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細胞結合配列を用いた易吸収性ペプチド製剤の設計

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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）
（総合）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

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研究要旨　アルギニンーグリシンーアスパラギン酸（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様々な抗ペプチド抗体を誘導できることを確認した。また *in silico* でマルチアグレトープ型 T 細胞エピトープを選択し、さらに既存の免疫記憶を利用することによって、抗原性が弱いペプチドに対しさらに効率良く抗体を誘導することに成功した。これらの手法を用いてアミロイドβペプチドの N 末端に特異的な抗体を誘導するペプチドを設計し、アルツハイマー病ワクチン療法の実現に近づいた。

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。

本研究事業では以下の点に関して研究を進めることとした。まず細胞結合配列を用い、その抗原性を増強する基本設計法の検証を進める。さらに抗原性の非常に弱いペプチド、抗体を誘導しづらいペプチドに対して、抗体誘導を可能にする手法に関して研究を行う。また、細胞結合後のペプチドの局所動態に関して、蛍光ラベルで標識したペプチドを用いて検討を行う。さらに、化学合成が困難な４０アミノ酸残基長のペプチドの生物学的生産方法に関して低分子タンパク質の発現をモデル系として若干の検討を行う。

B. 研究方法

モデルペプチドとして、主に“う蝕細菌”由来のSmUやオボアルブミン由来のOVAp

をB細胞エピトープとして用い、細胞結合配列を導入したペプチド設計の一般性を検証した。N末端側から細胞結合配列の代表であるアルギニン、グリシン、アスパラギン酸（RGD）、MHC-II分子によって提示されT細胞に認識されるT細胞エピトープ配列（T epitope）、細胞内の消化小包で切断を受けると考えられるリジンリンカー（KK）、抗体を誘導するための任意のアミノ酸配列（B epitope）、すなわち「RGD-T epitope-KK-B epitope」、という配列をもつペプチドを用いることで、様々なペプチド配列に対する抗体をアジュバントなしで効率良く誘導できるか、マウスの免疫実験にて検証した。

一方で、研究の実用化、社会への還元を意識し、いくつかの重要疾患の治療や予防につながるペプチドの設計とその検証実験も行った。具体的には、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、同じくインフルエンザウイルスのM2タンパク質の部分配列をB epitopeに使用したペプチドを設計しウイルス中和効果をマウス免疫実験にて検証した。さらに重要疾患であるアルツハイマー病の治療、予防につながるペプチドワクチンの設計を行った。アルツハイマー病のワクチン療法は、脳内に蓄積するアミロイドβペプチド（Ab）を患者に免疫し、抗アミロイド抗体を誘導することでその可溶化を行い、疾患の治療につなげる方法である。実際にアミロイドペプチド全長を用いた臨床試験が行われ免疫療法の有効性が確認されたが、数パーセントのヒトに細胞性免疫による脳炎が生じるという深刻な副作用も報告された。脳炎を誘導しない新しい免疫療法の開発が望まれている。本研究では治療に最も効果が高いとされるアミロイドβペプチドのN末端配列に対する抗体誘導を行うためのペプチドを設計、合成してマウスの免疫実験を行った。

様々な免疫実験の結果、免疫原性が非常に弱いために抗体誘導ができないB epitope。が多数存在することが明らかになってきた。そこで、より強い抗体誘導を可能にするため、各種データベースを用いたペプチドの*in silico*デザインを行った。市販のワクチンに含まれるタンパク質（具体的にはBCG（ツベルクリンタンパク質画分）由来のMpt64、三種混合ワクチンに含まれるジフテリア毒素及び破傷風毒素）から様々なHLA-IIに対して結合が予測されるペプチド配列を抽出し、それらをT epitopeとして採用した。加えて、人々が既に持っている免疫記憶を利用してペプチドの免疫原性を飛躍的に高める手法を検討した。人々の体内にはBCGや3種混合ワクチンなどの接種によって、ワクチン由来のT細胞エピトープを認識するT細胞が免疫記憶として多数存在している。この記憶T細胞をペプチド抗体の誘導に利用するのである。実際にマウスにツベルクリンタンパク質画分やジフテリア破傷風混合ワクチンをあらかじめ免疫した後に、Mtp、DiTox、TeTox1、TeTox3などのワクチン由来のT epitopeを利用したペプチドを免疫した。また、記憶T細胞メモリー効果をヒトに応用できるか、BCGおよび3種混合ワクチンの接種経験のあるボランティアから末梢血を採取し、T epitopeペプチドを添加したときの*in vitro*リンパ球増殖試験を行った。

細胞結合配列を持つペプチドが細胞内に取り込まれ、MHC-II分子によって効率良く提示されるのか、*in vitro*アッセイを試み

た。RGD 配列を付加したペプチドに蛍光ラベルを行い、BALB/c 由来のマクロファージ系細胞に与え、ペプチドの動態を顕微鏡下で観察した。また、N 末端を FLC、C 末端を TMR ラベルし、単独では超波長側の蛍光を発し、細胞に取り込まれてリジンリンカーが切断されると短波長側の蛍光を発する（FRET 効果）ペプチドを作成し、その動態を顕微鏡下で観察した。

ペプチドの生物学的な生産法に関して、植物細胞における生産の検討を進めた。まず、実績のある植物培養細胞を用いた抗体生産系を軸に、長鎖ペプチドに近い大きさの低分子タンパク質断片として Fab の L および H 鎖の生産を行い、その精製を試みた。

（倫理面への配慮）

マウスを用いた免疫実験に関しては、動物愛護観点に則り、最小限の実験で最大の効果が得られるよう心掛けた。実験遂行に関しては、あらかじめ動物実験委員会の審査を受け、倫理上問題がないと承認を得て行った（科学院動物実験委員会承認番号 17-001）。また、ヒト末梢血を用いた実験に関しても同様に倫理委員会の審査を受け、倫理上問題が無いように配慮して研究を行った（承認番号 NIPH-IBRA#05006）。ペプチドの生物学的な生産系に関する研究は、過去に国立感染症研究所 DNA 組換え実験計画審査を受け許可を得て（機 16-005）作成したタバコ培養細胞を用いた Fab 生産系（組換え生物ではない）を用いた精製実験等を行うことで実施した。

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。

続いてインフルエンザウイルスから B epitope を採用し、抗インフルエンザペプチドワクチンを試作した。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、HA1、M2 を用いてマウス免疫実験を行った結果、抗 HA1 および抗 M2 ペプチド抗体が誘導できた。しかし、実際にインフルエンザワクチンとしての利用を考えると、ウイルスの中和のためには、より強い抗体誘導能が必要であることが示唆された。同様に、ペプチドワクチンの実用化を考え、アルツハイマー病治療に効果があることが知られている、抗 Ab 抗体の誘導を試みた。ところが、Ab の抗原性が弱く、全く抗体誘導が見られなかった。これらの結果から、実用化のためにはより強い免疫誘導法の開発が求められた。

抗体誘導の強さを決める要因としては、B epitope そのものの性質以外に、より強い T epitope を使用することが考えられる。従来用いていた T epitope は、多様な MHC-II への対応を主眼においたマルチアグレトープ型の OMP、という人工的にデザインした配列、または天然のマルチアグレトープ配列である HIV 由来の Gag(298-312)などである。そこで新たに、抗体誘導能に優れていると考えられる既存のワクチンから、

マルチアグレトープ型の T epitope を公共のデータベースを用いて *in silico* の手法で抽出した。ツベルクリン由来エピトープ、Mpt、ジフテリア毒素由来エピトープ、DiTox、破傷風毒素由来エピトープ、TeTox1、TeTox3 を設計し、これらを用いて Ab 抗体誘導用ペプチド、RGD-Mpt-KK-Ab, RGD-DiTox-KK-Ab, RGD-TeTox1-KK-Ab, RGD-TeTox3-KK-Ab、を合成し、不完全アジュバントを用いてC57BL/6に免疫した。その結果DiToxを用いたペプチドで非常に強い Ab 抗体の誘導が確認できた。さらに、アジュバントを用いずに鼻腔免疫を行ったところ、C57BL/6 においてDiTox ペプチド、BALB/c において DiTox、TeTox1 ペプチドで抗 Ab 抗体の誘導が確認できた。新しい T epitope はワクチンによって生じる免疫記憶、記憶 T 細胞を利用できるはずであるため、ペプチドを免疫する２週間前に市販のジフテリア破傷風２種混合ワクチン、あるいはツベルクリン画分を結核菌死菌体を含んだ完全アジュバントで免疫を行った。その結果 BALB/c では 3 種のペプチド、C57BL/6 では DiTox ペプチドを用いたペプチドにおいて、ペプチド単独での抗体誘導に比べ、抗 Ab 抗体誘導が顕著に増強されることを確認した。それぞれのペプチドの T 細胞エピトープ部分のみを、マウスリンパ球幼若化試験に用い、DiTox による幼若化を確認した。さらにボランティア数名から末梢血を採取し、Mtp、DiTox、TeTox1、TeTox3 それぞれの T 細胞エピトープペプチドによるリンパ球増殖試験を行った結果、個々人により反応性に差があるが、有意な細胞増殖が確認された。

マウスマクロファージ様培養細胞に、蛍光ラベルしたペプチドを作用させたところ、ペプチドが数分のうちに細胞膜上に付着し、数十分の時間で細胞内に取り込まれるのが観察された。細胞内局在はエンドソーム、リソソームのマーカーと一致したが、ペプチド配列上細胞結合配列の有無によって大きな差は見いだせなかった。また、細胞内でのペプチドの消化から MHC-II による細胞表面への提示を FRET 解析によって確認を試みたが、FRET 効果が低いためか、あるいは消化効率が低いためか明瞭な結果が得られなかった。

ペプチド生産系のモデルとして FabL、H 鎖のタバコ培養細胞による生産を試みた。発現量増強のため、ER 滞留シグナルである KDEL 配列を C 末端に付加したものと付加しないものとで比較を行った。その結果、低分子タンパク質は細胞内へ蓄積させるより、細胞外へ分泌させた方がより効率の良い生産が期待できることが明らかになった。

D. 考察

まず、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。しかし細胞結合配列の効果だけでは免疫ができない弱いペプチドも存在する。そこで、新たに各種データベースの情報を用いて *in silico* で Mtp、DiTox、TeTox1、TeTox3 という T epitope を設計した。これらを用いてマウス免疫実験を行った結果、特に DiTox が非常に強い抗体誘導能を示し、その効果は２種混合ワクチンの

免疫記憶によってさらに増強されることが明らかとなった。他のエピトープに関してもマウスでは DiTox ほどの効果は見られなかったが、ヒトでは効果が期待できる可能性もある。特に、Mpt においては本来 BCG を接種した後に効果を見るべきものであったが、BCG を用いた場合感染実験となり、既存の施設では対応できなかったため、ツベルクリン画分と完全アジュバントで代用することにしたため、その効果を完全に評価できているとはいえない。事実、ヒト抹消血を用いた増殖試験では、Mtp に反応しているケースも見受けられる。アルツハイマー病のワクチン療法を考えた時には抗アミロイド抗体を強く誘導することが第一に求められるが、そのためには個々人が持つメモリーT 細胞のうち、より強いものを利用することが成功の鍵を握る。従って、これら４種のペプチドはもちろん、他にもいくつかの T 細胞エピトープを準備しておき、個々人に合ったエピトープを *in vitro* アッセイで選択して用いるという手法も考えられる。また、治療に先んじて BCG や２種混合ワクチンの再接種によって、メモリーT 細胞を増やすことも実用的な手法であろう。一方、我々のペプチドは MHC-II をターゲットとする上に、アジュバントなしの鼻腔免疫により抗体誘導を行うため、従来のアルツハイマー病の副作用である脳炎を引き起こす可能性が考えにくい。このようなことからも、実用的な治療法開発に直結した研究成果を出せたのではないかと考えている。

一方で、ペプチドの細胞局所の動態解析は思った成果が得られなかった。これは、もともと異物の取り込み能が高いマクロファージ系の細胞を用いたために、細胞結合配列の効果を見ることができなかったことが、大きな原因である。一方で、エンドソーム活性が低い細胞を用いた場合にはペプチドの取込みそのものが弱く、可視化が困難になるなど問題が多い。*In vivo* での粘膜への投与から抗原提示細胞への取込み、T 細胞への提示といった動態を再現するためには、複数種の細胞を混合培養するなど、更なる工夫が不可欠であると考えられる。

長鎖ペプチドの生産系に関しては、分泌系が有望であるという結果が得られた。従来からバクテリアなどでペプチドを発現させた場合には、細胞内ですみやかに分解されてしまうことが示唆されており、分泌系の利用は有力な候補の一つであった。現在、主任研究者の得意とするタバコ培養細胞を用いた発現系をモデルとして検討を行っているが、分泌系を用いれば様々な宿主において高効率のペプチドの生産が可能かもしれない。

E. 結論

細胞結合配列を用いた、ペプチドの基本設計を基に、任意の配列をもつペプチドの免疫原性を増強する新しいアプローチ、*in silico* によるマルチアグレトープ型 T 細胞エピトープの設計、宿主のもつ免疫記憶の利用を完成させた。本ペプチド設計を用いて、アルツハイマー病ワクチン療法のためのペプチド設計を行い、マウスにおいて有望な結果を得た。実際にヒトリンパ球増殖実験を行った結果、*in silico* の設計、免疫記憶の利用がヒトにおいて有効である可能性が示唆された。これらの結果は予想を上回るものであり、ヒト臨床用のワクチンに近付くことができたのではないかと考えている。

今後はアルツハイマー病のワクチン療法の実用化、またその他の様々な疾患にペプ

チド設計を適用し、研究成果の社会還元を進めていきたい。

F. 研究発表

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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.

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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-

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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 stepwise 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 TYEAALKQYEADL, 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: ISQAVHAAHAEINE) 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

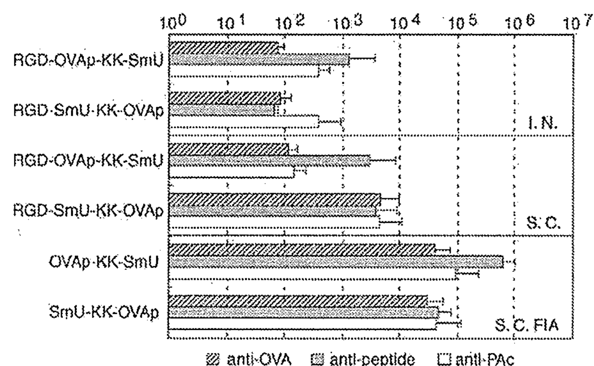

Fig. 1. Serum titres induced by intranasal and subcutaneous immunization with peptides. BALB/c mice were intranasally (I.N.) immunized with 50 μg of RGD added peptides, followed by two booster doses at two-week intervals. One hundred micrograms of peptide with FIA was used for subcutaneous (S.C.) immunization 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.

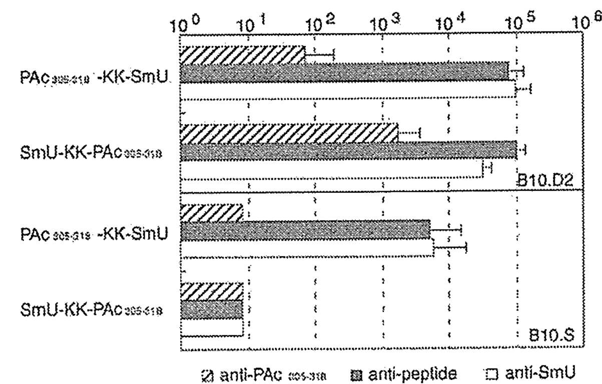

Fig. 2. Serum titres induced by subcutaneous immunization of peptides. B10.D2 and B10.S mice after subcutaneous immunization using peptides with FIA followed by a booster dose. Average serum titres are shown for anti-PAc305-318 (hatched-box), anti-peptide antigen (grey-box) and anti-SmU (open-box) with SD bars for each group.

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

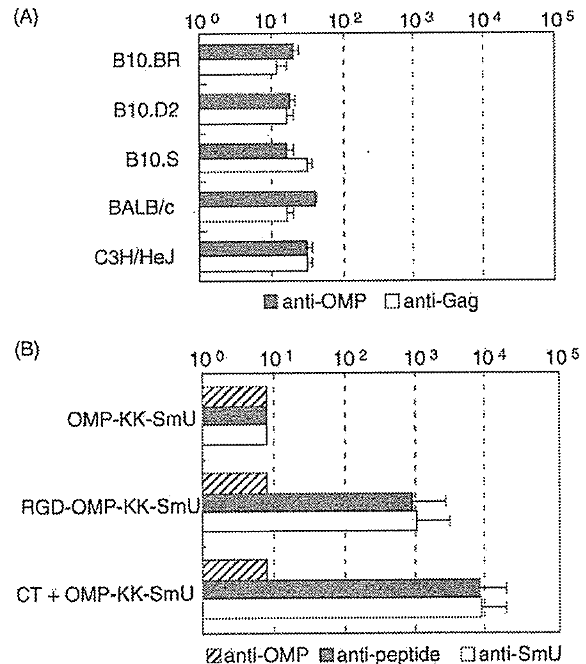

Fig. 3. Effect of multi-agretope type peptide on peptide antigens. (A) Each group of mice was immunized subcutaneously with FIA, followed by a booster dose, then specific antibody titres were determined by ELISA. Average titres are shown for anti-OMP (grey-box), and anti-Gag (open-box) with SD bars for 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.

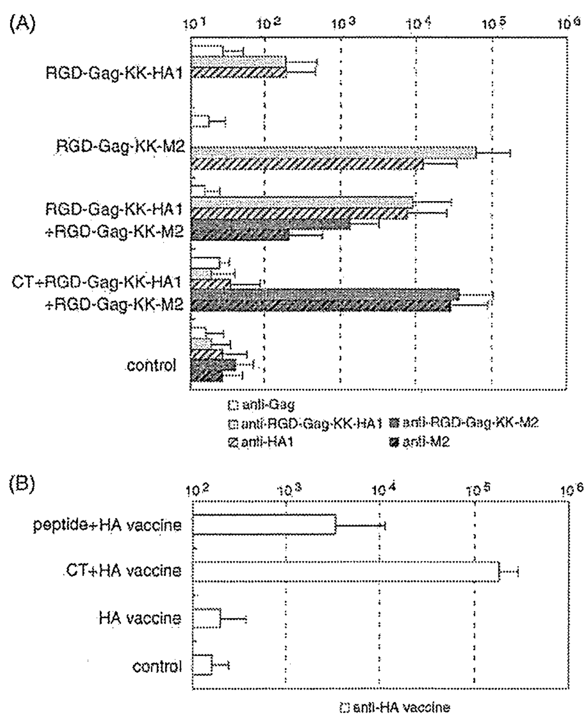

Fig. 4. Intranasal immunization with influenza vaccines. (A) B10.A mice were intranasally immunized with HA1 and M2 peptide vaccines, either with or without CT. Average titres are shown for anti-Gag (open-box), anti-HA1 (light grey-box), anti-RGD-Gag-KK-HA1 (hatched light grey-box), anti-M2 (dark grey-box) 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.

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 multi-agretope 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.

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Expert Opinion

1. Introduction
2. Molecular farming
3. How to manage the transgenic plant industry
4. Expert opinion
5. Conclusion

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 biopharmaceuticals 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.

Keywords: antibody, biopharmaceutical, developing country, GM crop, transgenic plant

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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 biotechnology. 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

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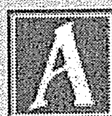

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over possible immunogenicity in humans. Bakker *et al.* [8] succeeded in expressing the human-type β1,4-galactose residue by introducing human galactosyltransferase into plants. Other workers have succeeded in producing recombinant human proteins in 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.

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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

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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].

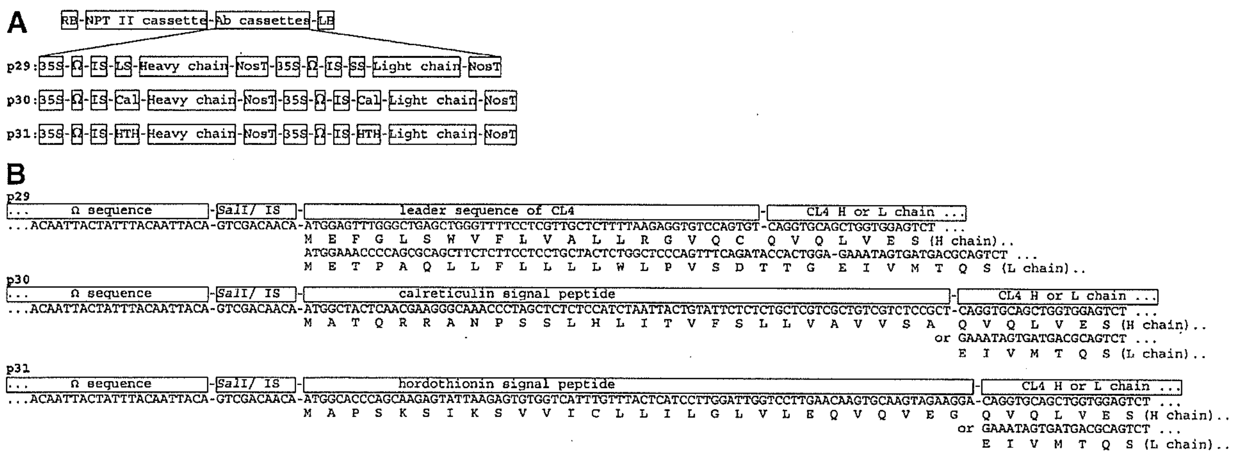

Fig. 1. Schematic representation of expression cassettes of the CL4 antibody. **A**: The antibody cassettes (Ab cassettes) were cloned into the T-DNA region separated from the binary vector, pBI101, by the right border (RB) and left border (LB) sequences. The Ab cassette of p29 binary vector contained 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 *Sal*I 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.