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(54)【発明の名称】う蝕の予防の免疫学的方法

(57)【要約】
ヒトのう蝕は、う蝕原性生物（例えば、エス・ミュータンス（Ｓ．　ｍｕｔａｎｓ））の表面抗原に結合するヒトまたはヒト化マウスモノクローナルＩｇＧおよびＩｇＭ抗体の経口摂取により、予防または治療される。これらがう蝕原性生物に結合すると、遺伝子的に免疫系はその破壊を引き起こす。好適な実施形態において、う蝕原性生物に対するモノクローナル抗体は、所望の抗体をコードするＤＮＡ配列により形質転換された食用植物（果実および野菜を含む）により産生される。抗体は、植物を食べることにより適用される。

【特許請求の範囲】
【請求項１】
う蝕原性生物に特異的に結合し、哺乳動物に体液性免疫応答を誘発するモノクローナル抗体の経口投与を含んでなる、哺乳動物のう蝕の治療および予防方法であって、モノクローナル抗体は、治療される哺乳動物以外の種から得られる、方法。
【請求項２】
う蝕の治療および予防方法であって、モノクローナル抗体が：
ａ）　少なくとも１つのう蝕原性生物を哺乳動物宿主に接種する工程；
ｂ）　少なくとも１つのう蝕原性生物の表面抗原に特異的な抗体を分泌する哺乳動物宿主から、ハイブリドーマを同定する工程；および
ｃ）　上記工程ｂ）のモノクローナル抗体からの相補性決定領域と、治療される哺乳動物からの定常ドメインとを含むキメラモノクローナル抗体を調製する工程、により産生される、方法。
【請求項３】
請求項２のう蝕の治療および予防法であって、調製工程は：
ａ）　上記請求項２の哺乳動物宿主から得られるハイブリドーマにより分泌されるモノクローナル抗体の相補性決定領域を発現に関してコードする核酸配列；および
ｂ）　治療される哺乳動物のＩｇＧクラスとＩｇＭクラスよりなる群から選択される抗体の定常領域を発現に関してコードする核酸配列、
とを含む核酸構築体の合成をさらに含む、方法。
【請求項４】
キメラモノクローナル抗体は、上記請求項３の核酸構築体で形質転換された真核生物宿主により発現される、請求項３のう蝕の治療および予防方法。
【請求項５】
モノクローナル抗体は、上記請求項４の核酸構築体で形質転換された真核生物宿主からの組織の経口摂取により投与される、請求項４の哺乳動物のう蝕の治療および予防方法。
【請求項６】
治療される哺乳動物はヒトであり、他の種はマウスである、請求項１のう蝕の治療および予防方法。
【請求項７】
う蝕原性生物に特異的に結合し、哺乳動物から体液性免疫応答を誘発するモノクローナル抗体の投与を含んでなる、哺乳動物のう蝕の治療および予防方法。
【請求項８】
請求項７のう蝕の治療および予防方法であって、モノクローナル抗体は：
ａ）　少なくとも１つのう蝕原性生物を哺乳動物宿主に接種する工程；
ｂ）　少なくとも１つのう蝕原性生物の表面抗原に特異的な抗体を分泌する哺乳動物宿主からハイブリドーマを同定する工程；および
ｃ）　上記工程ｂ）のモノクローナル抗体からの相補性決定領域と、治療される哺乳動物からの定常ドメインとを含むキメラモノクローナル抗体を調製する工程、により産生される、方法。
【請求項９】
請求項８のう蝕の治療および予防方法であって、調製工程は：
ａ）　上記請求項２の哺乳動物宿主から得られるハイブリドーマにより分泌されるモノクローナル抗体の相補性決定領域を発現に関してコードする核酸配列；および
ｂ）　治療される哺乳動物のＩｇＧクラスとＩｇＭクラスよりなる群から選択される抗体の定常領域を発現に関してコードする核酸配列、
とを含む少なくとも１つの核酸構築体の調製をさらに含む、方法。
【請求項１０】
キメラモノクローナル抗体は、上記請求項９の核酸構築体で形質転換された真核生物宿主により発現される、請求項９のう蝕の治療および予防方法。

【請求項１１】
モノクローナル抗体は、上記請求項９の核酸構築体で形質転換された真核生物宿主からの組織の経口摂取により投与される、請求項９の哺乳動物のう蝕の治療および予防方法。
【請求項１２】
哺乳動物宿主はマウスであり、治療される哺乳動物はヒトである、請求項８のう蝕の治療および予防方法。
【請求項１３】
真核生物は植物である、請求項５のう蝕の治療および予防方法。
【請求項１４】
真核生物はアブラナ属（Ｂｒａｓｓｉｃａ）の種の植物である、請求項５のう蝕の治療および予防方法。
【請求項１５】
真核生物は植物である、請求項１１のう蝕の治療および予防方法。
【請求項１６】
真核生物はアブラナ属（Ｂｒａｓｓｉｃａ）の種の植物である、請求項１１のう蝕の治療および予防方法。
【請求項１７】
治療される哺乳動物がイヌまたはネコである、請求項８のう蝕の治療および予防方法。
【発明の詳細な説明】
【０００１】
発明の背景
本出願は、う蝕の治療と予防のための免疫学的方法に関する。本発明は、幼児や決断力が無く充分に教育されていない集団のような、確立されたセルフケアの原理を応用する能力も動機も持たない患者に特に適用される。
【０００２】
う蝕（虫歯）と歯周病は、おそらく世界で最も一般的な慢性疾患である。歯の空洞の存在は、細菌感染の結果である。すなわちう蝕の存在は、正しくは歯の石灰化組織の局所的破壊を引き起こす感染性微生物疾患と見なされる。
【０００３】
ストレプトコッカス・ミュータンス（Ｓｔｒｅｐｔｏｃｏｃｃｕｓ ｍｕｔａｎｓ）は、ヒトの虫歯の主要な原因である。エス・ミュータンス（Ｓ． ｍｕｔａｎｓ）が歯垢中に多数存在し、複合糖を代謝すると、生じる有機酸が歯の表面の脱灰を引き起こす。その結果は、う蝕病変であり、これは一般的に窩洞として知られている。他の生物（例えば、ラクトバシラス（Ｌａｃｔｏｂａｃｃｉｌｌｉ）とアクチノミセス（Ａｃｔｉｎｏｍｙｃｅｓ））は、窩洞病変の進行と形成に関与していると考えられている。虫歯を引き起こすこれらの生物は、本明細書において「う蝕原性生物」と呼ぶ。
【０００４】
歯の損傷部分を除去したり詰め物をして修復すると、う蝕原性生物による口腔感染による損傷を、少なくとも一時的には止めることができる。しかし「ｄｒｉｌｌ ａｎｄ ｆｉｌｌ」アプローチは、起因細菌を排除していない。正しい口腔の清潔さが歯垢（ここにう蝕原性生物が増殖し、歯の表面を攻撃する）の蓄積を抑える。しかし歯のセルフケアは、特に自分でケアすることができない集団、またはセルフケアの正しい方法についての知識が無い集団では、限界がある。フッ素イオンの投与は、う蝕の発生を減少させるが、根絶はしないことが証明されている。
【０００５】
う蝕の発症にう蝕原性生物が関与しているという圧倒的な証拠を考えると、古典的な抗菌療法により症状を緩和する多くの試みがなされていることは驚くべきことではない。抗微生物剤の欠点は、う蝕原性生物に対して選択的ではないことである。非特異的殺菌剤の投与は、口腔に通常棲息している微生物のバランスを乱し、これが予測できない結果を招くが、病原性微生物のための環境が作り出されることがある。さらに、抗微生物剤の長期投

与は、これらに耐性の微生物を選択することになることが知られている。従って、虫歯を治療するために抗微生物剤を長期にかつ集団的に使用することは、現実的ではない。

【０００６】

エス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）または他のう蝕原性生物に対して能動免疫応答を誘発するためにヒトに予防接種することも、今は現実的ではない。このアプローチの１つの欠点は、予防接種が、主にＩｇＧとＩｇＭ抗体の産生を誘発することであるが、これらは唾液中に分泌されない。唾液中に存在するほとんどの抗体はＩｇＡイソタイプであり、これは、リンパ球や補体成分に結合できるが、これらを活性化して細菌を死滅させることは無い。従って予防接種は、免疫系を誘発して口の中のう蝕原性生物を死滅させることができる抗体を産生すると考えられない。予防接種により誘導された、口腔中のＩｇＧまたはＩｇＭイソタイプ抗体の力価を、選択的に上昇させる方法は知られていない。

【０００７】

動物やヒトの虫歯を予防するために、マウスで作成したモノクローナルＩｇＡ抗体を使用して、エス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）に対して患者を受動的に免疫する多くの試みが報告されている。ＩｇＡは多価抗体であるため、単一のＩｇＡ分子がいくつかの異なる抗原性部位に結合し、細菌の凝集を引き起こす。しかし細菌の表面抗原へのＩｇＡの結合は、細菌を死滅させない。むしろ、凝集は、細菌が歯の表面に結合することを阻害すると考えられている。このアプローチの別の欠点は、マウス（すなわち、異種）抗体をヒトに繰り返し投与すると、抗体に対する免疫応答を誘発する可能性があることである。

【０００８】

ＩｇＡ抗体と異なり、ＩｇＧとＩｇＭクラスの抗体は殺菌作用を有する。う蝕原性生物の表面上に存在する抗原に対するＩｇＧまたはＩｇＭ抗体の結合は、２つの異なる機構（補体介在細胞溶解と抗体依存性細胞障害）により細菌細胞の破壊を引き起こし得る。いずれの場合も、ある微生物に選択的に結合する抗体は、免疫系による破壊について、そのような細胞を標的とする。補体介在細胞溶解と抗体依存性細胞障害の両方とも、ＩｇＧとＩｇＭクラスの抗体により仲介されるヒト免疫応答の一部である。

【０００９】

抗体結合の所望の細胞障害作用を誘発するために、う蝕原性生物に対するモノクローナル抗体は、ヒトの免疫系により認識されなければならない。異種の哺乳動物免疫系から応答を引き起こす抗体が産生される、多くの異なる技術がある。１つの例は、異種生物の抗原特異的結合ドメインをコードする配列を取り込むように修飾された、ヒト抗体をコードする核酸構築体である。

【００１０】

ヒトのう蝕を治療するためのそのような遺伝子操作したモノクローナル抗体の産生と投与は、特に革新的な解決を受けやすい問題を提起する。モノクローナル抗体の産生についての先行技術の方法は、培養培地中でハイブリドーマを増殖させ、次に所望の抗体を抽出および精製する。これらの工程は、本発明の好適な実施形態では、食用植物または動物（真核生物）中で抗体を発現することにより、大幅に単純化される。抗体は、植物または動物の生成物（例えば、その中で抗体が変性していない果実、野菜またはミルク）の経口摂取により投与される。この投与法は、改善している虫歯でコンプライアンスが不要になる可能性がある。

【００１１】

好適な実施形態の要約

う蝕は、う蝕原性生物（例えば、エス・ミュータンス（Ｓ．　ｍｕｔａｎｓ））の表面抗原に結合する、ヒトまたはヒト化マウスモノクローナルＩｇＧおよびＩｇＭ抗体の経口摂取により予防または治療し得る。遺伝子操作したモノクローナル抗体は、う蝕原性生物に結合する時、ヒトの免疫系のエフェクター機構に参加して、微生物を破壊させる。好適な実施形態において、う蝕原性生物に対するモノクローナル抗体は、所望の抗体をコードするＤＮＡ配列により形質転換された食用植物（果実および野菜を含む）により産生される。抗体は、植物を食べることにより適用される。

【００１２】
好適な実施形態の詳細な説明
１．　モノクローナル抗体の調製
モノクローナル抗体技術は、すばらしい特異性を持った抗体供給源の調製を可能にする。特異的分子構造に結合するモノクローナル抗体は、今日標準的方法と考えられている技術を使用して産生することができる。
【００１３】
本発明で使用されるモノクローナル抗体は、う蝕原性生物の表面抗原に対して作成されるものである。表面抗原は、細胞の表面に表示される物質である。そのような抗原は、体液中に存在する抗体がアクセスできる。本発明においてう蝕原性生物の表面抗原は、う蝕を引き起こす微生物の表面に存在する。う蝕病変の発症における細菌活性の役割は、充分解明されているが、特定の微生物とう蝕の発症との間の因果関係は、完全には確立されていない。今日までエス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）のみがう蝕と関係するとされている。しかしラクトバシラス（Ｌａｃｔｏｂａｃｃｉｌｌｉ）とアクチノミセス（Ａｃｔｉｎｏｍｙｃｅｓ）の種もまた、特にう蝕病変の活動的な進行に関与していると考えられている。う蝕病変を発症する微生物は、本発明に従って調製され使用されるモノクローナル抗体の潜在的な標的である。
【００１４】
本発明の実施で使用されるモノクローナル抗体のさらなる要件は、う蝕原性生物に対して選択的であることである。う蝕原性生物ならびに非う蝕原性生物上に存在する抗原に対するモノクローナル抗体は、口腔内の菌叢の構成の非特異的変化を引き起こすことがある。そのような変化の結末はあまり理解されていない。
【００１５】
従って好適なモノクローナル抗体は、う蝕原性生物の表面抗原に対するものである。すなわち好適なモノクローナル抗体は、う蝕を引き起こす微生物に特異的に結合する。
【００１６】
本発明の範囲は、ヒトの虫歯の予防に限定されないことは明瞭に理解される。本発明のモノクローナル抗体は、他の哺乳動物（例えば、ペットとして家畜化されているもの）の免疫系のエフェクター応答を行うように遺伝子操作することができる。
【００１７】
モノクローナル抗体は、マウスまたは他の哺乳動物宿主を、う蝕原性生物から単離した細胞壁物質で免疫することにより調製される。好適な実施形態において、う蝕原性生物は、ｃ型エス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）（ＡＴＣＣ２５１７５）である。細胞壁中に存在する分子の免疫原性は、当該分野で公知の種々の方法により増強することができる。好適な実施形態において、そのような分子の免疫原性は、単離した細胞物質をホルマリンで変性することにより増強される。細胞壁タンパク質を修飾して免疫原性を増強する他の方法は、本発明の範囲内である。典型的には宿主は、単離した細菌細胞断片を一回またはそれ以上注射されて、抗体の力価を増加させた後、屠殺およびクローニングされる。
【００１８】
宿主の脾臓細胞を採取し、ＫｏｈｌｅｒとＭｉｌｓｔｅｉｎの先駆的な仕事以来標準法となった技術を使用して、限界希釈法によりクローニングする。好適な実施形態において、生存しているハイブリドーマは、う蝕原性生物に対する抗体について、ホルマリン化細菌細胞物質でコートしたマイクロタイタープレートに対して、ＥＬＩＳＡアッセイによりスクリーニングされる。陽性の上清を、さらにスクリーニングして、う蝕原性生物に対する親和性が最も大きい抗体を分泌するクローンを同定する。好適な実施形態において、バックグランドより少なくとも３倍高い力価を有するクローンが、エス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）からの変性細胞壁物質に対して、免疫沈降法を使用して再度スクリーニングされる。好適な実施形態において、エス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）株ＡＴＣＣ２５１７５、ＬＭ７、ＯＭＺ１７５およびＡＴＣＣ３１３７７にのみ検出できる形で結合する３つのクローンが同定された。これらのクローンは、アメリカンタイプカルチ

ャーコレクションに寄託して、寄託番号ＨＢ－１２５６０、１２５９９および１２５５８を受けた。

【００１９】

２．　ヒト免疫系からエフェクター応答を誘発することができるモノクローナル抗体の調製

う蝕の予防のための免疫学的方法を開発する従来の試みは、異種抗体を使用した。例えば、Ｌｅｈｎｅｒ、ＵＳ Ｐａｔｅｎｔ Ｎｏ．５，３５２，４４６は、マウスで作製したエス・ミュータンス（Ｓ．ｍｕｔａｎｓ）表面抗原に対するモノクローナル抗体の、サルにおけるこれらの細菌の増殖を阻害するための使用に関する。さらに最近は、Ｍａら、Ｎａｔｕｒｅ Ｍｅｄｉｃｉｎｅ，４５（５）６０１－６（１９９８）は、タバコ植物で発現した、エス・ミュータンス（Ｓ．ｍｕｔａｎｓ）に対する遺伝子操作した分泌性モノクローナル抗体を使用して、ヒトで同様の結果を報告した。このアプローチの欠点は、１）異種抗体の投与は、有害な微生物を凝集させるが、そのような抗体は免疫応答を誘発しないため、これらを死滅させることはない；および２）抗体の繰り返し投与は、患者に抗体に対する免疫応答を誘発することがある。好適なアプローチは、組換え法を使用して、う蝕原性生物の表面抗原に対して特異的に作成されるキメラ抗体分子を調製することであり、これはまた、標的微生物に結合することによりヒトの免疫系（ヒトで使用する場合）にエフェクター応答を誘発するであろう。これは、う蝕原性生物に特異的なマウスモノクローナル抗体からの種々の領域または相補性決定領域（「ＣＤＲ」）を、治療される哺乳動物からのＩｇＧおよび／またはＩｇＭクラスの抗体に挿入することにより行われる。治療される哺乳動物がヒトである時、抗体は「ヒト化」されたと言う。

【００２０】

う蝕原性生物の表面抗原に対するヒトまたはヒト化モノクローナル抗体をコードする核酸配列を得るには、種々の方法がある：１）う蝕原性生物に対するモノクローナル抗体を産生するマウスハイブリドーマを単離し、これらの抗体をコードするマウス遺伝子をクローニングする；２）精製したう蝕原性生物を使用して、ヒトＢリンパ球から作製したファージディスプレイランダムライブラリーをスクリーニングし、う蝕原性生物に特異的な抗体をコードする遺伝子を得る；３）重度感染患者から回収したＢリンパ球を使用して、う蝕原性生物に対するモノクローナル抗体を産生するヒトハイブリドーマを単離し、これらの抗体をコードするヒト遺伝子をクローニングする；または４）精製したう蝕原性生物を使用して、ｉｎ ｖｉｔｒｏでヒトＢリンパ球と脾臓細胞を免疫し、次に融合させて、ハイブリドーマを形成させ、不死化細胞株を作製する。必要な技術は、当業者に公知である。

【００２１】

本発明の好適な実施形態において、マウスモノクローナル抗体は「ヒト化」される。ＰＣＲまたはサザンブロット技術を使用して、う蝕原性生物の細胞表面抗原に特異的な抗体を分泌するマウスハイブリドーマの可変ドメインをコードするＤＮＡ断片を単離する。遺伝子クローニング技術を使用して、ヒトＩｇＧとＩｇＭ免疫グロブリンの可変領域を、対応するマウス可変領域またはＣＤＲで置換する。この遺伝子操作の結果は、う蝕原性生物の表面抗原に選択的に結合するが、その定常領域を介してヒト免疫系と相互作用して、体液性免疫応答を誘発する可変ドメインを有するキメラ抗体分子である。

【００２２】

３．　モノクローナル抗体の投与

臨床的使用に充分な量のモノクローナル抗体を調製するためには、ＩｇＧまたはＩｇＭをコードする配列でトランスフェクションした所望の細胞株を増殖させなければならない。既存の技術が、組織培養でのモノクローナル抗体の大規模増殖を可能にする。トランスフェクションした細胞株は、モノクローナル抗体を組織培養培地中に分泌するであろう。分泌されたモノクローナル抗体は回収され、ゲル濾過およびタンパク質化学の関連技術で精製される。

【００２３】

実験的研究で、エス・ミュータンス（Ｓ．ｍｕｔａｎｓ）に対するモノクローナル抗体

は、歯の表面に直接適用されている。うがい薬の摂取、またはチューインガムによる適用もまた提唱されている。現在好適な代替法は、食用植物（例えば、バナナまたはブロッコリー）中で本発明のモノクローナル抗体を発現することである。本発明により形質転換された植物を食べることにより、歯の表面および口の別のところに存在するう蝕原性生物に抗体が適用される。また、植物性および動物性の他の微生物を形質転換して、本明細書に記載のモノクローナル抗体を発現し、その結果そのような抗体が例えばミルクを飲むことにより摂取されることも企図される。

【００２４】

実施例

１．　エス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）に対するマウスモノクローナル抗体の産生

ｃ型エス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）株ＡＴＣＣ２５１７５を、ＢＨＩ培地中で対数期まで増殖させ、リン酸緩衝化生理食塩水（ＰＢＳ）（ｐＨ７．２）を用いて３０００×ｇで５分遠心分離して２回洗浄する。ペレットを１％ホルマリン／０．９％　ＮａＣｌに再懸濁し、室温で３０分混合し、０．９％　ＮａＣｌで２回洗浄する。Ｂａｌｂ／ｃマウス（８～１０週齢）を、フロイント不完全アジュバント（ＦＩＡ）で乳化したホルマリン化した完全なエス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）細菌の約10^8個の全細胞を含有する１００　ＦＩの抗原を腹腔内投与して免疫する。３～５週後、マウスに抗原（ＦＩＡ中10^8個の全細胞の細菌）の第２回投与を行う。融合の３日前に、マウスに、食塩水中10^8個の全細胞を静脈内投与して追加免疫する。

【００２５】

標準的組織培養培地は、２ｍＭ　Ｌ－グルタミン、１ｍＭピルビン酸ナトリウム、および１０ｍＭヘペスを補足したＲＰＭＩ１６４０（Ｇｉｂｃｏ）培地であり、さらに１００μｇ／ｍｌペニシリンと１００μｇ／ｍｌストレプトマイシンを１０％牛胎児血清とともに含有する。ＨＡＴ（１００μｇヒポキサンチン、０．４μＭアミノプテリン、１６μＭチミジン）を含有する培地中で、ハイブリッドを選択する。アミノプテリンを除去した後、ＨＴ（１００μｇヒポキサンチン、１６μＭチミジン）を培養培地中で２週間維持する。ハイブリドーマのクローニング中に、組織培養物に追加の増殖因子として、ＯＰＩ（１ｍＭオキザロ酢酸、０．４５ｍＭピルビン酸および０．２Ｕ／ｍｌウシインスリン）を加える。ＬｉｄｄｅｌｌとＣｒｙｅｒ（Ａ　Ｐｒａｃｔｉｃａｌ　Ｇｕｉｄｅ　ｔｏ　Ｍｏｎｏｃｌｏｎａｌ　Ａｎｔｉｂｏｄｉｅｓ，ジョンワイリーアンドサンズ（Ｊｏｈｎ　Ｗｉｌｅｙ　ａｎｄ　Ｓｏｎｓ）、Ｃｈｉｃｈｅｓｔｅｒ，Ｅｎｇｌａｎｄ，１９９１）が報告した方法に従って、ハイブリドーマを作成する。ＮＳＩ／Ａ９４．１マウスミエローマ細胞株を、融合のパートナーとして使用し、５％CO_2で３７℃で撹拌培養で増殖させ、対数増殖期で維持した後、融合する。

【００２６】

エス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）に対する種特異的モノクローナル抗体のスクリーニングのために、以下のアプローチがとられる。最初のスクリーニングをＥＬＩＳＡアッセイを使用して行い、これは、エス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）に結合する抗体を含有する培養上清について選択する。ホルマリン化細菌をＰＢＳでOD_{600}＝０．５に希釈し、あらかじめ０．０２ｍｇ／ｍｌポリ－Ｌ－リジン１００μｌで４時間インキュベートした９６ウェルＰＶＣ　ＥＬＩＳＡプレートのウェル中に二重測定で加える（１００μｌ）。これらの抗原被覆プレートを、湿潤箱中で４℃で一晩インキュベートし、次にＰＢＳで３回洗浄し、ＰＢＳ中の０．５％胎児牛血清でブロックし、４℃に保存する。１００μｌの成熟ハイブリドーマ上清を抗原プレートの適当なウェルに加え、室温で１時間インキュベートし、ＰＢＳ－０．０５％ツイーン２０で３回洗浄し、結合した抗体を、ＰＢＳ－１％胎児牛血清で１：１０００希釈したアルカリホスファターゼと結合した多価ヤギ抗マウスＩｇＧ抗体を添加して、検出する。炭酸バッファー（１５ｍＭ　Na_2CO_3、３５ｍＭ　NaH_2CO_3、１０ｍＭ　$MgCl_2$、ｐＨ９．６）中の基質である１ｍｇ／ｍｌのｐ－ニトロフェニルリン酸を加えた後、１５分後の発色を、ＥＩＡリー

ダーで４０５ｎｍで測定する。次に、陽性の上清（対照の３倍より高い）を免疫沈降アッセイ（１００μｌの細菌と１００μｌの上清を混合する）を行って、エス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）と強い陽性の反応性を有するものを選択する。寄託したクローンはこの方法で調製された。

【００２７】

２．　エス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）に対するヒト化モノクローナル抗体をコードするマウス／ヒトキメラ遺伝子の作成

マウスモノクローナル抗体をヒト化する方法の１つをここに説明する。マウスハイブリドーマ細胞株のゲノムＤＮＡを、ＱＩＡａｍｐシステム（キアゲン（Ｑｕｉａｇｅｎ）、Ｖａｌｅｎｃｉａ、ＣＡ）を使用して単離する。種々の制限酵素で消化した後、ＤＮＡ断片を電気泳動により０．８％アガロースゲルで分画し、ニトロセルロース膜に移す。サザンブロッティングを行い、免疫グロブリン遺伝子を同定する。重鎖遺伝子を、Ｊ３とＪ４セグメントおよびエンハンサー領域を含むマウスＩｇＧ重鎖遺伝子からのＤＮＡ断片とプローブ結合させる。軽鎖遺伝子は、Ｊ１－５セグメントを含有するマウスＩｇＧ軽鎖遺伝子からのＤＮＡ断片とプローブ結合させる。

【００２８】

サザンブロット解析で同定した選択したサイズのＤＮＡ制限断片を、アガロースゲルから、Ｑｉａｇｅｎ　ＤＮＡクリーンアップおよびゲル抽出システムを使用して精製する。ＤＮＡを、ラムダ－Ｚａｐ１１ベクター（ストラタジーン（Ｓｔｒａｔａｇｅｎｅ））に連結して、ラムダファージ中のこれらのマウスハイブリドーマの重鎖および軽鎖ライブラリーを構築する。前述のようにライブラリーを重鎖および軽鎖Ｊ領域プローブを用いてスクリーニングする。陽性クローンのＤＮＡを単離し、サブクローニングし、配列決定する。最良の正確度を達成するために、センス鎖とアンチセンス鎖の両方を配列決定する。ＢＬＡＳＴ検索を行って、ヌクレオチド配列をアミノ酸配列に翻訳し、それを既存の抗体遺伝子と比較する。重鎖の可変領域を同定し、サブクローニングし、ヒトＩｇＧ重鎖定常領域をコードするＤＮＡ断片およびミコフェノール酸に対する耐性を与えるＥｃｏｇｐｔ遺伝子を含有する発現ベクターに挿入する。軽鎖の可変領域も同定し、サブクローニングし、ヒトＩｇＧ軽鎖定常領域をコードするＤＮＡ断片およびＧ４１８に対する耐性を与えるｎｅｏ遺伝子を含有する別の発現ベクターに挿入する。

【００２９】

３．　形質転換した生物中のエス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）に対するモノクローナル抗体の発現

ａ）　動物細胞でヒトまたはヒト化モノクローナル抗体を産生する

ヒトＩｇＧ遺伝子の重鎖および軽鎖を、リポフェクション試薬（ＢＲＬ、Ｇｒａｎｄ　Ｉｓｌａｎｄ，　ＮＹ）を使用して、動物細胞株（例えばＳＰ２／０）中に、別々に導入または同時トランスフェクションする。トランスフェクションした細胞を、３７℃で５％ＣＯ２雰囲気中で、１×亜鉛選択培地中で２４時間インキュベートし、次に１０％胎児牛血清含有培地中でインキュベートする。４８時間インキュベーション後、細胞をマイクロタイタープレートに移し、Ｇ４１８とミコフェノール酸を含有する選択培地中で増殖させる。薬剤耐性細胞の上清を採取し、ＥＬＩＳＡまたは上記の沈降アッセイを使用して、エス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）に対する免疫反応性についてスクリーニングする。

【００３０】

ｂ）　食用植物中でヒトまたはヒト化モノクローナル抗体を産生する

トランスジェニック植物は、大量の外来タンパク質を非常に低コストで製造するための非常に有用なシステムであることが認識されている。エス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）に対するヒトまたはヒト化モノクローナル抗体を食用植物（野菜または果実）中で発現することは、存在するう蝕を治療し、将来の細菌感染を予防するために、口に植物または植物抽出物を直接適用することを可能にする。トランスジェニックな食用植物の選択には、特に限定されないが、ジャガイモ、トマト、ボロッコリーおよびバナナがある。

【００３１】
本明細書にトランスジェニックアラビドプシス（Ａｒａｂｉｄｏｐｓｉｓ）を製造する方法が記載され、これは、アブラナ属（Ｂｒａｓｓｉｃａ）の種に密接に関連する食用植物である、一般的な野菜（例えば、キャベツ、カリフラワーおよびブロッコリー）を含む。これは、この植物について多くの遺伝的および生化学的手段が開発されているため、選択される。この植物でＩｇＧを発現するのにいくつかの方策がある。１つの方策は、まず重鎖と軽鎖をコードするヒトＩｇＧ遺伝子を２つの別個のトランスジェニック株に導入することである。２つの遺伝子は、遺伝子交配および選択により一緒にされる。他の方法は連続的形質転換であり、ここでは、１つのＩｇＧ遺伝子で形質転換されたトランスジェニック株が、第２の遺伝子で再形質転換される。あるいは、重鎖と軽鎖とをコードする遺伝子が、同じＴ－ＤＮＡ形質転換ベクター中の２つの異なるクローニング部位に、２つのプロモーターの制御下でクローン化され、両方の遺伝子の発現は、単一の構築体を植物に形質転換することにより行われる。技術的には、別々の形質転換法は、最も簡単な方法であり、これは通常、より高い抗体収率を与える。従って我々はここに、この方策を提示する。同様の技術を使用して、他の植物を形質転換することが可能である。

【００３２】
ヒトＩｇＧ遺伝子の重鎖と軽鎖をコードするＤＮＡ断片は、アグロバクテリウム・ツメファシエンス（Ａｇｒｏｂａｃｔｅｒｉｕｍ ｔｕｍｅｆａｃｉｅｎｓ）のＴｉプラスミド中に別々にクローン化される。このプラスミドは、アラビドプシス・タリアーナ（Ａｒａｂｉｄｏｐｓｉｓ ｔｈａｌｉａｎａ）中のＩｇＧのヒト重鎖と軽鎖、アグロバクテリウム・ツメファシエンス（Ａｇｒｏｂａｃｔｅｒｉｕｍ ｔｕｍｅｆａｃｉｅｎｓ）中の抗生物質マーカー、およびアラビドプシス（Ａｒａｂｉｄｏｐｓｉｓ）中の形質転換選択のための除草剤耐性遺伝子とを発現するプロモーターを含有する。アグロバクテリウム・ツメファシエンス（Ａｇｒｏｂａｃｔｅｒｉｕｍ ｔｕｍｅｆａｃｉｅｎｓ）株は、これらのプラスミドで形質転換され、抗生物質選択下で後期対数期まで増殖され、Ｂｅｔｈｔｏｌｄら（Ｃ．Ｒ．Ａｃａｄ．Ｓｃｉ．Ｐａｒｉｓ Ｌｉｆｅ Ｓｃｉ．３１６：１１９４－１１９９，１９９３）が記載した浸透培地中に再懸濁する。

【００３３】
アグロバクテリウム・ツメファシエンス（Ａｇｒｏｂａｃｔｅｒｉｕｍ ｔｕｍｅｆａｃｉｅｎｓ）を含有するＴｉプラスミドによるアラビドプシス（Ａｒａｂｉｄｏｐｓｉｓ）の形質転換は、真空浸透により行われる。アラビドプシス（Ａｒａｂｉｄｏｐｓｉｓ）の植物全体を細菌懸濁液中に浸漬する。この方法は、真空チャンバー中で行われる。５分の真空（約４０ｃｍ水銀）が４サイクル行われる。各適用後、真空を解除し直ちに繰り返す。浸透後、植物を増殖チャンバー中で２４時間水平に維持する。次に植物を成熟するまで増殖させ、その種子を採取する。採取した種子を、最初の一対の真の葉が出るまで、非選択培地中で発芽させる。この段階で、植物に除草剤Ｂａｓｔａを１５０ｍｇ／ｌ水の濃度で噴霧する。形質転換したＴｉプラスミドを含有するアラビドプシス（Ａｒａｂｉｄｏｐｓｉｓ）は、除草剤に耐性であり、非形質転換植物は脱色され死滅する。そのような選択は、植物の第２世代まで続く。耐性植物について、総ゲノムＤＮＡを単離し、ＩｇＧ遺伝子の重鎖と軽鎖とをコードするＤＮＡ断片とプローブ結合させる。陽性形質転換体の植物抽出物を調製し、ヒトＩｇＧタンパク質の発現について、ウェスタンブロットにより、ヒトＩｇＧの定常領域の重鎖と軽鎖に対する抗体を使用してスクリーニングする。

【００３４】
ヒトＩｇＧ重鎖を発現する植物を、ヒトＩｇＧ軽鎖を発現する植物と有性交配させて、両方の鎖を発現する子孫を作り出す。ウェスタンブロッティング法を使用して、重鎖と軽鎖の両方をスクリーニングする。陽性の形質転換体からの抽出物を採取し、上記のＥＬＩＳＡまたは沈降アッセイを使用してエス・ミュータンス（Ｓ．ｍｕｔａｎｓ）に対する免疫反応性についてスクリーニングする。

【００３５】
４．　ヒトう蝕を治療または予防するためのエス・ミュータンス（Ｓ．ｍｕｔａｎｓ）

に対するヒトまたはヒト化モノクローナル抗体の使用
上記試験がうまく完了すると、エス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）に対するヒト化モノクローナル抗体が得られる。この植物組織の効力を試験する。
【００３６】
エス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）に対するモノクローナル抗体を含有する植物組織抽出物を、精製したヒト補体成分または精製したヒト多形核好中球の存在下および非存在下で、種々の濃度のエス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）と混合する。２時間インキュベーション後、混合物をＢＨＩプレートに蒔いて、細菌活性を調べる。
【００３７】
Ｗｏｌｉｎｓｋｙら（Ｊ．　Ｄｅｎｔ．　Ｒｅｓ．　７５：８１６－８２２，　１９９６）が開発した人工的プラーク形成システムを使用して、モノクローナル抗体を含有する植物組織抽出物を使用して、発現されたモノクローナル抗体が、唾液または人工的歯科エナメル上の歯垢中のエス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）を死滅させる能力を調べる。同様の方法を使用して、歯垢の形成を防ぐ能力を調べる。
【００３８】
動物細胞または植物により産生したこれらのモノクローナル抗体を使用して、ヒトの臨床試験が行われている。ヒトボランティアを、エス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）に対するこれらのヒトモノクローナル抗体有りでまたは無しで処置した。次に唾液と歯垢中のエス・ミュータンス（Ｓ．　ｍｕｔａｎｓ）のレベルを調べる。モノクローナル抗体の処理との関係における、現在と将来のう蝕の間の相関も調べる。
【００３９】
前記実施例は例示のみが目的であり、請求項に示す本発明の範囲を決して限定するものではない。

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(54) **Title:** IMMUNOLOGIC METHOD FOR THE PREVENTION OF DENTAL CARIES

(57) **Abstract:** Dental caries in man may be prevented or treated by oral ingestion of human or humanized mouse monoclonal IgG and IgM antibodies that bind to surface antigens of cariogenic organisms, such as *S. mutans*. The genetically immune system when they bind to cariogenic organisms, resulting in their destruction. In a preferred embodiment, monoclonal antibodies to cariogenic organisms are produced by edible plants, including fruits and vegetables, transformed by DNA sequences that code on expression for the desired antibodies. The antibodies are applied by eating the plants.

IMMUNOLOGIC METHOD FOR THE PREVENTION OF DENTAL CARIES

Background of the Invention

This application relates to an immunologic methodology for the treatment and prevention of dental caries. This invention has special application to patients who are without the ability or motivation to apply established principles of self care, such as very young children, the infirm and poorly educated populations.

Dental caries (tooth decay) and periodontal disease are probably the most common chronic diseases in the world. The occurrence of cavities in teeth is the result of bacterial infection. Hence the occurrence of dental caries is properly viewed as an infectious microbiological disease that results in localized destruction of the calcified tissues of the teeth.

Streptococcus mutans is believed to be the principal cause of tooth decay in man. When *S. mutans* occurs in large numbers in dental plaque, and metabolizes complex sugars, the resulting organic acids cause demineralization of the tooth surface. The result is carious lesions, commonly known as cavities. Other organisms, such as *Lactobaccilli* and *Actinomyces* are also believed to be involved in the progression and formation of carious lesions. Those organizations that cause tooth decay are referred to herein as "cariogenic organisms."

Removal of the damaged portion of a tooth and restoration by filling can, at least temporarily, halt the damage caused by oral infection with cariogenic organisms. However, the "drill and fill" approach does not eliminate the causative bacterial agent. Proper oral hygiene can control the accumulation of dental plaque, where cariogenic organisms grow and attack the tooth surfaces.

However, dental self-care has its limits, particularly in populations that are unable to care for themselves, or where there is a lack of knowledge of proper methods of self care. Administration of fluoride ion has been shown to decrease, but not eliminate the incidence of dental caries.

In view of the overwhelming evidence of the involvement of cariogenic organisms in the pathogenesis of dental caries, it is not surprising that there have been a number of different attempts to ameliorate the condition using traditional methods of anti-microbial therapy. The disadvantage of antimicrobial agents is that they are not selective for cariogenic organisms. Administration of non-specific bacteriocidal agents disturbs the balance of organisms that normally inhabit the oral cavity, with consequences that cannot be predicted, but may include creation of an environment that provides opportunities for pathogenic organisms. In addition, long term use of antimicrobial agents is known to select for organisms that are resistant to them. Hence long term and population-wide use of antimicrobial agents to prevent tooth decay is not practical.

Vaccination of humans to elicit an active immune response to *S. mutans*, or other cariogenic organisms, is also not a practical solution at this time. One drawback of this approach is that vaccination elicits production of predominantly IgG and IgM antibodies, but they are not secreted into saliva. The majority of antibodies present in saliva are of the IgA isotype, which can bind to, but cannot activate lymphocytes or complement components to kill bacteria. Accordingly, vaccination is not believed likely to be capable of producing antibodies that can trigger the immune system to kill cariogenic organisms in the mouth. There is no known method for selectively increasing the titer of vaccination induced antibodies of the IgG or IgM isotypes in the oral cavity.

There have been a number of reported attempts to passively immunize patients to *S. mutans* using monoclonal IgA antibodies raised in mice to prevent tooth decay in animals and in man. Because IgA is a multivalent antibody, a single molecule of IgA can bind to several different antigenic sites, resulting in clumping of bacteria. However, binding of IgA to bacterial surface antigens does not

kill the bacteria. Rather, clumping is believed to hinder the ability of bacteria to bind to tooth surfaces. Another drawback of this approach is that repeated administration of mouse (i.e., heterologous) antibodies to humans has the potential to evoke an immune response to the antibodies.

Unlike IgA antibodies, antibodies of the IgG and IgM classes have bacteriocidal effects. Binding of IgM or IgG antibodies to antigens present on the surface of cariogenic organisms may result in the destruction of the bacterial cells by two separate mechanisms: complement mediated cell lysis and antibody-dependent cell-mediated cytotoxicity. In either case, antibodies that selectively bind to certain microbial organisms target just those cells for destruction by the immune system. Both complement mediated cell lysis and antibody-dependent cell mediated cytotoxicity are part of the humoral immune response that is mediated by antibodies of the IgG and IgM classes.

In order to elicit the desired cytotoxic effect of antibody binding, monoclonal antibodies to cariogenic organisms must be recognized by the human immune system. There are a number of different technologies by which antibodies that will trigger a response from a heterologous mammalian immune system can be produced. One example is a nucleic acid construct that codes on expression for a human antibody modified to incorporate sequences encoding the antigen specific binding domain from heterologous organisms.

Production and administration of such genetically engineered monoclonal antibodies to treat dental caries in man poses issues susceptible to a particularly innovative solution. Prior art methods for production of monoclonal antibodies involve growing hybriclomas in culture media, followed by extraction and purification of the desired antibody. These steps are significantly simplified in a preferred embodiment of the invention by expressing the antibodies in edible plants or animals (eukaryotes). The antibodies are administered upon oral ingestion of d plant or animal products, such as fruits, vegetables or milk wherein the antibodies are not denatured. This mode of administration has the potential for obviating compliance issues in ameliorating tooth decay.

Summary of the Preferred Embodiments

Dental caries may be prevented or treated by oral ingestion of human or humanized mouse monoclonal IgG and IgM antibodies that to bind surface antigens of cariogenic organisms, such as S. mutans. The genetically engineered monoclonal antibodies engage the effector apparatus of the human immune system when they bind to cariogenic organisms, resulting in their destruction. In a preferred embodiment, monoclonal antibodies to cariogenic organisms are produced by edible plants, including fruits and vegetables, transformed by DNA sequences that code on expression for the desired antibodies. The antibodies are applied by eating the plants.

Detailed Description of the Preferred Embodiments

1. Preparation of Monoclonal Antibodies

The monoclonal antibody technique permits preparation of a source of antibodies with extraordinary specificity. Monoclonal antibodies that bind to specific molecular structures can be produced using what are today considered standard techniques.

The monoclonal antibodies that may be used in this invention are those that are directed to surface antigens of cariogenic organisms. Surface antigens are substances that are displayed on the surface of cells. Such antigens are accessible to antibodies present in body fluids. In the context of the present invention, surface antigens of cariogenic organisms are present on the surface of organisms that cause dental caries. While the role of bacterial activity in the genesis of carious lesions is well defined, establishing a cause and effect relationship between a particular organism and the occurrence of dental caries has not been completely successful. To date, only *S. mutans* has been definitively associated with dental caries. However, species of the *Lactobacilli and Actinomyces* are also believed to be involved, particularly with the active progression of carious lesions. Any organism that can produce a carious lesion is a potential target for the monoclonal antibodies prepared and used in accordance with this invention.

A further requirement of the monoclonal antibodies that may be used in the practice of the present invention is that they are selective for cariogenic organisms. Monoclonal antibodies directed to antigens present on cariogenic as well as non-cariogenic organisms may produce non-specific alterations in the makeup of the flora within the oral cavity. The consequences of such changes are not understood.

Accordingly, the preferred monoclonal antibodies are directed to surface antigens of cariogenic organisms. That is to say, the preferred monoclonal antibodies bind specifically to organisms that cause dental caries.

It should be clearly understood that the scope of the present invention is not limited to the prevention of tooth decay in man. Monoclonal antibodies in accordance with the present invention can be genetically engineered to engage the effector response of the immune system of other mammals, such as those that are domesticated as pets.

Monoclonal antibodies are prepared by immunizing mice or other mammalian hosts with cell wall material isolated from cariogenic organisms. In a preferred embodiment, the cariogenic organisms are type c *S. mutans* (ATCC25175). The immunogenecity of molecules present in cell walls may be enhanced by a variety of techniques known in the art. In a preferred embodiment, immunogenecity of such molecules is enhanced by denaturation of the isolated cell material with formalin. Other techniques for modifying cell wall proteins to enhance immunogenecity are within the scope of this invention. Typically, hosts receive one or more subsequent injections of isolated bacterial cell fragments to increase the titer of antibodies prior to sacrifice and cloning.

Spleen cells from hosts are harvested and cloned by limiting dilution using techniques that have become standard since the pioneering work of Kohler and Milstein. In a preferred embodiment, surviving hybridomas are screened for antibody directed to cariogenic organisms by ELISA assay against microtiter plates coated with formalinized bacterial cell material. Positive supernatants may be subjected to further screening to identify clones that secrete antibodies with the greatest affinity for the cariogenic organisms. In a preferred embodiment, clones with titers at least three times higher than background are screened again using an immunoprecipitation against denatured cell wall material from *S. mutans.* In a preferred embodiment, three clones were identified which bound detectably only to *S. mutans* strains ATCC25175, LM7, OMZ175 and ATCC31377. These clones were deposited with the American Type Culture Collection, receiving Deposit Numbers HB-1 2560, 12599 and 12558.

2. Preparation of Monoclonal Antibodies Capable Of Eliciting An Effector Response From Human Immune System

Previous efforts to develop an immunological method for the prevention of dental caries employed heterologous antibodies. For example, Lehner, United States patent 5,352,446, referred to use of monoclonal antibodies to *S. mutans* surface antigens raised in mice in inhibiting the proliferation of those bacteria in monkeys. More recently, Ma et al. *Nature Medicine*, 45(5) 601-6 (1998), reported similar results in humans, using a genetically engineered secretory monoclonal antibody to *S. mutans* expressed in tobacco plants. Drawbacks to this approach include 1) administration of heterologous antibodies may aggregate the offending organisms, but will not kill them because such antibodies will not elicit an immune response; and 2) repeated administration of the antibody may elicit an immune response from the patient to the antibody. A preferable approach is to use recombinant techniques to prepare chimeric antibody molecules directed specifically to surface antigens of cariogenic organisms, that will also elicit an effector response from the human immune system (when used in man) upon binding to the target organism. This can be accomplished by inserting variable regions or complementarity determining regions ("CDR's") from mouse monoclonal antibodies that are specific to cariogenic organisms into antibodies of the IgG and/or IgM classes from the mammal to be treated. When the mammal to be treated is man, the antibodies are said to be "humanized."

There are various ways to obtain nucleic acid sequences that code on expression for human or humanized monoclonal antibodies to surface antigens of cariogenic organisms: 1) Isolating mouse hybridomas which produce monoclonal antibodies against cariogenic organisms and cloning mouse genes that code on expression for those antibodies; 2) Using purified cariogenic organisms to screen a phage display random library made from human B lymphocytes to obtain genes that encode antibodies specific for cariogenic organisms; 3) Isolating human hybridomas that produce monoclonal antibodies against cariogenic organisms, using B lymphocytes recovered from heavily

infected patients and cloning the human genes encoding for these antibodies; or 4) immunizing human B lymphocytes and spleen cells *in vitro* using purified cariogenic organisms, followed by fusion to form hybriclomas to create immortal cell lines. The techniques required are known to those skilled in the art.

In the presently preferred embodiment of the invention, mouse monoclonal antibodies are "humanized." Using the PCR or Southern blot technique, DNA fragments encoding the variable domains of mouse hybridomas secreting antibody specific to cell surface antigens of cariogenic organisms are isolated. Using gene cloning techniques, the variable regions of human IgG and IgM immunoglobulin are replaced with the corresponding mouse variable regions or CDR's. The result of this genetic engineering is a chimeric antibody molecule with variable domains that selectively bind to surface antigens of cariogenic organisms, but which interacts with the human immune system through its constant regions to trigger a humoral immune response.

3. Administration of Monoclonal Antibodies

In order to prepare a sufficient quantity of monoclonal antibodies for clinical use, the desired cell line transfected with IgG or IgM encoding sequences must be propagated. Existing technology permits large scale propagation of monoclonal antibodies in tissue culture. The transfected cell lines will secrete monoclonal antibodies into the tissue culture medium. The secreted monoclonal antibodies are recovered and purified by gel filtration and related techniques of protein chemistry.

In experimental studies, monoclonal antibodies to *S. mutans* have been applied directly to the surface of teeth. Application by ingestion of mouthwash, or by chewing gum has also been proposed. A presently preferred alternative is to express the monoclonal antibodies of the present invention in edible plants, such as banana or broccoli. Eating plants transformed in accordance with this invention will result in application of the antibodies to cariogenic organisms present on tooth surfaces, and elsewhere in the mouth. It is also contemplated that other organisms,

both plant and animal may be transformed to express the monoclonal antibodies described herein, so that such antibodies may be ingested, for example, by drinking milk.

Examples

1. Producing mouse monoclonal antibodies against *S. mutans*

Type c *S. mutans* strain ATCC25175 are grown to log phase in BHI medium and washed twice with phosphate buffered saline, pH 7.2 (PBS), by centrifugation at 3000xg for 5 min. The pellet is resuspended in 1% formalin/0.9% NaCl, mixed at room temperature for 30 min and washed twice with 0.9% NaCl. BALB/c mice (8-10 weeks) are immunized intraperitoneally with 100 Fl of the antigen containing approximately 10^8 whole cells of formalinized intact *S. mutans* bacteria emulsified with Freund's incomplete adjuvant (FIA). After 3-5 weeks, mice will receive a second dose of antigen (10^8 whole cells of bacteria in FIA). Three days prior to fusion, the mice are boosted intravenously with 10^8 whole cells in saline.

The standard tissue culture media is RPMI 1640 (Gibco) medium supplemented with 2 mM L-glutamine, 1mM sodium pyruvate, and 10 mM HEPES and containing 100 μg/ml penicillin and 100 / μg/ml streptomycin with 10% fetal calf serum. Hybrids are selected in media containing HAT (100μg Hypoxanthine, 0.4. μM Aminopterin; 16 μM Thymidine). HT (100 μg Hypoxanthine; 16 μM Thymidine) is maintained in the culture medium for 2 weeks after aminopterin is withdrawn. OPI (1 mM oxaloacetate, 0.45 mM pyruvate and 0.2 U/ml bovine insulin) is added as an additional growth factor to the tissue culture during cloning of hybriclomas. Hybriclomas are raised according to the procedure reported by Liddell and Cryer (A Practical Guide to Monoclonal Antibodies, John Wiley & Sons, Chichester, England, 1991). The NSl/A94.1 mouse myeloma cell line is used as the fusion partner and grown in spinner cultures in 5% C02 at 37°C and maintained in log phase of growth prior to fusion.

The following approach is used for screening for species-specific monoclonal antibodies against *S. mutans*. The initial screening is performed using an ELISA assay, which selects for the culture supernatants containing antibodies that bind to *S. mutans*. Formalinized bacteria are diluted in PBS to

OD_{600} = 0.5, and added to duplicate wells (100μl) in 96 well PVC ELISA plates preincubated for 4 h with 100 μl of 0.02 mg/ml Poly-L-lysine. These antigen-coated plates are incubated overnight at 4°C in a moist box then washed 3 times with PBS and blocked with 0.5% fetal calf serum in PBS and stored at 4°C. 100 μl of mature hybridoma supernatants are added to the appropriate wells of the antigen plates, incubated for 1 h at room temperature, washed 3 times with PBS-0.05% tween 20, and bound antibody is detected by the addition of polyvalent goat-anti-mouse IgG antibody conjugated with alkaline phosphatase diluted 1:1000 with PBS-1% fetal calf serum. After the addition of the substrate, 1 mg/ml p-nitrophenyl phosphate in carbonate buffer (15 mM Na_2CO_3, 35 mM NaH_2CO_3, 10 mM $MgCl_2$ pH 9.6), the color developments after 15 min is measured in an EIA reader at 405 nm. The positive supernatants (3 fold higher than control) are then subjected to the immunoprecipitation assay (mixing 1 00μl bacteria with 100μl supernatant) to screen for those with strong positive reactivity with *S. mutans*. The deposited clones were prepared according to this method.

2. Generating mouse/human chimeric genes which encode humanized monoclonal antibodies against *S. mutans*.

Described here is one of the ways to humanize mouse monoclonal antibodies. Genomic DNA of mouse hybridoma cell lines is isolated using the QIAamp system (Qiagen, Valencia, CA). After digestion with various restriction enzymes, DNA fragments are fractionated through 0.8% agarose gel by electrophoresis and transferred to a nitrocellulose membrane. Southern blotting is performed to identify the immunoglobulin gene. The heavy chain gene is probed with a DNA fragment from a mouse IgG heavy-chain gene that includes the J3 and J4 segments and the enhancer region. The light chain gene is probed with a DNA fragment from a mouse IgG light-chain gene containing J1-5 segments.

DNA restriction fragments of the selected size identified through Southern blot analysis are purified from agarose gel using Qiagen DNA Clean-up and Gel extraction system. The DNA is ligated into the Lambda-Zap 11 vector

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3. Expressing Monoclonal Antibodies to S. mutans In Transformed Organisms

a) Producing human or humanized monoclonal antibodies in animal cells

The heavy and light chain of a human IgG gene are separately introduced or cotransfected into an animal cell line (such as SP2/0) using a lipofection reagent (BRL, Grand Island, NY). The transfected cells are incubated at 37°C in a 5% C02 atmosphere in 1x zinc option medium for 24 h and then in medium containing 10% fetal bovine serum. After 48 h incubation, the cells are transferred to a microtiter plate and grown in selection medium containing G418 and mycophenolic acid. The supernatants of drug-resistant cells are collected and screened for immuno-reactivity against *S. mutans* using the ELISA or precipitation assays mentioned above.

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Presented here are the procedures to produce transgenic Arabidopsis, an edible plant closely related to *Brassica* species including common vegetables such as cabbage, cauliflower and broccoli. It is chosen because many genetic and biochemical tools have been well developed for this plant. There are several strategies to express IgG in this plant. One strategy is to first introduce the human IgG genes encoding the heavy chain and light chain to two separate transgenic lines. The two genes are brought together by genetic crossing and selection. Other methods involve sequential transformation, in which transgenic lines transformed with one IgG gene are re-transformed with the second gene. Alternatively, genes encoding the heavy chain and light chain are cloned into two different cloning sites in the same T-DNA transformation vector under the control of two promoters, and the expression of both genes can be achieved by the transformation of a single construct to plant. Technically, the separate transformation method is the simplest one and it usually results in higher antibody yield. Therefore, we present this strategy here. It is possible to transform other plants using similar techniques.

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With the successful completion of the above studies, humanized monoclonal antibodies against *S. mutans* are obtained. The plant tissue is tested for efficacy.

Plant tissue extracts containing monoclonal antibodies to *S. mutans* are mixed with various concentrations of *S. mutans* in the presence and absence of purified human complement components or purified human polymorphonuclear neutrophilic leukocytes. After a two hour incubation, the mixtures are plated onto BHI plates to examine the bactericidal activity.

Using the artificial plaque formation system developed by Wolinsky et al. (J. Dent. Res. 75:816-822, 1996), plant tissue extracts containing monoclonal antibodies are used to examine the ability of the expressed monoclonal antibodies ability to kill *S. mutans* in saliva or in existing dental plaques on artificial dental enamel. Analogous techniques are used to examine the ability to prevent the formation of dental plaques.

Human clinical trials are performed using these monoclonal antibodies produced through animal cells or plants. Human volunteers are treated with or without these human monoclonal antibodies against *S. mutans*. Then the level of *S. mutans* in saliva and in dental plaques is examined. The correlation between present and future dental caries in relationship with treatment of monoclonal antibodies is also examined.

It should be understood that the foregoing examples are for illustrative purposes only, and are not intended to limit the scope of applicants' invention which is set forth in the claims appearing below.

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(54) Title: IMMUNOLOGIC METHOD FOR THE PREVENTION OF DENTAL CARIES

(57) Abstract: Dental caries in man may be prevented or treated by oral ingestion of human or humanized mouse monoclonal IgG and IgM antibodies that bind to surface antigens of cariogenic organisms, such as*S. mutans*. The genetically immune system when they bind to cariogenic organisms, resulting in their destruction. In a preferred embodiment, monoclonal antibodies to cariogenic organisms are produced by edible plants, including fruits and vegetables, transformed by DNA sequences that code on expression for the desired antibodies. The antibodies are applied by eating the plants.

IMMUNOLOGIC METHOD FOR THE PREVENTION OF DENTAL CARIES

Background of the Invention

This application relates to an immunologic methodology for the treatment and prevention of dental caries. This invention has special application to patients who are without the ability or motivation to apply established principles of self care, such as very young children, the infirm and poorly educated populations.

Dental caries (tooth decay) and periodontal disease are probably the most common chronic diseases in the world. The occurrence of cavities in teeth is the result of bacterial infection. Hence the occurrence of dental caries is properly viewed as an infectious microbiological disease that results in localized destruction of the calcified tissues of the teeth.

Streptococcus mutans is believed to be the principal cause of tooth decay in man. When *S. mutans* occurs in large numbers in dental plaque, and metabolizes complex sugars, the resulting organic acids cause demineralization of the tooth surface. The result is carious lesions, commonly known as cavities. Other organisms, such as *Lactobacilli* and *Actinomyces* are also believed to be involved in the progression and formation of carious lesions. Those organizations that cause tooth decay are referred to herein as "cariogenic organisms."

Removal of the damaged portion of a tooth and restoration by filling can, at least temporarily, halt the damage caused by oral infection with cariogenic organisms. However, the "drill and fill" approach does not eliminate the causative bacterial agent. Proper oral hygiene can control the accumulation of dental plaque, where cariogenic organisms grow and attack the tooth surfaces.

However, dental self-care has its limits, particularly in populations that are unable to care for themselves, or where there is a lack of knowledge of proper methods of self care. Administration of fluoride ion has been shown to decrease, but not eliminate the incidence of dental caries.

In view of the overwhelming evidence of the involvement of cariogenic organisms in the pathogenesis of dental caries, it is not surprising that there have been a number of different attempts to ameliorate the condition using traditional methods of anti-microbial therapy. The disadvantage of antimicrobial agents is that they are not selective for cariogenic organisms. Administration of non-specific bacteriocidal agents disturbs the balance of organisms that normally inhabit the oral cavity, with consequences that cannot be predicted, but may include creation of an environment that provides opportunities for pathogenic organisms. In addition, long term use of antimicrobial agents is known to select for organisms that are resistant to them. Hence long term and population-wide use of antimicrobial agents to prevent tooth decay is not practical.

Vaccination of humans to elicit an active immune response to *S. mutans*, or other cariogenic organisms, is also not a practical solution at this time. One drawback of this approach is that vaccination elicits production of predominantly IgG and IgM antibodies, but they are not secreted into saliva. The majority of antibodies present in saliva are of the IgA isotype, which can bind to, but cannot activate lymphocytes or complement components to kill bacteria. Accordingly, vaccination is not believed likely to be capable of producing antibodies that can trigger the immune system to kill cariogenic organisms in the mouth. There is no known method for selectively increasing the titer of vaccination induced antibodies of the IgG or IgM isotypes in the oral cavity.

There have been a number of reported attempts to passively immunize patients to *S. mutans* using monoclonal IgA antibodies raised in mice to prevent tooth decay in animals and in man. Because IgA is a multivalent antibody, a single molecule of IgA can bind to several different antigenic sites, resulting in clumping of bacteria. However, binding of IgA to bacterial surface antigens does not

kill the bacteria. Rather, clumping is believed to hinder the ability of bacteria to bind to tooth surfaces. Another drawback of this approach is that repeated administration of mouse (i.e., heterologous) antibodies to humans has the potential to evoke an immune response to the antibodies.

Unlike IgA antibodies, antibodies of the IgG and IgM classes have bacteriocidal effects. Binding of IgM or IgG antibodies to antigens present on the surface of cariogenic organisms may result in the destruction of the bacterial cells by two separate mechanisms: complement mediated cell lysis and antibody-dependent cell-mediated cytotoxicity. In either case, antibodies that selectively bind to certain microbial organisms target just those cells for destruction by the immune system. Both complement mediated cell lysis and antibody-dependent cell mediated cytotoxicity are part of the humoral immune response that is mediated by antibodies of the IgG and IgM classes.

In order to elicit the desired cytotoxic effect of antibody binding, monoclonal antibodies to cariogenic organisms must be recognized by the human immune system. There are a number of different technologies by which antibodies that will trigger a response from a heterologous mammalian immune system can be produced. One example is a nucleic acid construct that codes on expression for a human antibody modified to incorporate sequences encoding the antigen specific binding domain from heterologous organisms.

Production and administration of such genetically engineered monoclonal antibodies to treat dental caries in man poses issues susceptible to a particularly innovative solution. Prior art methods for production of monoclonal antibodies involve growing hybriclomas in culture media, followed by extraction and purification of the desired antibody. These steps are significantly simplified in a preferred embodiment of the invention by expressing the antibodies in edible plants or animals (eukaryotes). The antibodies are administered upon oral ingestion of d plant or animal products, such as fruits, vegetables or milk wherein the antibodies are not denatured. This mode of administration has the potential for obviating compliance issues in ameliorating tooth decay.

Summary of the Preferred Embodiments

Dental caries may be prevented or treated by oral ingestion of human or humanized mouse monoclonal IgG and IgM antibodies that to bind surface antigens of cariogenic organisms, such as S. mutans. The genetically engineered monoclonal antibodies engage the effector apparatus of the human immune system when they bind to cariogenic organisms, resulting in their destruction. In a preferred embodiment, monoclonal antibodies to cariogenic organisms are produced by edible plants, including fruits and vegetables, transformed by DNA sequences that code on expression for the desired antibodies. The antibodies are applied by eating the plants.

Detailed Description of the Preferred Embodiments

1. Preparation of Monoclonal Antibodies

The monoclonal antibody technique permits preparation of a source of antibodies with extraordinary specificity. Monoclonal antibodies that bind to specific molecular structures can be produced using what are today considered standard techniques.

The monoclonal antibodies that may be used in this invention are those that are directed to surface antigens of cariogenic organisms. Surface antigens are substances that are displayed on the surface of cells. Such antigens are accessible to antibodies present in body fluids. In the context of the present invention, surface antigens of cariogenic organisms are present on the surface of organisms that cause dental caries. While the role of bacterial activity in the genesis of carious lesions is well defined, establishing a cause and effect relationship between a particular organism and the occurrence of dental caries has not been completely successful. To date, only *S. mutans* has been definitively associated with dental caries. However, species of the *Lactobaccili* and *Actinomyces* are also believed to be involved, particularly with the active progression of carious lesions. Any organism that can produce a carious lesion is a potential target for the monoclonal antibodies prepared and used in accordance with this invention.

A further requirement of the monoclonal antibodies that may be used in the practice of the present invention is that they are selective for cariogenic organisms. Monoclonal antibodies directed to antigens present on cariogenic as well as non-cariogenic organisms may produce non-specific alterations in the makeup of the flora within the oral cavity. The consequences of such changes are not understood.

Accordingly, the preferred monoclonal antibodies are directed to surface antigens of cariogenic organisms. That is to say, the preferred monoclonal antibodies bind specifically to organisms that cause dental caries.

It should be clearly understood that the scope of the present invention is not limited to the prevention of tooth decay in man. Monoclonal antibodies in accordance with the present invention can be genetically engineered to engage the effector response of the immune system of other mammals, such as those that are domesticated as pets.

Monoclonal antibodies are prepared by immunizing mice or other mammalian hosts with cell wall material isolated from cariogenic organisms. In a preferred embodiment, the cariogenic organisms are type c *S. mutans* (ATCC25175). The immunogenecity of molecules present in cell walls may be enhanced by a variety of techniques known in the art. In a preferred embodiment, immunogenecity of such molecules is enhanced by denaturation of the isolated cell material with formalin. Other techniques for modifying cell wall proteins to enhance immunogenecity are within the scope of this invention. Typically, hosts receive one or more subsequent injections of isolated bacterial cell fragments to increase the titer of antibodies prior to sacrifice and cloning.

Spleen cells from hosts are harvested and cloned by limiting dilution using techniques that have become standard since the pioneering work of Kohler and Milstein. In a preferred embodiment, surviving hybridomas are screened for antibody directed to cariogenic organisms by ELISA assay against microtiter plates coated with formalinized bacterial cell material. Positive supernatants may be subjected to further screening to identify clones that secrete antibodies with the greatest affinity for the cariogenic organisms. In a preferred embodiment, clones with titers at least three times higher than background are screened again using an immunoprecipitation against denatured cell wall material from *S. mutans*. In a preferred embodiment, three clones were identified which bound detectably only to *S. mutans* strains ATCC25175, LM7, OMZ175 and ATCC31377. These clones were deposited with the American Type Culture Collection, receiving Deposit Numbers HB-1 2560, 12599 and 12558.

2. Preparation of Monoclonal Antibodies Capable Of Eliciting An Effector Response From Human Immune System

Previous efforts to develop an immunological method for the prevention of dental caries employed heterologous antibodies. For example, Lehner, United States patent 5,352,446, referred to use of monoclonal antibodies to *S. mutans* surface antigens raised in mice in inhibiting the proliferation of those bacteria in monkeys. More recently, Ma et al. *Nature Medicine*, 45(5) 601-6 (1998), reported similar results in humans, using a genetically engineered secretory monoclonal antibody to *S. mutans* expressed in tobacco plants. Drawbacks to this approach include 1) administration of heterologous antibodies may aggregate the offending organisms, but will not kill them because such antibodies will not elicit an immune response; and 2) repeated administration of the antibody may elicit an immune response from the patient to the antibody. A preferable approach is to use recombinant techniques to prepare chimeric antibody molecules directed specifically to surface antigens of cariogenic organisms, that will also elicit an effector response from the human immune system (when used in man) upon binding to the target organism. This can be accomplished by inserting variable regions or complementarity determining regions ("CDR's") from mouse monoclonal antibodies that are specific to cariogenic organisms into antibodies of the IgG and/or IgM classes from the mammal to be treated. When the mammal to be treated is man, the antibodies are said to be "humanized."

There are various ways to obtain nucleic acid sequences that code on expression for human or humanized monoclonal antibodies to surface antigens of cariogenic organisms: 1) Isolating mouse hybridomas which produce monoclonal antibodies against cariogenic organisms and cloning mouse genes that code on expression for those antibodies; 2) Using purified cariogenic organisms to screen a phage display random library made from human B lymphocytes to obtain genes that encode antibodies specific for cariogenic organisms; 3) Isolating human hybridomas that produce monoclonal antibodies against cariogenic organisms, using B lymphocytes recovered from heavily

infected patients and cloning the human genes encoding for these antibodies; or 4) immunizing human B lymphocytes and spleen cells *in vitro* using purified cariogenic organisms, followed by fusion to form hybriclomas to create immortal cell lines. The techniques required are known to those skilled in the art.

In the presently preferred embodiment of the invention, mouse monoclonal antibodies are "humanized." Using the PCR or Southern blot technique, DNA fragments encoding the variable domains of mouse hybridomas secreting antibody specific to cell surface antigens of cariogenic organisms are isolated. Using gene cloning techniques, the variable regions of human IgG and IgM immunoglobulin are replaced with the corresponding mouse variable regions or CDR's. The result of this genetic engineering is a chimeric antibody molecule with variable domains that selectively bind to surface antigens of cariogenic organisms, but which interacts with the human immune system through its constant regions to trigger a humoral immune response.

3. Administration of Monoclonal Antibodies

In order to prepare a sufficient quantity of monoclonal antibodies for clinical use, the desired cell line transfected with IgG or IgM encoding sequences must be propagated. Existing technology permits large scale propagation of monoclonal antibodies in tissue culture. The transfected cell lines will secrete monoclonal antibodies into the tissue culture medium. The secreted monoclonal antibodies are recovered and purified by gel filtration and related techniques of protein chemistry.

In experimental studies, monoclonal antibodies to *S. mutans* have been applied directly to the surface of teeth. Application by ingestion of mouthwash, or by chewing gum has also been proposed. A presently preferred alternative is to express the monoclonal antibodies of the present invention in edible plants, such as banana or broccoli. Eating plants transformed in accordance with this invention will result in application of the antibodies to cariogenic organisms present on tooth surfaces, and elsewhere in the mouth. It is also contemplated that other organisms,

both plant and animal may be transformed to express the monoclonal antibodies described herein, so that such antibodies may be ingested, for example, by drinking milk.

Examples

1. Producing mouse monoclonal antibodies against *S. mutans*

Type c *S. mutans* strain ATCC25175 are grown to log phase in BHI medium and washed twice with phosphate buffered saline, pH 7.2 (PBS), by centrifugation at 3000xg for 5 min. The pellet is resuspended in 1% formalin/0.9% NaCl, mixed at room temperature for 30 min and washed twice with 0.9% NaCl. BALB/c mice (8-10 weeks) are immunized intraperitoneally with 100 Fl of the antigen containing approximately 10^8 whole cells of formalinized intact *S. mutans* bacteria emulsified with Freund's incomplete adjuvant (FIA). After 3-5 weeks, mice will receive a second dose of antigen (10^8 whole cells of bacteria in FIA). Three days prior to fusion, the mice are boosted intravenously with 10^8 whole cells in saline.

The standard tissue culture media is RPMI 1640 (Gibco) medium supplemented with 2 mM L-glutamine, 1mM sodium pyruvate, and 10 mM HEPES and containing 100 μg/ml penicillin and 100 / μg/ml streptomycin with 10% fetal calf serum. Hybrids are selected in media containing HAT (100μg Hypoxanthine, 0.4. μM Aminopterin; 16 μM Thymidine). HT (100 μg Hypoxanthine; 16 μM Thymidine) is maintained in the culture medium for 2 weeks after aminopterin is withdrawn. OPI (1 mM oxaloacetate, 0.45 mM pyruvate and 0.2 U/ml bovine insulin) is added as an additional growth factor to the tissue culture during cloning of hybriclomas. Hybriclomas are raised according to the procedure reported by Liddell and Cryer (A Practical Guide to Monoclonal Antibodies, John Wiley & Sons, Chichester, England, 1991). The NSI/A94.1 mouse myeloma cell line is used as the fusion partner and grown in spinner cultures in 5% C02 at 37°C and maintained in log phase of growth prior to fusion.

The following approach is used for screening for species-specific monoclonal antibodies against *S. mutans*. The initial screening is performed using an ELISA assay, which selects for the culture supernatants containing antibodies that bind to *S. mutans*. Formalinized bacteria are diluted in PBS to

OD_{600} = 0.5, and added to duplicate wells (100μl) in 96 well PVC ELISA plates preincubated for 4 h with 100 μl of 0.02 mg/ml Poly-L-lysine. These antigen-coated plates are incubated overnight at 4°C in a moist box then washed 3 times with PBS and blocked with 0.5% fetal calf serum in PBS and stored at 4°C. 100 μl of mature hybridoma supernatants are added to the appropriate wells of the antigen plates, incubated for 1 h at room temperature, washed 3 times with PBS-0.05% tween 20, and bound antibody is detected by the addition of polyvalent goat-anti-mouse IgG antibody conjugated with alkaline phosphatase diluted 1:1000 with PBS-1% fetal calf serum. After the addition of the substrate, 1 mg/ml p-nitrophenyl phosphate in carbonate buffer (15 mM Na_2CO_3, 35 mM NaH_2CO_3, 10 mM $MgCl_2$ pH 9.6), the color developments after 15 min is measured in an EIA reader at 405 nm. The positive supernatants (3 fold higher than control) are then subjected to the immunoprecipitation assay (mixing 1 00μl bacteria with 100μl supernatant) to screen for those with strong positive reactivity with *S. mutans*. The deposited clones were prepared according to this method.

2. Generating mouse/human chimeric genes which encode humanized monoclonal antibodies against *S. mutans*.

Described here is one of the ways to humanize mouse monoclonal antibodies. Genomic DNA of mouse hybridoma cell lines is isolated using the QIAamp system (Qiagen, Valencia, CA). After digestion with various restriction enzymes, DNA fragments are fractionated through 0.8% agarose gel by electrophoresis and transferred to a nitrocellulose membrane. Southern blotting is performed to identify the immunoglobulin gene. The heavy chain gene is probed with a DNA fragment from a mouse IgG heavy-chain gene that includes the J3 and J4 segments and the enhancer region. The light chain gene is probed with a DNA fragment from a mouse IgG light-chain gene containing J1-5 segments.

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(Stratagene) to construct heavy- and light-chain libraries of these mouse hybridomas in lambda phage. The libraries are screened with heavy- and light-chain J-region probes as mentioned above. DNA of the positive clones is isolated, subcloned and sequenced. To achieve the best accuracy, both sense and antisense strands are sequenced. BLAST search is employed to translate the nucleotide sequence into the amino acid sequence and compare it with the existing antibody genes. The variable region of the heavy-chain is identified, subcloned and inserted into an expression vector which contains a DNA fragment encoding the human IgG heavy chain constant region and the *Ecogpt* gene providing resistance to mycophenolic acid. The variable region of the light-chain is also identified, subcloned and inserted into another expression vector which includes a DNA fragment encoding the human IgG light chain constant region and the neo gene giving resistance to G418.

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The heavy and light chain of a human IgG gene are separately introduced or cotransfected into an animal cell line (such as SP2/0) using a lipofection reagent (BRL, Grand Island, NY). The transfected cells are incubated at 37°C in a 5% C02 atmosphere in 1x zinc option medium for 24 h and then in medium containing 10% fetal bovine serum. After 48 h incubation, the cells are transferred to a microtiter plate and grown in selection medium containing G418 and mycophenolic acid. The supernatants of drug-resistant cells are collected and screened for immuno-reactivity against *S. mutans* using the ELISA or precipitation assays mentioned above.

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The DNA fragments encoding the heavy and light chains of a human IgG gene are separately cloned into a Ti plasmid of *Agrobacterium tumefaciens*. The plasmid contains a promoter to express human heavy and light chains of IgG in *Arabidopsis thaliana*, an antibiotic marker for selection in *Agrobacterium tumefaciens* and an herbicide resistance gene for transformation selection in *Arabidopsis*. An *Agrobacterium tumefaciens* strain is transformed

with these plasmids, grown to late log phase under antibiotic selection, and resuspended in infiltration medium described by Bethtold et al. (C.R. Acad. Sci. Paris Life Sci. 316:1194-1199, 1993).

Transformation of *Arabidopsis* by Ti-plasmid containing *Agrobacterium tumefaciens* is performed through vacuum infiltration. Entire plants of *Arabidopsis* are dipped into the bacterial suspension. The procedure is performed in a vacuum chamber. Four cycles of 5 min vacuum (about 40 cm mercury) are applied. After each application, the vacuum is released and reapplied immediately. After infiltration, plants are kept horizontally for 24 h in a growth chamber. Thereafter, the plants are grown to maturity and their seeds are harvested. The harvested seeds are germinated under unselective growth condition until the first pair of true leaves emerged. At this stage, plants are sprayed with the herbicide Basta at concentration of 150 mg/l in water. The *aribidopsis* plants containing transformed Ti plasmids are resistant to the herbicide while the untransformed plants are bleached and killed. Such a selection continues to the second generation of the plants. For the resistant plants, total genomic DNA is isolated and probed with the DNA fragments encoding heavy and light chains of the IgG gene. The plant extracts from the positive transformants are prepared and screened for the expression of human IgG protein with Western blot using antibodies against heavy and light chains of constant regions of human IgG.

The plants expressing human IgG heavy chain are sexually crossed with plants expressing human IgG light chain to produce progeny expressing both chains. Western blotting is used to screen the both heavy and light chains. Extracts from positive transformants are collected and screened for immuno-reactivity against *S. mutans* using the ELISA or precipitation assays mentioned above.

4. Using human or humanized monoclonal antibodies against *S. mutans* to treat or prevent human dental caries

With the successful completion of the above studies, humanized monoclonal antibodies against *S. mutans* are obtained. The plant tissue is tested for efficacy.

Plant tissue extracts containing monoclonal antibodies to *S. mutans* are mixed with various concentrations of *S. mutans* in the presence and absence of purified human complement components or purified human polymorphonuclear neutrophilic leukocytes. After a two hour incubation, the mixtures are plated onto BHI plates to examine the bactericidal activity.

Using the artificial plaque formation system developed by Wolinsky et al. (J. Dent. Res. 75:816-822, 1996), plant tissue extracts containing monoclonal antibodies are used to examine the ability of the expressed monoclonal antibodies ability to kill *S. mutans* in saliva or in existing dental plaques on artificial dental enamel. Analogous techniques are used to examine the ability to prevent the formation of dental plaques.

Human clinical trials are performed using these monoclonal antibodies produced through animal cells or plants. Human volunteers are treated with or without these human monoclonal antibodies against *S. mutans*. Then the level of *S. mutans* in saliva and in dental plaques is examined. The correlation between present and future dental caries in relationship with treatment of monoclonal antibodies is also examined.

It should be understood that the foregoing examples are for illustrative purposes only, and are not intended to limit the scope of applicants' invention which is set forth in the claims appearing below.

CLAIMS

What is claimed is:

1. A method for treatment and prevention of dental caries in a mammal comprising oral administration of a monoclonal antibody that specifically binds to a cariogenic organism, and which elicits a humoral immune response from the mammal, wherein the monoclonal antibody is derived from a species other than the mammal to be treated.

2. The method for treatment and prevention of dental caries of claim 1 wherein the monoclonal antibody is produced by the steps of:

a) inoculating a mammalian host with at least one cariogenic organism;

b) identifying hybridomas from the mammalian host that secrete antibodies specific to surface antigens of at least one cariogenic organism; and

c) preparing a chimeric monoclonal antibody comprising complementarity-determining regions from the monoclonal antibody of step b) above and a constant domain from the mammal to be treated.

3. The method for treatment and prevention of dental caries of claim 2 wherein the step of preparing further comprises synthesis of a nucleic acid construct comprising:

a) a nucleic acid sequence that codes on expression for a complementarity determining region of the monoclonal antibody secreted by the hybridomas derived from the mammalian host of claim 2 above; and

b) a nucleic acid sequence that codes on expression for a constant region of an antibody selected from the group of class IgG and class IgM of the mammal to be treated.

4. The method for treatment and prevention of dental caries of claim 3 wherein the chimeric monoclonal antibody is expressed by a eukaryotic host that has been transformed with the nucleic acid construct of claim 3 above.

5. The method for treatment and prevention of dental caries in a mammal of claim 4, wherein the monoclonal antibody is administered by oral ingestion of tissue from a eukaryotic host transformed with the nucleic acid construct of claim 4 above.

6. The method for treatment and prevention of dental caries of claim 1 wherein the mammal to be treated is man, and the other species is mouse.

7. A method for treatment and prevention of dental caries in a mammal comprising administration of a monoclonal antibody that specifically binds to a cariogenic organism, and which elicits a humoral immune response from the mammal.

8. The method for treatment and prevention of dental caries of claim 7 wherein the monoclonal antibody is produced by the steps of:

a) inoculating a mammalian host with at least one cariogenic organism;

b) identifying hybridomas from the mammalian host that secrete antibodies specific to surface antigens of at least one cariogenic organism; and

c) preparing a chimeric monoclonal antibody comprising complementarity-determining regions from the monoclonal antibody of step b) above and a constant domain from a mammal to be treated.

9. The method for treatment and prevention of dental caries of claim 8 wherein the step of preparing further comprises preparation of at least one nucleic acid construct that includes:

a) a nucleic acid sequence that codes on expression for a complementarity determining region of the monoclonal antibody secreted by the hybridomas derived from the mammalian host of claim 2 above; and

b) a nucleic acid sequence that codes on expression for a constant region of an antibody selected from the group of class IgG and class IgM of the mammal to be treated.

10. The method for treatment and prevention of dental caries of claim 9 wherein the chimeric monoclonal antibody is expressed by a eukaryotic host that has been transformed with the nucleic acid construct of claim 9 above.

11. The method for treatment and prevention of dental caries in a mammal of claim 9, wherein the monoclonal antibody is administered by oral ingestion of tissue from a eukaryotic host that has been transformed with the nucleic acid construct of claim 9 above.

12. The method for treatment and prevention of dental caries of claim 8, wherein the mammalian host is a mouse, and the mammal to be treated is man.

13. The method for treatment and prevention of dental caries of claim 5 wherein the eurkaryote is a plant.

14. The method for treatment and prevention of dental caries of claim 5 wherein the eurkaryote is a plant of the species *Brassica*.

15. The method for treatment and prevention of dental caries of claim 11 wherein the eurkaryote is a plant.

16. The method for treatment and prevention of dental caries of claim 11 where the eurkaryote is a plant of the species *Brassica*.

17. The method for treatment and prevention of dental caries of claim 8, wherein the mammal to be treated is a dog or a cat.

【国際調査報告】

INTERNATIONAL SEARCH REPORT

ational Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/40 A61P31/04 A61K7/16 //C07K16/12,C07K16/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHARMA ARUN K ET AL: "Transgenic plants for the production of edible vaccines and antibodies for immunotherapy." CURRENT SCIENCE, vol. 77, no. 4, 25 August 1999 (1999-08-25), pages 524-529, XP001002438 Bangalore abstract page 524, right-hand column, paragraph 2 -page 525, left-hand column, paragraph 2 page 527, left-hand column, paragraph 3 -right-hand column, paragraph 1 page 528, left-hand column, paragraph 2 -right-hand column, paragraph 1 --- -/--	1-13,15

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

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"E" earlier document but published on or after the international filing date
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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
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4B064 AG27 CA02 CA10 CA11 CA19 CA20 CC24 DA01
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4H045 AA30 CA11 DA76 EA20 FA71 FA72 FA74