドの動態を可視化することを試みているが、実験条件の検討段階であり、はっきりした結果が得られていない。

ヒト抗体のような医療用タンパク質をタバコ培養細胞にて産生できる。技術的にはペプチドの生産にも適用可能であるが、このような生産系が有用であるか問題点を議論し論文としてまとめた。

D．考察

マウスを用いた免疫実験の結果を見る限り、細胞結合配列を付加したペプチドは一般的に高い免疫原性をもち、リポソームなどのベクターを用いることなく鼻腔免疫が可能であると考えられる。また、OMP や Gag などのマルチアグレトープタイプの T 細胞エピトープをもつペプチドを N 末端に配置することで、MHC-II 型の異なる系統に抗体誘導が可能なペプチドが設計可能である。このとき、B 細胞エピトープとしては弱い配列を採用することで、C 末端のペプチドに特異的な抗体の誘導が期待できる。同様の性質を備える、ヒト用の T 細胞エピトープを用いれば、ヒトに使用可能なペプチドの設計が十分可能であろう。問題はどのようにしてヒト用エピトープを決定するかである。ひとつの手法としては、今年度から試みを始めている培養細胞を用いたペプチドの動態を可視化する実験系を用いることが考えられる。ペプチドが細胞に取り込まれ、リソソームで消化された後、MHC(HLA)-II 分子にローディングされ HLA-ペプチド複合体が細胞表面に提示される。これが可視化できればペプチドがヒト用の T 細胞エピトープを持つか否か、HLA-II を持つヒト細胞を多数そろえることでアッセイ可能になる。

RGD をはじめとする細胞結合配列の生体内における挙動は複雑で、細胞接着の研究者にとっても全体像は把握できていないのが現状である。したがって、単純化した in vitro 系で局所的なペプチドの挙動をシュミレーションすることが重要であると考えられる。

E．結論

細胞結合配列を付加することで、ペプチドの免疫原性は増強され、さらに、細胞結合配列をもつペプチドは鼻腔粘膜への投与によって血清抗体を誘導可能になる。実用的なペプチドワクチンの設計としては、N 末端に細胞結合配列、続いて T 細胞エピトープ、リジンリンカーを挟んで C 末端に B 細胞エピトープを持つ。このとき T 細胞エピトープには多種の MHC-II に対応したマルチアグレトープ型で、かつ B 細胞エピトープとしては弱いペプチドが望ましい。

F．研究発表

1．論文発表

Yano A, Onozuzka A, Asahi-Ozaki Y, Imai S, Hnada N, Miwa Y, Nisizawa T. An ingenious design for peptide vaccines. Vaccine (2005) 23(17-18): 2322-2326.

Yano A, Takekoshi M. Transgenic plant-derived pharmaceuticals –the practical approach? Expert Opinion on Biological Therapy (2004) 4(10):1565-1568.

Yano A, Maeda F, Takekoshi M. Transgenic Tobacco Cells Producing the Human Monoclonal Antibody to Hepatitis B Virus Surface Antigen.

Journal of Medical Virology (2004). 73(2):208-215.

２．学会発表
Yano A, Miwa Y, Onozuka A, Nisizawa T. The ingenious peptide design for vaccines and adjuvants. 4th World Congress on Vaccines and Immunisation. (Tsukuba Science City) Sep-Oct, 2004.

G．知的所有権の取得状況
１．特許取得　なし
２．実用新案登録　なし
３．その他　なし

別紙4

研究成果の刊行に関する一覧表レイアウト（参考）

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
なし							

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yano A, Onozuzka A, Asahi-Ozaki Y, Imai S, Hnada N, Miwa Y, Nisizawa T.	An ingenious design for peptide vaccines.	Vaccine	23(17-18)	2322-2326	2005
Yano A, Takekoshi M.	Transgenic plant-derived pharmaceuticals - the practical approach?	Expert Opinion on Biological Therapy	4(10)	1565-1568	2004
Yano A, Maeda F, Takekoshi M.	Transgenic Tobacco Cells Producing the Human Monoclonal Antibody to Hepatitis B Virus Surface Antigen.	Journal of Medical Virology	73(2)	208-215	2004

ELSEVIER

Vaccine

An ingenious design for peptide vaccines

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Abstract

For humoral immunization, it may be possible to make effective and safe peptide vaccines for various diseases by selection of proper B-cell epitopes. However, a lack of T-cell epitopes on short peptides, such as those associated with major histocompatibility complex (MHC)-restriction, is a major problem for peptide vaccine development. We propose a solution for the design of peptide vaccines that involves induction of broadly reactive T-cell epitopes via agretopes. The strategy involves positioning multi-agretope type peptides on the N-terminal side of a di-lysine linker and B-cell epitopes on the C-terminal side. The addition of the arginine–glysine–aspartate (RGD)-motif to the N terminus of the peptide enhances its immunogenicity, and enables nasal immunization without adjuvants.
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Keywords: RGD-motif; Multi-agretope; MHC-restriction

1. Introduction

The advantage of a peptide vaccine is that it can induce immune responses to a specific sequence of amino acids. However, the vaccine peptide must contain at least one major histocompatibility complex (MHC) binding motif (agretope) as an antigen, since antigens are presented by the immune system as a short peptide that binds to the MHC. Subsequently, the T-cell receptors recognize the peptide as a T-cell epitope on the MHC molecules [1–3]. For a humoral immune response, a peptide must have B-cell epitopes to induce specific antibodies and at least one agretope/T-cell epitope for presentation by MHC class II (MHC-II) molecules [1–3]. However, there are over 10 haplotypes of MHC-II in human [1] and the agretopes are different for each haplotype. The MHC-restriction is the most critical impediment to the development of peptide vaccines.

A peptide vaccine has another disadvantage. The weak antigenicity of peptide vaccines dictates a need for use of strong adjuvants, such as Freund's, for induction of antibodies. Several approaches, such as liposome capsulation [4], MAPs [5], and lipopeptides [6] have been used to eliminate the need for strong adjuvants. We have introduced the arginine–glysine–aspartate (RGD)-motif into peptide antigens. The RGD-motif is the most representative cell attachment motif seems to enhance the binding of peptides to specific receptors. Antigenicity of the peptides was enhanced and nasal immunization by peptides was successful without adjuvants [7].

We have proposed a design for peptide vaccines that contain the RGD-motif, and the lysine linker (-KK-) that joins two peptides [7,8]. The lysine linker is the target sequence of the lyzosomal protease, cathepsin B, which is one of the important proteases for antigen processing in the context of MHC-II antigen presentation [2]. When two peptide antigens are joined with -KK-, we can avoid induction of antibodies to the amino acid sequence that is generated by joining of two peptides and most antibodies are reactive to each peptide [8]. In our previous report, we have noticed a bias between the peptides, depending upon whether they are on the N- or C-terminal side of -KK- for induction of antibodies [7]. In this study, we have investigated the reason for this bias and pro-

pose the use of the bias as a solution to the MHC-restriction problem.

2. Materials and methods

2.1. Peptide synthesis

All peptides used in this study were synthesized by a step-wise solid-phase procedure as described previously [7]. Peptides were verified by MALDI-TOF/MS (Voyager-DE™S TR work station: Applied Biosystems Foster City, CA) when necessary. The single-letter universally accepted notation for amino acids is used throughout the text.

2.2. Immunizations

BALB/c, B10.D2, B10.S, B10.A, and C3H/HeJ mice were obtained from Japan SLC Inc. (Shizuoka, Japan) and were used at 6 weeks of age to begin the immunization in all experiments. Groups of four to six mice were immunized intranasally with 50 µg of a peptide, either with or without 1 µg of cholera toxin (CT: Sigma-Aldrich, Missouri). A micropipettor was used to gently instill 4 µL of immunogen-containing saline solution into the nasal cavities of each mouse (2 µL into each nasal orifice). Two identical booster doses were given at 2-week intervals.

One group of mice was primed subcutaneously with 100 µg of peptides in 100 µL phosphate-buffered saline (PBS) either with or without 100 µL Freund's incomplete adjuvants (FIA: Pierce and Endogen: Funakoshi Co. Ltd., Tokyo, Japan). The same subcutaneous booster dose was given at 2-week intervals.

HA vaccine (split-product virus vaccine) was prepared from influenza virus A/PR/8/34 (A/PR8, H1N1) according to the method of Davenport et al. [9] at the Kitasato Institute (Saitama, Japan). Groups of BALB/c mice were intranasally immunized 1 µg of HA vaccine with 1 µg of peptide or 1 µg of CT. Four booster doses were given at 2-week intervals.

One week after the last booster dose, animals were bled and serum samples were prepared from clotted blood by centrifugation and stored individually with Complete™ protease inhibitor cocktail (Roche Diagnostics Japan, Tokyo) and 0.05% (w/v) of sodium azide.

2.3. ELISA assays

Protein antigens used for ELISA were BSA, OVA, and PAc. Recombinant PAc was isolated from *Streptococcus mutans* TK18 as described previously [10]. For the ELISA, 96-well microtiter H-plates (Sumitomo Bakelite, Tokyo, Japan) were coated with 2 µg/well peptide or 1 µg/well protein antigen in 100 µL of 50 mM carbonate buffer pH 9.6 and held overnight at 4 °C. All assays were performed with alkaline phosphatase and p-nitrophenyl phosphate systems as described previously [7]. The $OD_{405-620}$ was measured using

a microtiter plate reader (Multiskan BICHROMATIC, Labosystem, Helsinki, Finland). The ELISA antibody titer was expressed as the reciprocal of the highest dilution giving an $OD_{405-620}$ of 0.1 unit above that of the control wells without antigen.

3. Results and discussion

3.1. Investigation of B-cell epitopes

SmU, with the amino acid sequence TYEAALKQ-YEADL, is a minimum peptide antigen (PAc365–377) for the induction of antibodies that cross react with the cell surface protein antigen of *S. mutans* [10]. SmU has both a strong B-cell epitope and the helper T-cell epitope for H-2^d haplotype mice, but lacks the T-cell epitope for H-2^s haplotype [11]. OVAp is a peptide antigen (OVA323–336: ISQAVHAA-HAEINE) for induction of antibodies that are cross-reactive to ovoalbumin, which has strong epitopes for both B- and the T-cells of H-2^d haplotype mice [11]. To investigate the position bias of the peptide containing lysine linker, we synthesized the long peptides, SmU-KK-OVAp, OVAp-KK-SmU, RGD-SmU-KK-OVAp and RGD-OVAp-KK-SmU. We immunized mice both nasally and subcutaneously with the peptides, either with or without FIA. The serum titers to peptide antigens, PAc and OVA are shown in Fig. 1. In all cases, both SmU and OVAp were good antigens for BALB/c mice (H-2^d) and induced antibodies cross-reactive to PAc and OVA. When we focused on SmU, it was unclear whether the N- or C-terminal side of -KK- linker was the more favorable position for induction of antibodies to PAc. This was also true for OVAp. When the RGD-motif was added to the peptides
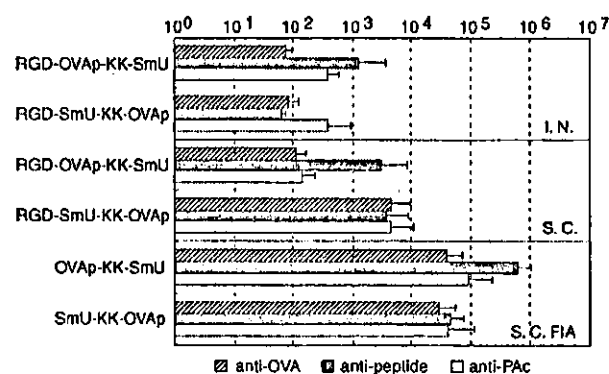

Fig. 1. Serum titres induced by intranasal and subcutaneous immunization with peptides. BALB/c mice were intranasally (I.N.) immunized with 50 µg of RGD added peptides, followed by two booster doses at two-week intervals. One hundred micrograms of peptide with FIA was used for subcutaneous (S.C.) immunization and immunization with RGD added peptides without adjuvant was followed by a booster dose given at a two-week interval. One week after the last booster dose, serum samples were collected and serum antibody titres were determined by ELISA. Average serum titers are shown for anti-OVA (hatched-box), anti-peptide antigen (grey-box) and anti-PAc (open box) with SD bars for each group.

and used for nasal immunization, SmU was a stronger antigen than OVAp, independent of its position. Those results clearly indicated that if both peptides, placed either on the N- or C-terminal side of -KK- linker, were strong enough as helper T-cell epitopes, antibody titers were dependent upon the strength of B-cell epitopes.

3.2. The position of T-cell epitopes and MHC-restriction

The effects of both the position and the strength of T-cell epitopes in the peptide, including the lysine linker, were examined. During the screening of SmU from the PAc, we had analyzed B- and T-cell epitopes of several peptides. PAc305–318 (NEADYQAKLTAYQT) has weaker B- and T-cell epitopes for H-2^d mice than SmU. However, unlike SmU, it has T-cell epitopes for H-2^s mice [11]. We have synthesized two peptides, SmU-KK-PAc305–318 and PAc305–318-KK-SmU that were used with FIA to subcutaneously immunize B10.D2 (H-2^d) and B10.S (H-2^s) mice (Fig. 2). B10.D2 mice responded with antibodies to both PAc305–318 and SmU in response to both peptides. SmU is a stronger B-cell epitope than PAc305–318, since anti-SmU titers are always higher than those of anti-PAc305–318. When we investigated the importance of the peptide position on either the N- or C-terminal side of the linker, the C-terminal position was best for induction of antibodies. B10.S mice produced antibodies only to the PAc305–318-KK-SmU. In this case, T-cell epitopes for H-2^s haplotype mice existed only on PAc305–318. Thus, we could conclude that T-cell epitopes should be placed on the N-terminal side of -KK- linker. The cause of this phenomenon is not clear. There may be molecular mechanisms that preferentially achieve MHC-II loading of peptides that are placed on the N-terminal side of the lyzosomal digestion site. Since SmU lacks T-cell epitopes for H-2^s haplotype, B10.S mice could not produce antibodies by immunization with SmU alone. However, we were able to induce antibodies to SmU by immunization with PAc305–318-KK-SmU.

This result shows a solution to MHC-restriction and points to the possibility of developing peptide vaccines for clinical use.

Thus, T-cell epitopes on the N-terminal side of -KK-linker were sufficient for induction of the antibodies. Therefore, if we placed the multi-agretope type peptide, broadly cross-reactive T-cell epitopes, such as T1 [12], Gag298-312 (KRWIILGLNKIVRMY)[13], or overlapping multi-agretope type peptide (OMP: LAVYWELLAKYLL-DRVQKVA) [7], on the N-terminal side of -KK- linker, we should be able to develop broadly effective peptide vaccines for human and verterinary use. However, peptides on the N-terminal side should not induce antibodies. We examined the ability of those peptides to induce antibodies by immunization of several types of mice. OMP and Gag298-312 with FIA were used to immunize B10.BR (H-2^k), B10.D2 (H-2^d), B10.S (H-2^s), BALB/c (H-2^d) and CH3/HeJ (H-2^k) mice and those peptides seemed to have very weak B-cell epitopes (Fig. 3A). Those peptides are suitable for T-cell epitopes in our peptide vaccines. We have synthesized peptides for induction of antibodies to SmU ((RGD-)OMP-KK-SmU) by immunization of B10.S mice (Fig. 3B). Intranasal immunization with OMP-KK-SmU alone did not induce specific antibodies because of its weak immunogenicity. Immunization with either RGD-added peptide alone or with CT induced
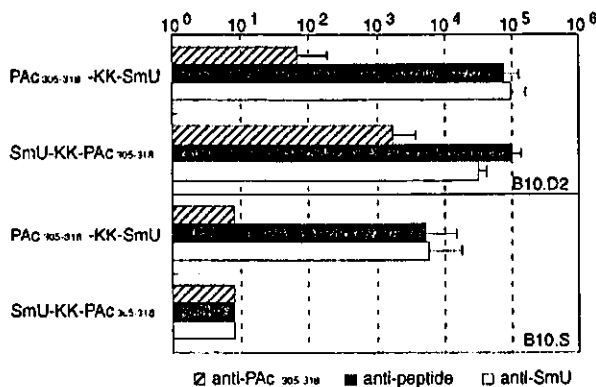

Fig. 2. Serum titres induced by subcutaneous immunization of peptides. B10.D2 and B10.S mice after subcutaneous immunization using peptides with FIA followed by a booster dose. Average serum titres are shown for anti-PAc305-318 (hatched-box), anti-peptide antigen (grey-box) and anti-SmU (open-box) with SD bars for each group.
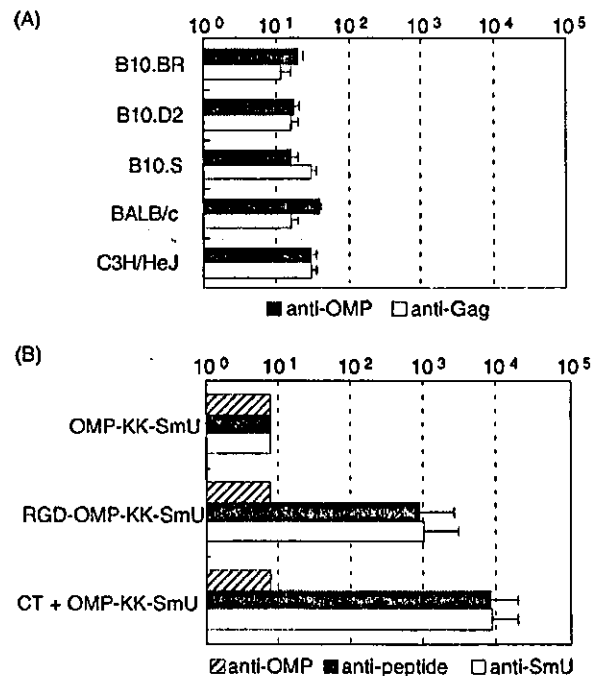
Fig. 3. Effect of multi-agretope type peptide on peptide antigens. (A) Each group of mice was immunized subcutaneously with FIA, followed by a booster dose, then specific antibody titres were determined by ELISA. Average titres are shown for anti-OMP (grey-box), and anti-Gag (open-box) with SD bars for each group. (B) B10.S mice were intranasally immunized either with or without CT. Average titres are shown for anti-OMP (hatched-box), anti-peptide antigen (grey-box), and anti-SmU (open-box) with SD bars for each group.

antibodies to SmU. OMP served as a T-cell for MHC-II of H-2^s mice in the same way as PAc305–318.

3.3. Application of the design of peptide vaccines

We have applied our design for peptide vaccines to an influenza vaccine. Several B-cell epitopes were reported that are involved in neutralization of the experimental strains of influenza virus A/PR8, HA1 (VTGLRNIPSIQSR) [14] and M2 (EVETPIRNEWGCRCNGSSD) [15]. We have synthesized peptide vaccines, RGD-Gag298-312-KK-HA1 and RGD-Gag298-312-KK-M2. In order to obtain high titer of antibodies, we immunized several mouse strains (data not shown) and B10.A was found to be the most reactive strain. Nasal immunization with peptides was repeated five times and serum titers were measured (Fig. 4A). In all cases, anti-HA1 and anti-M2 titers were specifically elevated. When two peptides were used for immunization at the same time, HA1 was a major antigen and when CT was used as mucosal adjuvant, M2 was major. It may be necessary to use additional approaches for the induction of antibodies to several peptides at the same time.

Our peptide vaccines with the RGD-motif were reported as being useful adjuvants for nasal immunization [7]. When soluble proteins, such as BSA or OVA, were used for intranasal immunization with a small amount of RGD peptide vaccine, antibodies to BSA or OVA were strongly induced. However, background level antibodies to the RGD peptide were detected. In Fig. 4B, we have intranasally immunized mice using HA vaccines either with or without RGD peptide (RGD-OMP-KK-OVAp). The RGD peptide was already confirmed to be a nasal adjuvant in BALB/c mice [7]. After a series of five nasal immunizations, serum titers to HA vaccine were elevated by addition of the peptide, but the effects were weaker than when CT was added. In order to develop clinically useful peptide vaccines and adjuvants, we have to select for strong B-cell epitopes and develop the methods for enhancing the adjuvanicity of peptides.

In conclusion, our approach to the design of a peptide vaccine enables intranasal immunization without the need for adjuvant and solves the problem of MHC-restriction. Specifically, the design places the RGD at the N-terminus, a multiagretope peptide at the N-terminal side of lysine linker, and a B-cell epitope at the C-terminus. We suggest that our design might be universally applicable to the development of peptide vaccines and adjuvants for intranasal vaccination.
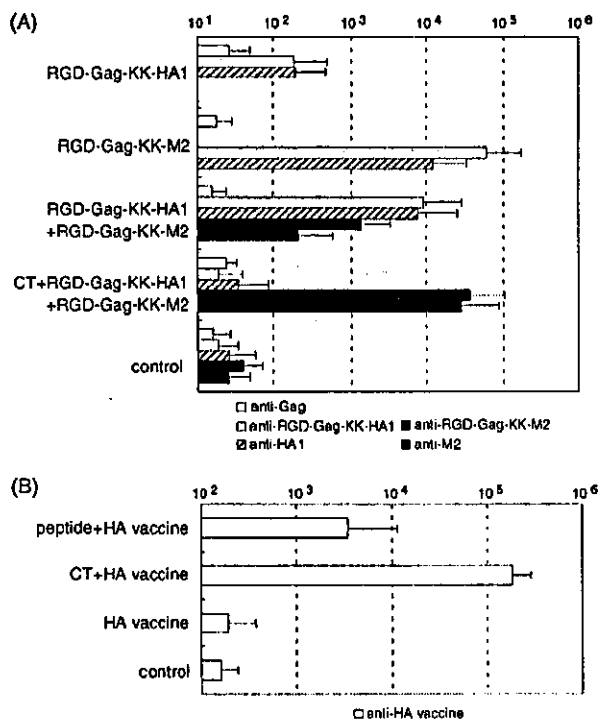
Fig. 4. Intranasal immunization with influenza vaccines. (A) B10.A mice were intranasally immunized with HA1 and M2 peptide vaccines, either with or without CT. Average titres are shown for anti-Gag (open-box), anti-HA1 (light grey-box), anti-RGD-Gag-KK-HA1 (hatched light grey-box), anti-M2 (dark grey-box) and anti-RGD-Gag-KK-M2 (hatched dark grey-box with SD bars for each group. (B) BALB/c mice were intranasally immunized with influenza HA vaccine (1 μg), with RGD-OMP-KK-OVAp (1 μg), or CT (1 μg), followed by four booster doses. Average anti-HA vaccine titres are shown as open-boxes with SD bars for each group.

References

[1] Margulies DH. The major histocompatibility complex. In: Paul WE, editor. Fundamental immunology. 4th ed. New York: Lippincott-Raven; 1999. p. 263–85.

[2] Lennon-Duménil AM, Bakker AH, Wolf-Bryant P, Ploegh HL, Lagaudrière-Gesbert C. A closer look at proteolysis and MHC-class-II-restricted antigen presentation. Curr Opin Immunol 2002;14(1):15–21.

[3] Bjorkman PJ. MHC restriction in three dimensions: a view of T cell receptor/ligand interactions. Cell 1997;89(2):167–70.

[4] Alving CR, Koulchin V, Glenn GM, Rao M. Liposomes as carriers of peptide antigens: induction of antibodies and cytotoxic T lymphocytes to conjugated and unconjugated peptides. Immunol Rev 1995;145:5–31.

[5] Tam JP. Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. Proc Natl Acad Sci USA 1988;85(15):5409–13.

[6] BenMohamed L, Wechsler SL, Nesburn AB. Lipopeptide vaccines—yesterday, today, and tomorrow. Lancet Infect Dis 2002;2(7):425–31.

[7] Yano A, Onozuka A, Matin K, Imai S, Hanada N, Nisizawa T. RGD motif enhances immunogenicity and adjuvanicity of peptide antigens following intranasal immunization. Vaccine 2003;22(2):237–43.

[8] Oishi Y, Onozuka A, Kato H, Shimura N, Imai S, Nisizawa T. The effect of amino acid spacers on the antigenicity of dimeric peptide-inducing cross-reacting antibodies to a cell surface protein antigen of *Streptococcus mutans*. Oral Microbiol Immunol 2001;16(1):40–4.

[9] Davenport FM, Hennessy AV, Brandon FM, Webster RG, Barrett CD, Lease GO. Comparisons of serologic and febrile responses in humans to vaccination with influenza A viruses or their hemagglutinins. J Lab Clin Med 1964;63:5–13.

[10] Senpuku H, Miyauchi T, Hanada N, Nisizawa T. An antigenic peptide inducing the cross-inhibiting antibodies against the interaction of *Streptococcus mutans* PAc with human salivary components. Infect Immun 1995;63(12):4695–703.

[11] Senpuku H, Iizima T, Yamaguchi Y, Nagata S, Ueno Y, Saito M, et al. Immunogenicity of peptides coupled with multiple T-cell epitopes of a surface protein antigen of *Streptococcus mutans*. Immunology 1996;88(2):275–83.

[12] Ahlers JD, Takeshita T, Pendleton CD, Berzofsky JA. Enhanced immunogenicity of HIV-1 vaccine construct by modification of the native peptide sequence. Proc Natl Acad Sci USA 1997;94(20):10856–61.

[13] Wilson CC, Palmer B, Southwood S, Sidney J, Higashimoto Y, Appella E, et al. Identification and antigenicity of broadly cross-reactive and conserved human immunodeficiency virus type 1-derived helper T-lymphocyte epitopes. J Virol 2001;75(9):4195–207.

[14] Rajnavölgyi É, Horváth A, Gogolák P, Tóth GK, Fazekas G, Fridkin M, et al. Characterizing immunodominant and protective influenza hemagglutinin epitopes by functional activity and relative binding to major histocompatibility complex class II sites. Eur J Immunol 1997;27(12):3105–14.

[15] Neirynck S, Deroo T, Saelens X, Vanlandschoot P, Jou WM, Fiers W. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nat Med 1999;5(10):1157–63.

General

Transgenic plant-derived pharmaceuticals – the practical approach?

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Production of biopharmaceuticals in transgenic plants would involve the creation of a new industry. Those transgenic plants, including staple food crops, could provide many benefits to people all over the world. However, the new industry might require a strict regulation system. It is probable that such a strict system would not be acceptable to Japan or to most developing countries. Many countries should use non-food crops for production of bio-pharmaceuticals and take on more simple systems. The new industry must develop strategies for promoting the benefits of transgenic plant-derived biopharmaceuticals on both the domestic and worldwide scales.

1. Introduction

Recombinant DNA technology has been available for ~ 30 years, and from it developed the biotechnology industry, which has grown steadily since its early days. As part of these developments, we have seen many associated scientific activities, such as the mapping of the human genome and, subsequently, postgenome projects [1,2]. The plant biotechnology industry has been a major player in this field and has created many transgenic plants, more popularly known as genetically modified (GM) crops. These crops could have an influential role to play in the present and future of bio-technology. One of the reasons behind this thinking is that GM crops have the potential to improve people's daily lives and health by providing a stable supply of food, materials and even pharmaceuticals, which would all be hallmarked by a consistent and reliable quality [3-5]. If we can steer plant biotechnology in the right direction, create effective GM crops and use them wisely, we will be able to solve one of the more difficult problems of 21st century – feeding the world's growing population.

2. Molecular farming

The biotechnology industry in industrialised countries is producing many biopharmaceuticals through the application of recombinant DNA technologies. In particular, > 200 monoclonal antibody (mAb)-based products are in clinical development, with many more in the preclinical stage [6]. If these trends continue, they could lead to new problems. Many biopharmaceuticals are bulk-produced in mammalian cell culture facilities. However, as the worldwide capacity of this means of manufacturing biopharmaceuticals is limited, plant biotechnology could provide an alternative [7]. Recent advances in plant biotechnology have made the production of human protein in plants a realistic possibility. However, it is thought that human-style glycosylation might be necessary for the successful development of therapeutic glycoproteins. Therefore, the role that plant-specific glycosylation may play in the development of recombinant mammalian glycoproteins in transgenic plants has raised a few questions

over possible immunogenicity in humans. Bakker *et al.* [8] succeeded in expressing the human-type β1,4-galactose residue by introducing human galactosyltransferase into plants. Other workers have succeeded in producing recombinant human proteins in plants that lack plant-type core-fucose and core-xylose residues [9]. One of the plant-based protein production systems, the moss reactor, has already realised production of human-type glycosylated protein [10]. Other plant-based systems are likely to follow.

Recently, the authors have reported that human mAb to hepatitis B virus (HBV) surface antigen (HBsAg) was produced by tobacco cells [11]. It will be possible to use this mAb as a substitute for anti-hepatitis B human immunoglobulin (HBIg) to prevent the transmission of HBV by combining its use with an HBV vaccine [12,13]. HBIg, which is prepared from the blood of HBsAg-positive donors, is essential for the control of HBV transmission. There are several advantages in substituting HBIg with mAb, namely, the consistent production of high-avidity antibody, good availability and no requirement for donor screening; similar advantages would extend to the use of passive immune treatments of other infectious diseases [14,15]. Plants are not usually affected by human infectious diseases; therefore, plant-derived materials are likely to contain less human infectious agents compared with materials derived from mammalian cells or human blood. If mAbs could be produced in transgenic plants, which were able to modify proteins by the addition of the human type of glycosylation, we could produce hepatitis B antibodies more cheaply and on a larger scale than HBIgs, which would also be safer as pharmaceuticals. Furthermore, such mAbs to infectious diseases would be of great benefit in developing countries where these diseases are a more serious problem than in industrialised countries.

3. How to manage the transgenic plant industry

The industrial systems necessary to produce biopharmaceuticals in transgenic plants are discussed in several journals [16-18], and draft guidance has already been proposed by the US FDA in 2002 [19]. One basic consideration is the selection of the plant species for production of pharmaceuticals. In the early stages of the development of transgenic or GM plants, food crops were often considered as ideal vehicles for protein production [3,4], and especially medicinal proteins. This was because such crops were naturally involved in the production and supply of proteins to their own storage organs (seeds or tubers). Food crops have been bred as safe (minimal content of toxins or allergens) and high-yielding, and their harvest, manufacturing and distribution systems are already in place and are cheaper to finance than those associated with pharmaceuticals. However, we now recognise that there are difficulties associated with the application of food crops to the wide-scale production of pharmaceuticals [20]. The potential for 'contamination' of non-GM crops by GM crops is one problem. This includes migration of the transgenes from the

modified varieties to their wild type, known as introgression, and the adulteration of a GM grain into non-GM grains in farms, factories and distribution processes. In the case of GM food crops designed for pharmaceuticals, only a small degree of contamination would be necessary to cause concern over adverse effects to the ecological and economical food chains. Therefore, management of the growing and manufacturing processes of GM crops must, of necessity, take this issue into account. As pharmaceuticals derived from bioengineered plants could have direct and beneficial consequences for human health [19], it is important to find ways around these problems by getting secure management systems in place. As a first step, the physical isolation of transgenic plant-based pharmaceutical production systems is required, followed by the introduction of genetic systems that would reduce the risk of contamination; systems being considered include gene use-restriction technology (GURT) [18,21]. In the US, case-by-case risk assessment analysis and management would be done by the government and the imposition of industrial regulation standards [16,17]. The implementation of wide-ranging monitoring systems for the possible contamination of staple foods by non-food GM crops might be required to guarantee food safety if the production of pharmaceuticals by GM food crops becomes an important source.

4. Expert opinion

In order to initiate the production of pharmaceuticals by transgenic crops in Japan, we need to prepare our domestic regulatory and advisory systems to deal with all the issues raised by the scientific communities and the general public. Japan has already enforced the mandatory labelling of GM foods [22]. However, this labelling is not aimed at the strict regulation of the possible contamination of GM crops, rather it is aimed at allowing the consumer a choice and acknowledging their right to know. At present, up to 5% (weight/weight) contamination of GM-derived ingredients is allowed for labelling of GM-free [22]. In November 2003, Japan adopted the Cartagena Protocol on Biosafety, and enforced the related laws. However, this protocol was aimed mainly at GM foods; plant-derived pharmaceuticals were not covered. In addition, the public acceptance of GM crops in Japan might not necessarily be improved by the adoption of these guidelines [23]. The production of pharmaceuticals in our stable food crops, which will require the enforcement of additional regulations in an already highly regulated food industry, is likely to cause annoyance and confusion in the industry and amongst consumers. Utilisation of non-food crops under the existing and accepted guidelines may be the solution.

In the case of developing countries, the use of non-food crops may also be feasible for several reasons. Implementation of strict regulations for any industry is not realistic in most developing countries, and trying to enforce the strict regulation of GM crops would be too heavy a burden in most cases. In addition, as many people are suffering from poverty and

hunger in these regions of the world [4,5,24,25], their food crops must be protected, as they are often the only source of food. Obviously, adverse contamination of their foods by transgenic introgression will increase the starvation in these impoverished countries. Nevertheless, the introduction of acceptable guidelines is necessary for even the majority of these countries, and, therefore, the utilisation of non-food crops for the production of biopharmaceuticals has a wide appeal. The production of pharmaceuticals using medicinal plants (e.g., antibodies from tobacco [11]), the retrieval of industrial materials from material plants (pulp obtained from trees with altered lignification processes [26]), and improving the nutritional qualities of existing and well-established crop plants (e.g., 'designer' nutrition in golden rice [27]) may provide a simple and easily acceptable starting point. If developing countries could export such raw materials for modern biopharmaceuticals, it will provide them with a good source of revenue to improve the lives of their general population.

5. Conclusion

The world is facing difficult times, with growing populations and dwindling resources, coupled with climate change, affecting many of the poorer, but often more heavily populated areas. Analysis of world trends has shown that greatly improved technologies will be necessary to avoid conflicts and

for a transition to a sustainable system [24,25]. As mentioned in the introduction, plant biotechnology offers a great potential in this respect. We must use these technologies in order to realise a sustainable way of life for all the people of the world. The production of biopharmaceuticals by transgenic plants may well be the trend of the next decade, and industrialised countries will reap the rewards. However, it is important that developing countries also benefit from the introduction of these new technologies. Therefore, we must develop strategies that pay full attention to promoting the benefits of bioengineering and biopharmaceuticals, not only on the domestic front, but also worldwide. Of particular relevance in this respect is the production of pharmaceuticals, including edible vaccines [28,29], which are undergoing development, and by which the parallel contamination of food crops must be prohibited. In order to realise the benefit of an edible vaccine, excellent and feasible strategies will also be necessary to ensure that it is distributed to the large numbers of people in need. We must advance, but at the same time keep our focus firmly on the future of the world and the needs of all its people.

Acknowledgements

Bibliography

Papers of special note have been highlighted as of considerable interest (••) to readers.

1. LANDER ES, LINTON LM, BIRREN B et al.: Initial sequencing analysis of the human genome. Nature (2001) 409(6822):860-921.

2. ABUIN A, HOLT KH, PLATT KA, SANDS AT, ZAMBROWICZ BP: Full-speed mammalian genetics: in vivo target validation in the drug discovery process. Trends Biotechnol (2002) 20(1):36-42.

3. SIMOENS C, VAN MONTAGU M: Genetic engineering in plants. Hum. Reprod. Update (1995) 1(6):525-542.

4. THE ROYAL SOCIETY OF LONDON et al.: Transgenic Plants and World Agriculture. National Academy Press, Washington DC, USA (2000):1-40.
•• Important report under the auspices of the international science societies. Can be accessed at http://books.nap.edu/html/transgenic/

5. VASIL IK: The science and politics of plant biotechnology – a personal perspective. Nat. Biotechnol (2003) 21(8):849-851.

6. FISCHER R, TWYMAN RM, SCHILLBERG S: Production of antibodies in plants and their use for global health. Vaccine (2003) 21(7-8):820-825.

7. GARBER K: Biotech industry faces new bottleneck. Nat. Biotechnol (2001) 19(3):184-185.

8. BAKKER H, BARDOR M, MOLTHOFF JW et al.: Galactose-extended glycans of antibodies produced by transgenic plants. Proc. Natl Acad. Sci. USA (2001) 98(5):2899-2904.

9. SRIRAMAN R, BARDOR M, SACK M et al.: Recombinant anti-hCG antibodies retained in the endoplasmic reticulum of transformed plants lack core-xylose and core-α(1,3)-fucose residues. Plant Biotechnol. J. (2004) 2(4):279-288.

10. DECKER E, RESKI R: The moss bioreactor. Curr. Opin. Plant Biol. (2004) 7(2):166-170.

11. YANO A, MAEDA F, TAKEKOSHI M: Transgenic tobacco cells producing the human monoclonal antibody to hepatitis B virus surface antigen. J. Med. Virol. (2004) 73(2):208-215.

12. BEASLEY PR, HWANG LY, STEVENS CE et al.: Efficacy of hepatitis B immune globulin for perinatal transmission of the hepatitis B virus carrier state: Final report of a randomized double-blind, placebo-controlled trial. Hepatology (1983) 3(2):135-141.

13. MULLER R, GUBERNATIS G, FARLE M et al.: Liver transplantation in HBs antigen (HBsAg) carriers: Prevention of hepatitis B virus (HBV) recurrence by passive immunization. Hepatology (1991) 13(1):90-96.

14. GALUN E, EREN R, SAFADI R et al.: Clinical evaluation (Phase I) of a combination of two human monoclonal antibodies to HBV: safety and antiviral properties. Hepatology (2002) 35(3):673-679.

15. KELLER MA, STIEHM ER: Passive immunity in prevention and treatment of infectious diseases. Clin. Microbiol. Rev. (2000) 13(4):602-614.

16. PETERSON RK, ARNTZEN CJ: On risk and plant-based biopharmaceuticals. Trends Biotechnol (2004) 22(2):64-66.

12

17. KAPUSCINSKI AR, GOODMAN RM, HANN SD *et al.*: Making 'safety first' a reality for biotechnology products. *Nat. Biotechnol.* (2003) 21(6):599-601.

18. MASCIA PN, FLAVELL RB: Safe and acceptable strategies for producing foreign molecules in plants. *Curr. Opin. Plant Biology* (2004) 7(2):189-195.

19. GUIDANCE FOR INDUSTRY: Drugs, biologics, and medical devices derived from bioengineered plants for use in humans and animals, draft guidance. Docket No. 02D-0324, CBER 200134. *Fed. Reg.* (2002) 67:57828-57829.

20. NO AUTHORS LISTED: Drugs in crops – the unpalatable truth. *Nat. Biotechnol.* (2004) 22(2):133.

21. STEWART CN JR, HALFHILL MD, WARWICK SI: Transgene introgression from genetically modified crops to their wild relatives. *Nat. Rev. Genet.* (2003) 4(10):806-817.

22. HINO A: Safety assessment and public concerns for genetically modified food products: the Japanese experience. *Toxicol. Pathol.* (2002) 30(1):126-128.

23. SMYTH S, PHILLIPS PW: Labeling to manage marketing of GM foods. *Trends Biotechnol.* (2003) 21(9):389-393.

24. RUTTAN VW: The transition to agricultural sustainability. *Proc. Natl. Acad. Sci. USA* (1999) 96(11):5960-5967.

25. KATE RW, PARRIS TM: Long-term trends and a sustainability transition. *Proc. Natl. Acad. Sci. USA* (2003) 100(14):8062-8067.

26. PILATE G, GUINEY E, HOLT K *et al.*: Field and pulping performances of transgenic trees with altered lignification. *Nat. Biotechnol.* (2002) 20(6):607-612.

27. YE X, AL-BABILI S, KLOTI A *et al.*: Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* (2000) 287(5451):303-305.

28. RASKIN I, RIBNICKY DM, KOMARNYTSKY S *et al.*: Plants and human health in the twenty-first century. *Trends Biotechnol.* (2002) 20(12):522-531.

29. MA JK, DRAKE PM, CHRISTOU P: The production of recombinant pharmaceutical proteins in plants. *Nat. Rev. Genet.* (2003) 4(10):794-805.

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Transgenic Tobacco Cells Producing the Human Monoclonal Antibody to Hepatitis B Virus Surface Antigen

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The recombinant human monoclonal antibody (MAb) against hepatitis B virus (HBV) surface antigen (HBsAg) was expressed in tobacco suspension cultures. The parental CL4MAb was produced by the Epstein–Barr virus (EBV) transformed human cell line TAPC301-CL4. The CL4MAb cDNA was introduced into tobacco suspension cells by *Agrobacterium* mediated transformation. The monoclonal antibodies (MAbs), B294 and B303, which were derived from CL4 and subsequently produced in plant cells were selected for study. After purification on Protein A columns, B294 and B303 MAbs had anti-HBs relative affinity constants similar to the parental CL4MAb. B303 MAb interacted with cell surface HBsAgs and showed complement-dependent cytotoxicity in a manner that was similar to anti-HBs human immunoglobulins (HBIg) that are used clinically. The results of this study point to the feasibility of producing MAbs to HBsAg in plants as an alternative to HBIg. *J. Med. Virol.* **73:208–215, 2004.** © 2004 Wiley-Liss, Inc.

KEY WORDS: recombinant antibody; transgenic plant; HBV; HBs

INTRODUCTION

Immune serum has been an important therapeutic agent for many infectious diseases since early in the 20th century [Casadevall and Scharff, 1994]. The administration of antibodies to rabies virus and respiratory syncytial virus are typical examples of passive immune therapy [Keller and Stiehm, 2000]. High titer hepatitis B virus (HBV)-neutralizing serum is also administered prophylactically against hepatitis B viral infection. The transmission of HBV occurs on sexual contact, parenteral exposure, such as accidental exposure to HBV in clinical settings, or maternal–neonatal transmission [Centers for Disease Control and Prevention, 2002]. Among the different transmission scenarios, it is the most important to prevent maternal–neonatal

transmission for control of HBV and a vaccination program for newborns is ongoing [Kane and Brookes, 2002]. The use of a combination of anti-HBs human immunoglobulins (HBIg) and subunit HBV vaccine effectively prevented the transmission and subsequent infection of infants [Beasley et al., 1983]. HBIg is also used prophylactically for prevention of hepatitis B recurrence in hepatitis B-seropositive liver transplantation recipients [Muller et al., 1991]. HBIg is prepared from the sera of HBsAg antibody positive donors and the safety is guaranteed by rigorous product standards. The pharmaceuticals derived from human plasma or animal cell cultures are produced by new technologies in developed countries, however, production standards have increased both manufacturing costs and prices to the consumer. The high-sensitive viral detection system is recommended for selection of healthy donors for human blood derived pharmaceuticals [Burnouf and Radosevich, 2000]. Donor and production controls HBIg of that use costly technologies are not practical for developing countries. Genetically modified plants, capable of producing recombinant protein therapeutics, such as human monoclonal antibodies (MAbs), would be good choices for a number of reasons. Plant cells have fewer human infectious agents than human plasma and mammalian cells. Only sucrose, minerals, and some plant hormones are needed to grow the plant tissue cultures. Plants grown in either the field or greenhouses require only light, water, and a few agrochemicals. Because plants do not need animal serum or other animal-derived nutrients, they are safe and inexpensive hosts for production of the recombinant pharmaceutical proteins such as MAbs [Fischer et al., 2003]. The steps

that are required in mammalian cells for expression of full-length antibodies, such as protein folding, assembly, endoplasmic reticulum (ER)-mediated glycosylation and Golgi-mediated glycan processing, also occur in plants [Ma and Hein, 1995; Rayon et al., 1998; Sanderfoot and Raikhel, 1999; Vitale and Denecke, 1999]. Many recombinant biopharmaceuticals, including antibodies, have been developed in recent years [Breedveld, 2000; Raskin et al., 2002; Davidov et al., 2003]. In theory, the production of these new drugs could be shifted from animal to plant cells and scaled to the desired level. However, the transgenic technology for generation of biopharmaceuticals requires additional research before it becomes acceptable in our society.

The human cell line TAPC301-CL4 originated from Epstein–Barr virus (EBV) transformed B-cell lines that were derived from peripheral blood of a healthy human volunteer with high titer anti-HBs (adr subtype). The CL4MAb (IgG1/kappa) produced by the TAPC301-CL4 cells neutralized the HBV (adr subtype) activity in vivo [Matsui, 1982]. However, the MAbs from EBV transformed cells are not recommended for human use. In this report, the cDNA of the whole CL4MAb was cloned and introduced into the genome of suspension cultures of tobacco cells. The CL4MAb was expressed in tobacco plant cultures, purified, and the properties were compared with those of the original CL4MAb secreted by the TAPC301-CL4 cells.

MATERIALS AND METHODS

Binary Vector Constructions

The three constructs used in this study are shown in Figure 1. Only the signal sequences differ among the three plasmids. The p29 contained the original leader sequences (LS) of CL4MAb gene. In p30, the LSs were

replaced with the synthesized DNA coding for the secretion sequence (SS) of calreticulin of *Nicotiana plumbaginfolia* [Borisjuk et al., 1998, 1999] that sorts proteins to the apoplastic spaces. In p31, the LSs were synthesized DNA coding for the SS of hordothionin of barley [Florack et al., 1994] that directs proteins to the ER pathways. All immunoglobulin chains were cloned between the cauliflower mosaic virus 35S promoter (35S) with the omega sequence (Ω: translational enhancer) and nopaline synthase terminator (NosT) [Luehrsen et al., 1992; Sheen et al., 1995]. The translation initiation sequences (IS) were changed from GTCGACATG to AACAATG for enhancement of antibody expression [Guerineau et al., 1992]. The heavy- and light-chain expression cassettes, arranged in tandem, constituted the antibody expression cassette (Ab cassette). The β-glucuronidase-NosT of pBI101 vector [Datla et al., 1992] was replaced with the Ab cassettes.

Transformation of Tobacco Suspension Culture

Recombinant binary vectors, p29, p30, and p31 were used to transform ElectroMAX™ *Agrobacterium tumefaciens* LBA4404 cells (Invitrogen Corp., Carlsbad, CA) by electroporation. Suspension cultures of BY-2 tobacco cells [Nagata et al., 1981] were transformed by standard methods [An, 1985] using co-cultivation with *A. tumefaciens* that had been transformed by p29, p30, and p31. Kanamycin resistant cells were selected on Murashige and Skoog (MS) plates containing 100 µg/ml kanamycin [Nagata et al., 1981]. Between 10 and 20 colonies from each of the transgenic lines, designated as the B29, B30, or B31 series, were inoculated into the MS medium. The transgenic cells were propagated for 7 days at 26°C in the dark, under the same conditions used for BY-2 cells [Nagata et al., 1981].

Fig. 1. Schematic representation of expression cassettes of the CL4 antibody. A: The antibody cassettes (Ab cassettes) were cloned into the T-DNA region separated from the binary vector, pBI101, by the right border (RB) and left border (LB) sequences. The Ab cassette of p29 binary vector contained original leader sequences (LSs) in the light and heavy chain of CL4 cDNA. Those LSs were replaced with secretion sequence (SS) of plants (p30: calreticulin (Cal) of *Nicotiana plumbaginifolia*, p31: hordothionin (HTH) of barley. The Ab cassettes contained cauliflower mosaic virus 35S promoter (35S) with omega sequence (Ω), restriction endonuclease SalI recognition site, initiation sequence (IS: AACA), nopaline synthetase terminator (NosT). B: DNA and deduced amino acid sequences of N-terminal region of the antibodies are indicated.

Small-Scale Extraction of Transgenic Tobacco Cells

To measure both the levels of total soluble protein (TSP) and expression of antibody, 1 ml of suspension culture was transferred into a 2 ml microtube. One-tenth ml of 10× complete protease inhibitor mix (Roche Diagnostics K.K., Tokyo, Japan) in 0.5 M Tris pH 7.5 and 0.15 g each of 0.1 and 1 mm diameter glass beads (Sigma-Aldrich Japan K.K., Tokyo, Japan) were added. Tubes were shaken at 30 Hz for 5 min by Mixer Mill MM300 (Qiagen K.K., Tokyo, Japan) and centrifuged at 15,000g for 10 min to obtain the plant extract supernatants. TSP concentrations were determined by Bradford protein assay (Bio-Rad Laboratories, Hercules, CA).

Preparative Scale Extraction of Transgenic Tobacco Cells

For antibody purification, large-scale (100 ml × n) tobacco suspension cultures were sampled at 10 days after initiation of culture. The cells and media were separated by vacuum filtration through the filter paper (Advantec, ToyoRoshi, Tokyo, Japan). Media were centrifuged for 60 min at 18,000g and the supernatants were filtered through 0.45 µm filters for loading to the liquid chromatography. Cells were frozen overnight and thawed in 2 volumes of (v/v) extraction buffer (50 mM Tris pH 7.5, 200 mM NaCl, 1× complete protease inhibitor cocktail). Cells were homogenized (Physcotron, Microtech Co., Tokyo, Japan) until over the 80% of cells were disrupted, as determined by microscopic observation. Cell debris was removed by centrifugation at 18,000g for 30 min. The protein in the supernatant was concentrated by 50% ammonium sulfate precipitation and the resulting pellet was suspended in phosphate-buffered saline (PBS). The suspension was filtered through 0.45 µm filters before liquid chromatography.

Liquid Chromatography

The filtered protein solutions were loaded onto a 10 ml Poros Protein A Plastic column (Applied Biosystems Japan Ltd., Tokyo, Japan) at a flow rate of 10 ml/min using a chromatography system (BIO CAD sprint, Applied Biosystems Japan Ltd.). After washing with 10 column volumes of PBS, bound protein was eluted with 5 column volumes of 0.1 M citrate pH 3.0/0.1 M NaCl and collected in 0.2 volumes of 1 M Tris for readjustment to a neutral pH. The buffer was changed to PBS, the eluate was concentrated using a 100 kDa molecular size cut-off filter (CentriPlus, Millipore Co., Billerica, MA), and filtered through 0.22 µm filters.

ELISA

ELISA plates (E.I.A/R.I.A; Costar, Corning, Inc., Acton, MA) were coated with 0.2 µg/well of purified adr subtype HBsAg (Meiji Dairies Corp., Tokyo, Japan) derived from huGK-14 hepatoma cells. Plates were blocked overnight with 3% skim milk (Wako, Tokyo, Japan) in PBS to inhibit non-specific binding. After washing with PBS containing 0.05% Tween-20 (PBST), 100 µl of either twofold serially diluted plant extracts or purified antibodies were added to the plates for 1 hour incubation at room temperature. Plates were rewashed with PBST, 100 µl of either horseradish peroxidase (HRP) or alkaline phosphatase (AP)-conjugated goat anti-human IgG specific antibody (Jackson Immuno-Research, West Grove, PA) at a dilution of 1:5,000 was added, and the plates were incubated for an additional 1 hr at room temperature. Unbound HRP-conjugate was washed away with PBST. Bound antibodies were detected by incubation for 30 min with tetramethylbenzidine base (TMB, Bio-Rad) and the optical density at 650 nm (OD$_{650}$) in each well was read. When AP conjugate was used, unbound conjugate was washed away with PBST, bound antibodies were detected by incubation for 1 hr at 37°C with p-nitrophenyl phosphate (pNPP) substrate (Bio-Rad), and the OD$_{405}$ was read. The ELISA antibody titer was expressed as the highest dilution giving an OD$_{405}$ of 0.1 U above that of the control wells without antigen.

ELISA Quantification of the Human IgG in Plant Extracts

ELISA plates were coated with anti-human IgG (0.5 µg/well; Jackson ImmunoResearch) then the test samples were added to the plates for 1 hr at 37°C following by blocking with 3% skim milk in PBS. Serially diluted human IgG (Jackson ImmunoReserch) was used as standard in all assays. After incubation, AP-conjugated anti-human IgG (Jackson ImmunoResearch) at a dilution of 1:5,000 was added and the plates were incubated for an additional 1 hr at room temperature. Plates were washed and incubated with pNPP substrate (Bio-Rad) for 1 hr at 37°C and the OD$_{405}$ was read.

Inhibition ELISAs

Affinity measurement was carried out by inhibition ELISA [Burton et al., 1991]. ELISA plates were coated overnight at 4°C with 0.2 µg/well of purified HBsAg. Wells were blocked with 3% skim milk in PBS. The amount of each MAb that gave half-maximum binding to HBsAg-coated plates was determined by ELISA. HBsAg was diluted by twofold steps from 2 µg/ml (the average MW of the proteins in the HBsAg manufactured by Meiji Dairies Corp. is 25.5 kDa) to 62.5 ng/ml in PBS with 3% skim milk and mixed with the same volume of 2× antibody solution. A 50 µl/well aliquot of the HBsAg-antibody mixture was added to the plates. The plates were incubated for 90–120 min at room temperature and washed three times with PBST before addition of a 1:5,000 dilution of HRP-conjugated goat anti-human IgG specific antibody (Jackson ImmunoResearch). After 1 hr of incubation, followed by washes, TMB (Bio-Rad) was added. The OD$_{650}$ was read after 30 min. The HBsAg concentration that gave 50% inhibition of maximum binding reflected the mAb affinity, which is

approximately the same as the binding constant [Burton et al., 1991].

Flow Cytometric Analysis

The hepatocellular carcinoma cell line PLC/PRF/5 (Alexander cell line), which expresses HBs antigens (adw) on the cell surface, was cultured in 3 cm plastic dishes (Asahi Technoglass Co., Chiba, Japan). When cells reached confluence, medium was discarded, 1 ml of each antibody was added to $2-5 \times 10^5$ cells/dish, and cultures were held on ice for 1.5 hr. After washing twice with PBS, a 1:10 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG specific antibody (ICN Pharmaceuticals, Inc., Aurora, OH) was added and plates were incubated for 30 min at 37°C. At the end of the incubation, cells were trypsinized, filtered through nylon mesh, and sorted by flow cytometry (FACSCalibur, BD, Franklin Lakes, NJ).

Microcytotoxicity Assay

Complement-dependent cytotoxicity activity was assayed to test the reactivity of the MAb Fc region with complement. The Alexander cells were cultured to confluency in a 96-well plate, incubated on ice for 1.5 hr with 50 µl of purified test antibody, followed by incubation with rabbit complement (Veritas, Tokyo, Japan). Ten microliters of Cell Counting Kit8 (DOJIN, Tokyo, Japan) was added and cell viability was estimated by measurement of OD_{450}, as specified by the manufacturer's protocol.

RESULTS

Generation of Transgenic Tobacco Cells

The constructs, designated p29, p30, and p31, are shown in Figure 1. To obtain the active full length IgG antibodies, protein fragments of heavy and light chain must be delivered to the secretary pathway through the ER. Three series of transgenic tobacco BY-2 suspension cells (B29, B30, and B31) were obtained by Agrobacterium-mediated transformation with the numerically corresponding binary plasmids. Small-scale protein extracts of 7 day cultures of several kanamycin resistant clones from liquid medium culture were used for ELISA. The B29-4, B30-3, and B31-7 cell lines, with the specific cell lines designated after the hyphen, were selected for further analysis because they gave OD values in ELISA and cell growth in the liquid medium that were higher than the other cell lines that were tested (data not shown).

The time course of the anti-HBs synthesis was determined by quantifying amounts of human IgG proteins in the total extracts of the culture that consisted of both the medium and cell extracts, and TSPs were quantified (Fig. 2). The anti-HBs titers and human IgG proteins showed the almost same time course of synthesis, with peak titers observed at 10 days after inoculation, but the time courses were different from that of TSP. The amounts of both IgG and TSP in the
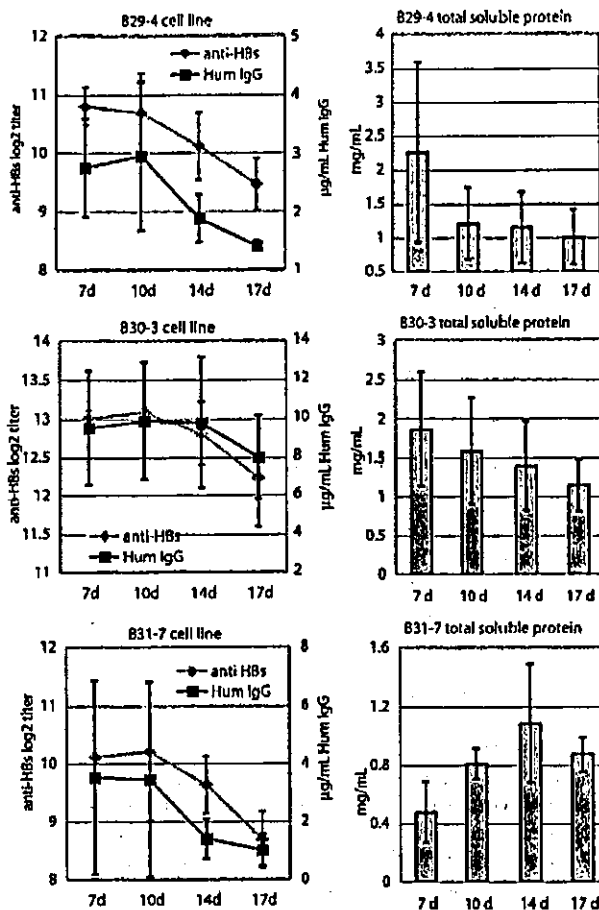

Fig. 2. Time course of antibody production in transgenic cell lines. B29-4, B30-3, and B31-7 cultures were sampled from 7 to 17 days after inoculation. Both anti-HBs titers and human IgG in samples were measured by ELISA. The amounts of total soluble proteins (TSP) were measured by Bradford assay. The results are presented as the mean ± SD (n = 3).

extracts of B30-3 cells were relatively stable from 7 to 17 days. In contrast, the B29-4 cells had rapid reductions in the amount of TSP after 10 days. The B31-7 cells also had reduced levels of IgG after 10 days, despite the peak of TSP detected at 10 days. The percentages of human IgG in the TSP were approximately 0.2% for B29-4, 0.6% for B30-3, and 0.25% for B31-7 cells. Despite the differences in the SSs for the three cell lines, the ratios of antibodies in the TSP were of same order, but the time points at which saturation density was reached were different. B30-3 cells grew well and rapidly reached saturation density. However, B31-7 cells grew slowly and did not reach saturation density until 10–14 days.

Purification of the Antibodies From Transgenic Tobacco Cells

Every 10 days the cells and medium from the cultured suspension cells were separated by paper filtration for purification of the recombinant MAb. Figure 3 shows the maximum yields of the preparative scale IgG purifica-
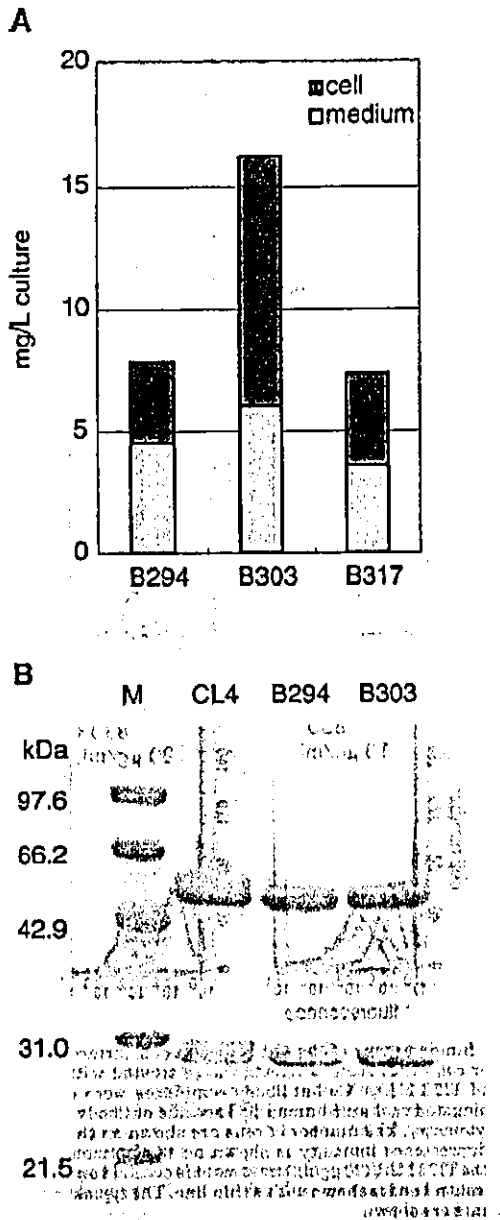
A

B

Fig. 3. Antibody purification from transgenic cell lines. A: IgG protein yields from B29-4 and B30-3 cultures. IgGs were purified from both cells and media of 10 day cultures with protein A columns. The B294 and B303 MAbs were quantified by ELISA. The maximum yield of the six times preparative scale purification procedures were indicated. B: SDS–PAGE analysis of purified antibodies. Purified CL4 (from culture medium), B294, and B303 MAbs (from medium and cell extracts) were analyzed using 12.5% SDS–PAGE. M, molecular weight marker.

tion from each of the tobacco cell lines cultured in n × 100 ml scale cultures for 10 days. The quantities of purified antibodies from medium were similar among the B29-4, B30-3, and B31-7 cell lines. However, the quantities of the antibodies recovered from the cells were varied. The maximum value after purification was 16.2 mg of human IgG from 1 L of a 10 day culture of B30-

3 cells. The Coomassie-stained SDS–PAGE of purified MAbs (10 µg each), parental CL4MAb, MAb B294, and MAb B303, from both medium and cell extracts is shown in Figure 3B. The total amount of antibody purified from B31-7 culture was insufficient for analysis. The major bands of heavy (H) and light (L) chains of the MAbs were almost all the same size. A difference among the three MAbs was the presence of weakly staining bands between the positions of the H and L chains. The positions of the extra bands were different for the original CL4 and the MAbs produced in plants. MAbs B294 and B303 showed similar patterns of extra bands. It was not clear whether the extra bands were the result of nonspecific binding of the plant proteins to Protein A column or degradation products of the IgGs.

Quantitative Analysis of Plant Produced Antibodies

The antigen binding activities were compared among the purified MAbs, parental CL4, B294, and B303 MAb. The MAbs were quantified by ELISA and then serially diluted over the same range as for quantification for comparison by ELISA of the HBs binding activities by ELISA (Fig. 4.). No obvious differences in antigen-binding were observed among the antibodies when tested from 0.3 µg/ml (=2.0 nM) to 73 pg/ml (=49 pM).

For a more detailed comparison among these MAbs, inhibition ELISA was done to determine the relative affinity constants (Fig. 5). In five independent assays, (data not shown) that used the HBsAg concentrations giving 50% inhibition, the relative affinity constants, which ranged from 1 to 1.26×10^{-8} M, were almost the same among MAbs B294, B303, and CL4.

Antibody Reaction With Cell Surface HBs

The Alexander cells were incubated with HBIg or HBs MAbs and the binding of antibodies was confirmed by flow cytometric analysis (FACS). Human cytomegalovirus (CMV) neutralizing MAb, TI23 (IgG1/kappa), was
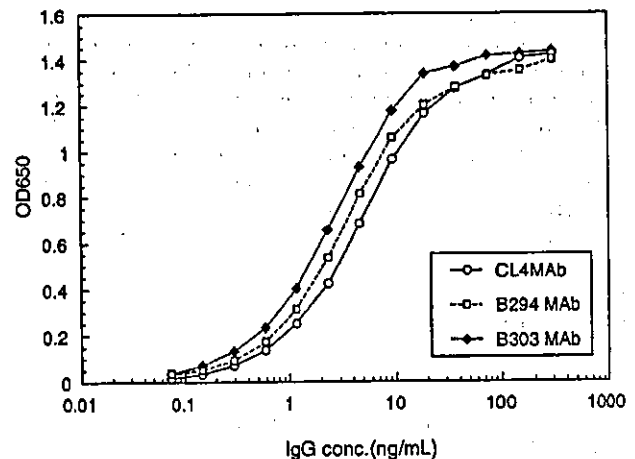

Fig. 4. Quantitative analysis of purified antibodies. Serially diluted solutions of the CL4, B294, and B303 MAbs were analyzed using the anti-HBsAg ELISA. The typical result of five experiments are shown.