厚生労働科学研究費補助金（医療技術実用化総合研究事業（臨床研究推進研究事業））
分担報告書

形成外科手術後の患者での皮膚創傷治癒に及ぼす補中益気湯の効果（予備的検討）

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研究要旨
補中益気湯の自然免疫ならびに栄養状態に及ぼす影響について研究における症例数などの決定のため、パイロット研究目的で少数例での補中益気湯の効果を検討した。研究の期間が短期間のため創部の感染の有無は問わなかった。その結果、感染を有した形成外科患者６名（男女各３名、平均年齢60歳±21歳）に補中益気湯7.5g分２～３で投与した。その結果３名で内服後気分不快があり中止した。残り３名は継続可能で、２名で創部の閉鎖ができた。さらに１名MRSA陰性化し、感染兆候がなかった。栄養状態は、継続可能症例のうち２名のアルブミンは正常範囲であった。１例は軽度低下していたが、創部の治癒後は正常化した。補中益気湯が内服可能例では、創傷治癒に好影響を及ぼす可能性が示唆された。

A.研究目的
「補中益気湯の自然免疫ならびに栄養状態に及ぼす影響について」の研究における症例数などの決定のため、パイロット研究目的で少数例での補中益気湯の効果を検討した。補中益気湯の投与期間が短期間のため、症例において創部の感染の有無は問わなかった。

B.研究方法
対象：感染などで創傷治癒の遅延した形成外科患者６名（男女各３名、平均年齢60歳±21歳）である。
方法：補中益気湯一日量 7.5g/日分２～３で投与した。内服期間は主治医が必要するまでとし制限は設けなかった。

（倫理面への配慮）
本研究にあたっては、「ヘルシンキ宣言」ならびに厚生労働省の「疫学研究に関する倫理指針」（平成14年６月14日策定、平成20年12月１日一部改訂）を遵守し行った。

C.研究結果
補中益気湯を６名に投与したところ、３名で内服後気分不快があり中止した。残り３名は継続可能で、２名が創部の閉鎖することができた。さらに１名MRSA陰性化し、２名では感染兆候は起こらなかった。
栄養状態は、継続可能症例のうち２名のア

ルブミンは正常範囲であった。1 例は軽度低下していたが、創部の治癒後は正常化した。

投与継続例を紹介する。

1）88 歳女性　MRSA 感染例。

X 年 1 月 14 日第 8，第 9 肋軟骨移植。感染なし。鼻背再建目的に頭頂骨を胸にバンキング中。鼻前頭縫合部に術後感染し，1 月 19 日より補中益気湯内服（7.5ｇ分２）。MRSA 検出され VCM、MEPM 併用。画像では膿瘍腔など明らかではない。1 月 24 日には感染した骨とプレートのみ摘出し、感染していない骨はそのまま留置しておいた。その後感染の再燃なく 2 月 5 日退院。外来においても引き続き内服し，2011 年 10 月 5 日に 30 日分処方終了した。その後転移性肝癌の疑いで当科追加手術は立ち消えとなっていたが、精査の末転移性肝癌の可能性は低いとのことで、X+1 年 4 月に修正手術予定。

2）44 歳女性：瘻孔形成

全外鼻再建用 expanded forehead flap 作成のため、額部にエキスパンダーを入れて膨らませている最中に，X 年 11 月 24 日より、皮膚の菲薄化のため瘻孔形成したため、X 年 11 月 26 日から、次の手術の X 年 12 月 21 日まで、抗生物質（フロモックス）内服と併用で補中益気湯（7.5ｇ分２）を内服した。培養は MSSA であったが、速やかに膿は漿液性となり、その後感染兆候は出なかった。瘻孔は閉鎖には至らなかった。

3）53 歳男性：瘻孔が自然閉鎖した耳介欠損に対し肋軟骨移植手術した例。

主訴は右耳介後部の皮下腫瘤。既往歴は特になし。

X-2 年 1 月頃より右耳介後部の皮下腫瘤を自覚した。近医受診し、徐々に増大したため、X-2 年 9 月精査加療目的で近医師より紹介受診となった。X-2 年 10 月腫瘤より生検施行。悪性腫瘍のため同月腫瘍切除を行った。切除後に耳介の一部残した。病理組織で断端が陰性であるのを確認後、僧帽筋皮弁で再建術を施行。術後経過は良好で 11 月末に退院となった。X-1 年 4 月（2 回目入院）耳鼻科で耳介切除された。再建後瘢痕拘縮があり形成外科で、耳介再建のため数回の手術施行。しかし X 年 4 月感染を生じた。小指頭大の皮下硬結で圧痛や熱感なし。全身の発熱なし。その後の耳起こし手術を X 年 2 月 18 日に施行後、５月 11 日に瘻孔を形成した。培養は MSSA。X 年５月補中益気湯 7.5ｇ分 3 と感受性のあるクラビットの内服開始。１週間後排膿は著減した。2 週後滲出少なくなっているが傷口はふさがりきってはいない。1 か月後の 6 月 8 日には瘻孔閉鎖した。6 月 22 日から補中益気湯のみ継続した。再建後の軟骨が残されている場合再発が考えられ、さらに 1 か月内服した。その後患者の強い要望で内服中止したが、その後の瘻孔再発を認めなかった。栄養状態は、アルブミン 4.2 g/dl と良好な方であった。この様な症例では難治の場合が多く、移植軟骨の摘出になることが多い。軟骨の摘出せずに傷が治癒した珍しい症例であったとの形成外科専門医の指摘もあった。

D. 考察

皮膚の創傷治癒は皮膚の損傷部位の生体の防御と修復反応である。これは圧力のかかることで生じる褥瘡、代謝の異常を生じさせる糖尿病、後天的な末梢動脈疾患や静脈還流不全などにより慢性化するといわれる。このような慢性創傷は高齢化、生活習慣病の蔓延に起因し、増加しており、外科的手技がいかに優れていても、それだけでは治癒に導くことができない。それに対して、手術後の創傷遅延は全身状態の評価・改善、基礎疾患の治療・ケアは、手術の検討時点で良好な状態になっているため、生じにくいはずである。しかし、実際は感染・遺物の残存などの急性な要因で創傷治癒が遅延する。また皮膚関連での創傷治癒が生

じやすいのは、高齢であるので年齢が高い患者ほど危険は高い。３名のうち、１名は８０歳台の高齢者であったが、その方でも、補中益気湯内服で改善した。また、、最後の症例の移植軟骨の例も経過として、軟骨の摘出を免れ、補中益気湯内服での治療効果がもっとも高かった印象であると専門医が述べている。補中益気湯内服が有用である可能性がある。
今回のパイロット研究の問題点としては、症例が少なくエビデンスとしては弱い点である。さらに感染改善には標準治療では抗生物質の併用が必須であるため、漢方薬の使用はあくまで、併用となる点である。そのため、効果が漢方薬の効果かの判定が難しい。そのため対照群の設定が重要で症例数が必要である。また、もう一つの問題点は補中益気湯の内服で半数の者が内服後の気分不快を訴えたため内服継続できず脱落した点がある。これらの点をどうするかを検討する必要性が判明した。術後患者の多くは、手術侵襲自体で全身状態が低下するので、その点では、補中益気湯の使用目標である「体力がおとろえて、元気がなく、衰弱の 傾向があり、食欲不振、倦怠、頭痛、悪寒、自汗、身熱、微熱などのあるものに用いる」に合致するものが多い。このため、脱落例があるが対照群を作ることで治療効果の有無を判定することが可能と考えられた。

E. 結論

形成外科患者に補中益気湯エキスを投与し、少数例で観察検討した。その結果、補中益気湯エキス内服持続できたものでは、創傷治癒に好影響を及ぼす可能性が示唆された。

F. 健康危険情報

総括研究報告書を参照

G. 研究発表

1. 論文発表　なし

2. 学会発表　なし

H.　知的財産権の出願・登録状況

1. 特許出願

なし

2. 実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金（医療技術実用化総合研究事業（臨床研究推進研究事業））
分担報告書

漢方薬によるワクチンアジュバント効果の検討と臨床応用

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日高　隆雄　同上　准教授
研究協力者　中島　彰俊、島　友子、同上

研究要旨
HPV ワクチン接種を行う女性を対象に、漢方薬が副作用軽減作用および抗体産生のアジュバント効果を持つかを検討中である。対象は 20 代の一般女性 40 人であり、無作為に十全大補湯エキス投与群と非投与群を割り付けし、ワクチン接種を行っている。調査項目は、症状日記を用いて副反応の軽減の有無：局所（注射部位）の特定した症状（疼痛、発赤、腫脹）および全身性の特定した症状（疲労、筋肉痛、頭痛、胃腸症状、関節痛、発疹、発熱、蕁麻疹）を調査し、血液検体を用いて生化学検査（BUN、Cre、GOT、GPT、γ-GTP）および免疫学的検査（HPV 抗体価）の測定を行う。現在 20 名が 2 群に分かれ投与を開始し、残り 20 名の参加を開始するところである。今後、漢方薬による副作用への影響および抗体産生へのアジュバンド効果を評価を行う予定である。

A.研究目的
「漢方薬によるワクチンアジュバント効果の検討と臨床応用」において、今回我々は、HPV ワクチン接種を行う女性を対象に、漢方薬が副作用軽減作用および抗体産生のアジュバント効果を持つかを検討する。HPV ワクチン（サーバリックス）は子宮頸癌の多くみられるHPV16,18型の2種類の感染予防に用いられ、子宮頸癌撲滅のため自治体等により HPV 接種費用の助成も行われている。しかし、10%以上の接種者に注射部位の発赤・痛みや、筋肉痛・関節痛を起こし、発疹・じんましん等を起こすことも知られている。そこで、HPV ワクチン接種を行う女性を対象に漢方薬を投与し、その副作用の軽減効果と抗体産生のアジュバント効果を検討する。

B.研究目的
1）対象は富山大学附属病院に関係する 20 代の一般女性とし、接種後の妊娠例は除外した。
2)対象を無作為に十全大補湯エキス投与群（一日量 7.5g/日）と非投与群の 2 群に分け、図 1 の如く実施した。
3）調査項目は、副反応の軽減の有無：局所（注射部位）の特定した症状（疼痛、発赤、腫脹)および全身性の特定した症状(疲労、筋肉痛、頭痛、胃腸症状、関節痛、発疹、発熱、蕁麻疹）に関して症状日記（別紙参

照）を被験者に記載してもらう形で調査する。血液検体は生化学検査（BUN、Cre、GOT、GPT、γ-GTP）および免疫学的検査（HPV 抗体価）の測定に用いた。
4）調査項目は、mean±S.D.で表し、有意差検定は投与群と非投与群間で repated measure ANOVA を用いておこない $P<0.05$ を有意と判定した。

（倫理面への配慮）
本研究は基礎研究であり、動物倫理として富山大学実験動物研究規則に準じて実施した。

C.研究結果
1）十全大補湯と非投与群の比較
参加者は十全大補湯エキス投与群 10 名（平均年齢 24.3±3.1 才）、非投与群 10 名（平均年齢 23.9±2.2 才）を開始しており、現在さらに各群 10 名ずつの参加を募っているところである。十全大補湯内服群は内服開始後 1.5 か月を経過しているが、現在のところ内服中止例は出ていない。非投与群の 2 名が妊娠のため、除外となっている。症状については記載中であり、副作用発現の解析は今後行う予定である。
2）抗体産生のアジュバント効果について
抗体投与前後で血清のストックを行っている。抗体測定のキットはコマーシャルベースには販売されていないため、国内で HPV 抗体価測定系が利用可能になった段階で、ストックした血清にて HPV 抗体価を測定し、第 1 群（コントロール群）および第 2 群（漢方内服群）で比較検討を予定している。

D.結論
HPV ワクチン接種は健常人女性に行うことから、漢方薬における副作用軽減作用は早期の評価項目として最も重要であり、本年度内にその結果は判明する予定である。一方で、海外での臨床試験によれば高い抗体価は 6.4 年維持されることは判明しているが、その効果が何年続くかは不明である。しかしながらワクチン効果の持続が最も重要な点であり、本研究では 3 年後までのフォローアップを行う予定としているが、その後の長期的なフォローアップを行うことも可能な点が、本研究の強みであると考えている。

E.結論
HPV ワクチン接種を行う健常人女性への副作用軽減調査および今後の長期フォローアップを行う体制が整い、現在実施中である。
F.健康危険情報
総括研究報告書を参照

G.研究発表
1. 論文発表
なし
2. 学会発表
1）なし

H. 知的財産権の出願・登録状況
1.特許出願
なし
2.実用新案登録
なし
3.その他
なし

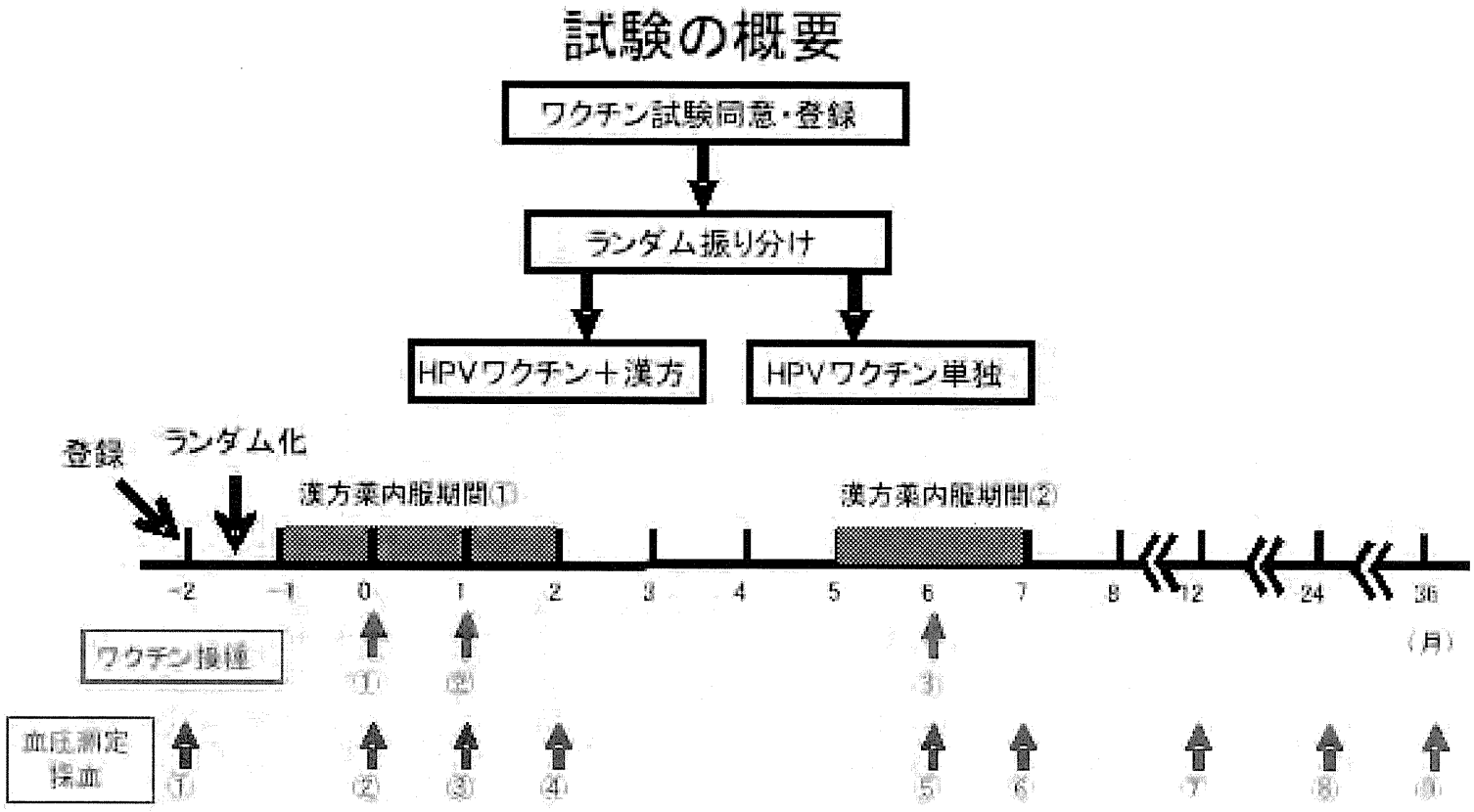

図1：ワクチンおよび漢方薬投与スケジュール

厚生労働科学研究費補助金（医療技術実用化総合研究事業（臨床研究推進研究事業））
分担報告書

漢方薬のインフルエンザワクチンアジュバント療法に関する臨床試験の計画及び統計解析に関する研究

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研究要旨
高齢者に対する漢方薬のインフルエンザワクチンアジュバント効果を検討したランダム化比較試験(N=38)を題材とし、漢方薬のアジュバント効果について検討した。比較した群は、補中益気湯（H群）、十全大補湯（J群）、そして非漢方薬群（N群）である。対象株はA/H1N1、A/H3N2、そしてB/H1の3種とした。有効性の評価指標としては、1）抗体価陽性率（40以上を陽性と定義）、2）陽転率（接種前抗体価<10かつ接種後抗体価が40以上、あるいは抗体価の上昇率が4倍以上で陽転と定義）の2種類を検討した。どちらも同様の結果であり、A/H3N2株に関して漢方薬のアジュバント効果は示唆

A. 研究目的

高齢者を対象とし、漢方薬（補中益気湯、十全大補湯）のインフルエンザワクチンアジュバント効果を検討するため、ランダム化比較試験が実施された。本研究の目的は、2種類の評価指標に関して、漢方薬のアジュバント効果を検討することである。

B. 研究方法

研究データは、高齢者に対するランダム化比較試験データである。補中益気湯6例、十全大補湯13例、非漢方薬群19例、全部で38例のデータを使用した。ランダム化であるにもかかわらず、症例数にインバランスを生じた理由は、補中益気湯を選択した施設では補中益気湯と非投与群を比較し、十全大補湯を選択した施設では十全大補湯と非投与群を比較したためである。すなわち、前者の施設では6例対6例（補中益気湯、非投与）の割付、後者の施設では13例対13例（十全大補湯、非投与）の割付となったためである。

対象株はA/H1N1、A/H3N2、そしてB/H1の3種を取り上げた。

有効性の評価指標としては、1）抗体価陽性率（40以上を陽性と定義）、2）陽転率（接種前抗体価<10かつ接種後抗体価が40以上、あるいは抗体価の上昇率が4倍以上で陽転と定義）の2種類を検討した。

（倫理面への配慮）

本研究は富山大学倫理委員会の承認済みの臨床研究情報を用いて遂行した。

C. 研究結果

図1に、インフルエンザA/H1N1株に関する抗体陽性率の推移を示した。Hは補中益気湯6例、Jは十全大補湯13例、Nは非

漢方薬群 19 例である。4～12 週にかけて、抗体陽性率は 3 群で違いは見られなかった。

図２は A/H3N2 株に関する結果である。8 週時に、漢方薬投与群で抗体陽性率が高い傾向が見られた（H: 46%, J: 33%, N: 16%)。漢方薬群での抗体陽性率は 42%（8/19)、非投与群では 16%（3/19）であった（P=0.074)。非有意ではあったが、抗体陽性率は２倍以上、漢方薬投与群で高かった。しかしながら、12 週時にはその差は縮まっていた（漢方薬群 21%, 非投与群 16%, P=0.59)。

図３は B/H1 株に関する結果である。これについては 3 群間で違いは見られなかった。

図４は、A/H1N1 株に関する抗体価陽性率の推移である。3 群の間にほとんど違いは認められなかった。接種前から陽性率が約 20%も見られ、4 週時には約 65%まで陽性率が上昇し、12 週にかけて約 10%低下している傾向が見られた。

図５は A/H3N2 株に関する結果である。4 週にかけて 3 群とも抗体価は上昇するも、漢方薬非投与群では 8～12 週にかけて著しく抗体価が下降する傾向が見られた。4 週から 8 週にかけての陰性反転率（4 週時に陽性だが、8 週時に陰性へ変化）をみると、漢方薬群では 10%（1/10)、非投与群では 30%(3/10)であった。すなわち、非投与群では一度抗体価は上がるが、それを維持しにくい傾向が見られた。ここでも 3 倍（10%対 30%）の違いがあったにもかかわらず、症例数が少なかったため統計学的には有意ではなかった（P=0.26)。

図６は B/H1 株に関する結果である。これについては 3 群間で違いは見られなかった。

D．考察

インフルエンザ株により、漢方薬のアジュバント効果には違いが見られた。予想通り、A/H3N2 株で漢方薬の効果が示唆される結果が得られたものの、症例数が少なすぎたため、それを立証する検出力に欠けていた。今後は、さらに症例数を増やしたうえで検証する必要があると思われる。
漢方薬は補中益気湯と十全大補湯を用いたが、わずかながら補中益気湯のほうでアジュバント効果は強いと示唆されたが、漢方薬の選択は施設により異なっていたため、施設の違いなのか、それとも漢方薬の種類の違いなのかは判別つかない。

E．結論

高齢者を対象として、漢方薬のインフルエンザワクチンアジュバント効果を検討したランダム化比較試験データを用いて、2 種類の評価指標に関して検討した。抗体価陽性率についても、陽転率についてもほぼ同様の結果であり、抗体価陽性化を高める漢方薬の効果は見られなかった。しかしながら、陽転を維持できるか否かについて、漢方薬を投与したほうが陽転維持効果の高い傾向が見られた。しかしながら、症例数が少なかったため、適切な検出力をもった臨床試験を今後実施することが望まれる。

F. 健康危険情報

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G. 研究発表

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2. 学会発表　なし

H.　知的財産権の出願・登録状況

1. 特許出願

なし

2. 実用新案登録

なし

3. その他

なし

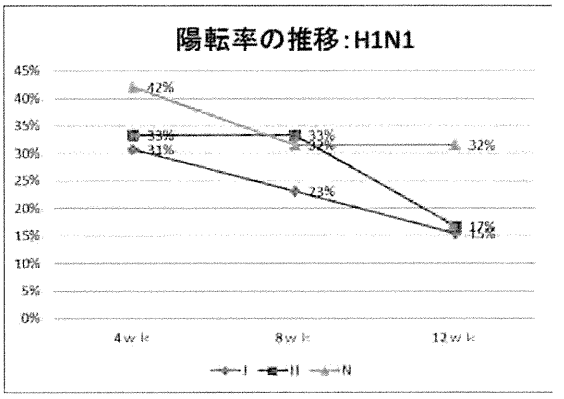
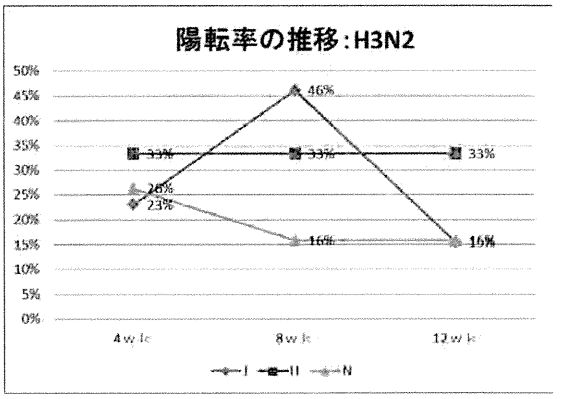


図１．A/H1N1株に関する陽転率の推移



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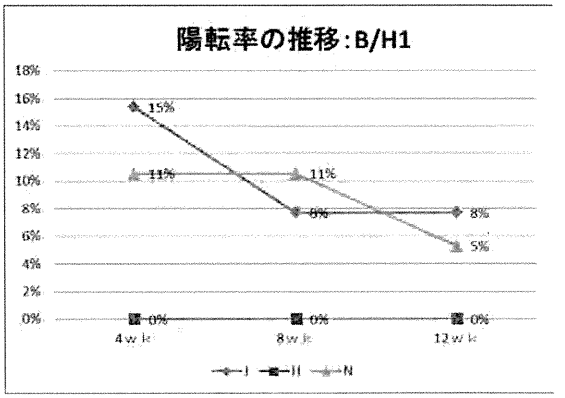

図３．B/H1株に関する陽転率の推移

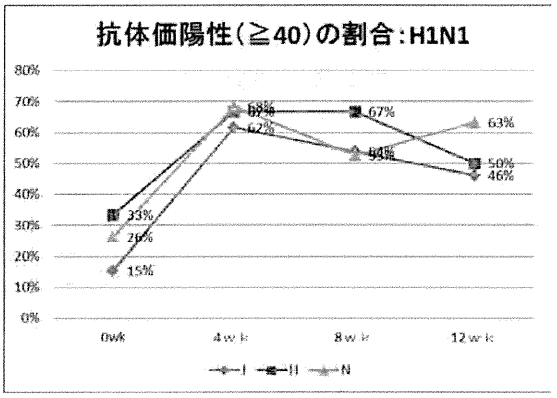

図４．A/H1N1株に関する抗体価陽性率の推移

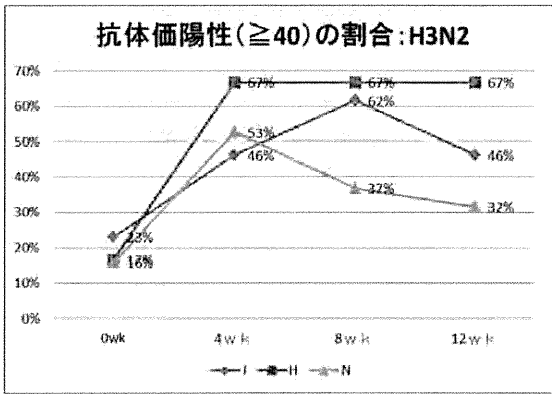

図５．A/H3N2株に関する抗体価陽性率の推移

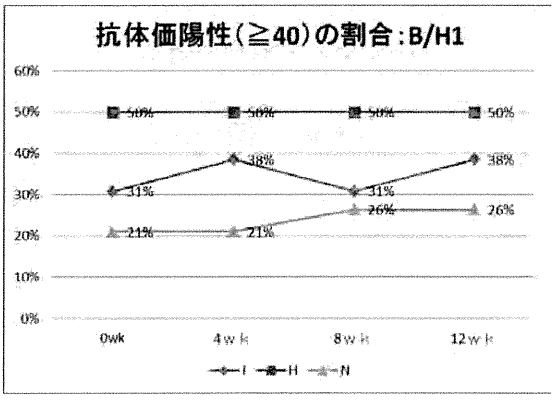

図６．B/H1株に関する抗体価陽性率の推移

研究成果の刊行に関する一覧表

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Review

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EXPERT REVIEWS

Novel vaccine development strategies for inducing mucosal immunity

Expert Rev. Vaccines 11(3), 367–379 (2012)

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To develop protective immune responses against mucosal pathogens, the delivery route and adjuvants for vaccination are important. The host, however, strives to maintain mucosal homeostasis by responding to mucosal antigens with tolerance, instead of immune activation. Thus, induction of mucosal immunity through vaccination is a rather difficult task, and potent mucosal adjuvants, vectors or other special delivery systems are often used, especially in the elderly. By taking advantage of the common mucosal immune system, the targeting of mucosal dendritic cells and microfold epithelial cells may facilitate the induction of effective mucosal immunity. Thus, novel routes of immunization and antigen delivery systems also show great potential for the development of effective and safe mucosal vaccines against various pathogens. The purpose of this review is to introduce several recent approaches to induce mucosal immunity to vaccines, with an emphasis on mucosal tissue targeting, new immunization routes and delivery systems. Defining the mechanisms of mucosal vaccines is as important as their efficacy and safety, and in this article, examples of recent approaches, which will likely accelerate progress in mucosal vaccine development, are discussed.

Keywords: delivery system • mucosal adjuvant • secretory IgA

Mucosal immune system

The mucosal immune system can be separated into inductive and effector sites based on the anatomical and functional properties. The migration of immune cells from mucosal inductive to effector tissues is the cellular basis for the common mucosal immune system (CMIS) (Figure 1). Thus, mucosal vaccination elicits immune responses in distant, multiple mucosal effector sites [1–5]. Mucosal inductive sites, including gut-associated lymphoreticular tissue (GALT) and nasopharyngeal-associated lymphoreticular tissue (NALT), collectively comprise a mucosa-associated lymphoreticular tissue (MALT) network for provision of a continuous source of memory B and T cells to mucosal effector sites [1,3–5]. The MALT contains T-cell zones, B cell-enriched areas containing a high frequency of surface IgA-positive ($sIgA^+$) B cells and a subepithelial area with APCs for the initiation of specific immune responses. The MALT is covered by a follicle-associated epithelium that consists of a subset of differentiated microfold (M) epithelial cells, columnar epithelial cells and lymphoid cells, which play a central role in the initiation of mucosal immune responses. M cells take up antigens (Ags) from the lumen of the intestinal and nasal mucosa and transport them to the underlying APCs, including dendritic cells (DCs). In addition, recent studies have now identified isolated lymphoid follicles (ILFs) in the mouse small intestine. The ILFs have been identified as a part of GALT and as such are a mucosal inductive tissue [6,7]. These ILFs mainly contain B cells, DCs and M cells in the overlying epithelium. In addition, most recent studies showed that tear duct-associated lymphoreticular tissue (TALT) and conjunctiva-associated lymphoreticular tissue (CALT) play a role as mucosal inductive tissues [8,9]. Mucosal effector sites, including the lamina propria regions of the GI, the upper respiratory (UR), and reproductive tracts, secretory glandular tissues and intestinal intraepithelial lymphocytes, contain Ag-specific mucosal effector cells such as IgA-producing plasma cells and B and T cells.

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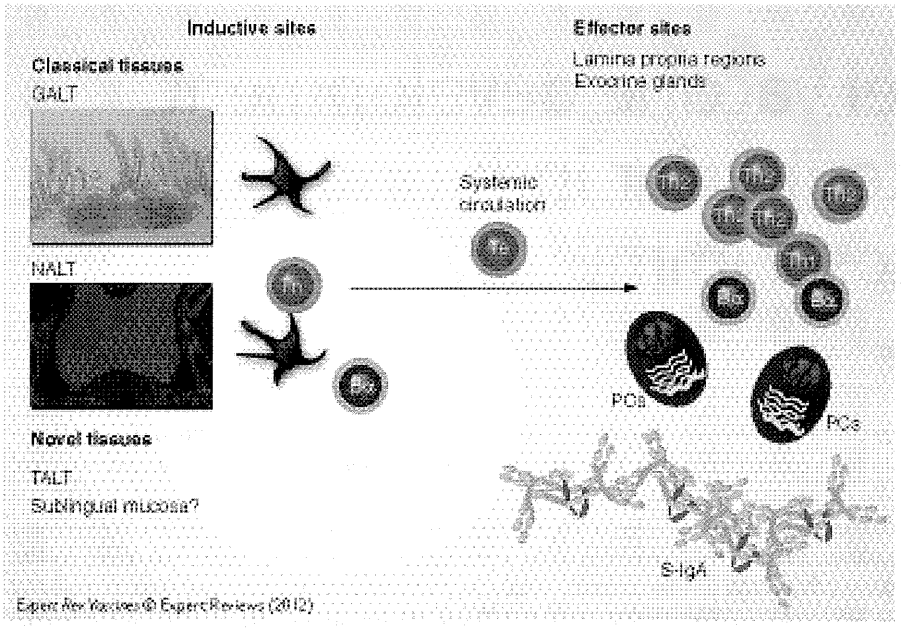

Figure 1. Concept of mucosal inductive and effector sites: when mucosal immunization is initiated, Ags are taken up by mucosal inductive tissues (GALT, NALT and TALT). This is an initial step for eliciting Ag-specific S-IgA Ab responses in mucosal effector tissues. DCs in mucosal inductive tissues play a major role as APCs for the activation of naive CD4+ T cells. In addition, ingested Ags activate IgA-committed B cells. Activated CD4+ T cells and IgA-committed B cells dispatch from mucosal inductive tissues and migrate into the mucosal effector tissues and subsequently interact for the terminal differentiation of IgA-committed B cells into IgA-producing plasma cells. In addition to the classical mucosal inductive tissues, the SL mucosa can initiate mucosal immune responses.
Bα: IgA-committed B cell; GALT: Gut-associated lymphoreticular tissue; MALT: Mucosa-associated lymphoreticular tissue; NALT: Nasopharyngeal-associated lymphoreticular tissue; PC: Plasma cell; Th1: Type 1 helper CD4+ T cell; Th2: Type 2 helper CD4+ T cell; Te: Effector CD4+ T cell; Tn: Naive CD4+ T cell.

Secretory (S)-IgA antibody (Ab) is a major player in the mucosal immune system and is locally produced in effector tissues [1,2,5,10–12]. The presence of Ag-specific S-IgA Abs at mucosal effector sites other than the inductive sites where initial Ag sampling occurred is definitive evidence for the CMIS. To this end, immunization of GALT or NALT effectively elicits Ag-specific mucosal IgA Ab responses in diverse mucosal effector tissues with some notable differences. Indeed, activated T cells in Peyer's patches (PPs) preferentially express α4β7 and CCR9 as gut-homing receptors for their migration into the intestinal lamina propria [13–16]. In this regard, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), the ligand for α4β7, mediates T-cell recruitment into the intestinal endothelium [17]. Furthermore, small intestinal epithelial cells express the CCR9 ligand, thymus-expressed chemokine. Recent studies demonstrated that retinoic acid-producing DCs in PPs and the mesenteric lymph nodes (MLNs) are key players in the enhancement of α4β7 and CCR9 expression by Ag-specific effector CD4+ T cells, which in turn guides their migration into the intestinal lamina propria [18]. In addition to mucosal T-cell homing, retinoic acid-producing DCs in PPs regulate T cell-independent IgA class switching and gut-homing receptor expression on B cells [19,20]. These findings clearly show that the CMIS exhibits distinct sites for induction and regulation of S-IgA Ab responses in mucosal effector tissues.

Although it has been shown that GALT and NALT share common features, it is also clear that a compartmentalization occurs between the oral and nasal immune systems [21–23]. Thus, oral immunization mainly elicits Ag-specific immune responses in the small intestine, in the proximal part of the large intestine, mammary and salivary glands, whereas nasal immunization induces mucosal immunity in the UR tract, nasal and oral cavities, and the cervicovaginal mucosa [21–23]. Furthermore, the organogenesis, lymphocyte trafficking and progression of immunosenescence in PPs and NALT are distinctly regulated [11,13,15,24–35]. Thus, the PPs develop between embryonic days 14 and 17 in an IL-7-IL-7Rα and LTα1β2-LTβR signaling cascade-dependent manner, whereas NALT organogenesis occurs postnatally in the absence of these cytokine cascades [28,32,34,35]. Furthermore, both Id2 and retinoic acid receptor-related orphan receptor-γt transcripts are essential for PP inducer cell development; however, NALT inducer cells require only Id2 [28,36–38]. In addition, activated T and B cells in PPs preferentially express α4β7 and CCR9 as gut-homing receptors, which help guide their migration back to the intestinal lamina propria [13,15]. In contrast, CD62L, α4β1 and CCR10 preferentially control the migration of T and B cells from NALT into the UR tract effector tissues [24,25,32,33]. The compartmentalization of GI and UR tract immune systems is also evident because distinct differences in mucosal aging occurred between the GI and UR tract immune systems [26,27,29–31]. Thus, age-associated alterations, including a reduction in number of PPs and the level of intestinal Ag-specific S-IgA Abs, occur in mice during aging [26,27]. Furthermore, mice lose oral tolerance, which represents another important mucosal immune regulatory function for maintaining systemic homeostasis to orally administered Ags during the aging process (6–12 months) [26,27,30,31]. In contrast, NALT shows a more intact immune response during aging (1-year-old mice), with signs of immunosenescence noted only in mice older than 2 years [26,27,29].

Because mucosal immunization induces not only Ag-specific mucosal S-IgA Abs but also systemic IgG Abs, developing mucosal vaccines could be used in much the same way as currently available licensed parenteral vaccines. Thus, mucosal vaccine delivery can induce systemic T-cell and Ab responses in peripheral lymphoid tissue, as is seen after parenteral vaccine delivery. However, simultaneous induction of mucosal immunity provides a dual protection

against pathogens. Furthermore, mucosal adjuvants and delivery systems are essential to induce Ag-specific immune responses in both mucosal and systemic compartments by avoiding induction of systemic unresponsiveness. This review focuses on several recent approaches to induce mucosal immunity to vaccines, with emphasis on mucosal tissue targeting, new immunization routes and delivery systems that are both effective and safe. As a mucosal targeting strategy, DCs and M cells are discussed as the two major targeting cell types. Although a large number of DC-targeting components have been studied as mucosal adjuvants, CpG oligodeoxynucleotides (CpG ODN) and Flt3 ligand (FL) are selected based on their effectiveness and safety. Importantly, the cellular and molecular mechanisms for these two DC-targeting mucosal adjuvants and an M cell-targeting vaccine delivery system have been well described. In contrast, the precise mechanisms for sublingual (SL) immunization, eye drops, and rice-based and nanogel delivery systems remain to be elucidated; however, the early results are promising. In summary, these novel strategies are attractive and exhibit high potential from a practical point of view. More extensive reviews, which include additional targeting strategies, adjuvants, and delivery systems, are provided. Some specific details are essential to understand the cellular and molecular mechanisms involved in using these novel vaccine strategies.

Targeting vaccines

Mucosal DCs

DCs play a central role in bridging the innate immune system with the adaptive immune system [39–42]. Thus, DCs are found throughout the body and are especially prominent at mucosal surfaces. Immature type DCs are enriched underneath the epithelium of mucosal inductive sites and are poised to capture Ags. When Ag uptake occurs, these DCs change their phenotype by expressing higher levels of MHC class II and costimulatory molecules and move to T-cell areas of inductive sites for Ag presentation. Thus, DCs and their derived cytokines play key roles in the induction of Ag-specific effector Th-cell responses. In this regard, targeting mucosal DCs is not only an effective strategy to induce mucosal immunity but also a safe approach, especially for nasal application, because vaccines mainly initiate immune responses through DCs in the absence of central nervous system toxicity.

Because of the recent progress in the understanding of innate immunity-associated molecules, toll-like receptor (TLR) ligands are now considered to be candidates as potent mucosal adjuvants. Among these, the TLR9 ligand CpG ODN is known to target professional plasmacytoid DCs for their activation, maturation and subsequent induction of Ag-specific Th1-type responses, including cytotoxic T lymphocytes (CTLs) [43,44]. It has been demonstrated that synthetic CpG ODNs can induce innate immune responses [45–48]. In this regard, CpG ODNs as effective immunomodulators, could target malignant tumors, and reduce allergic responses [49,50]. Furthermore, CpG ODNs have been used as potent adjuvants to elicit Ag-specific Ab and cell-mediated immune responses in mice and rats against both bacterial and viral Ags [51–58]. To this end, mucosal administration of CpG ODN exhibits potent adjuvant activity (Figure 2). Mucosal immunization with CpG ODN plus formalin-inactivated influenza virus, hepatitis B virus surface Ag, or tetanus toxoid effectively elicited vaccine-specific immunity in the mucosal compartment of mice [57–59]. CpG ODN as adjuvant mainly induces Th1-type responses. In this regard, CpG ODN could even switch a predominant Th2 into a Th1-type immune response pathway [60]. Although the detailed mechanisms of adjuvant activity of CpG ODN are still unclear, it has been demonstrated that CpG ODN enhanced MAPK-mediated IL-12 production by APCs [61]. Others also clearly showed that nasal immunization with the recombinant protective Ag of the anthrax lethal toxin and CpG ODN induced protective Ag-specific plasma IgG2a and mucosal S-IgA Ab responses with *in vitro* neutralizing activities [62].

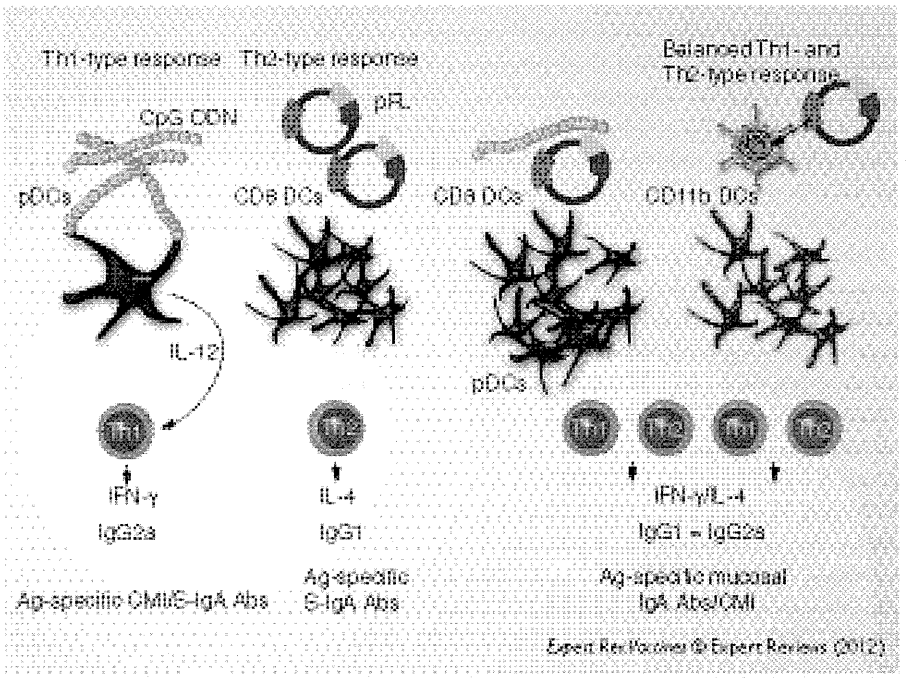

Figure 2. Nasal DC-targeting mucosal vaccines: nasal application of CpG ODN activates plasmacytoid DCs (pDC, B220$^+$ DCs) for the induction of Th1-type cytokine responses. Thus, CMI and cytotoxic T lymphocyte (CTL) activity can be elicited in addition to Ag-specific S-IgA Ab responses. In contrast, pFL as nasal adjuvant preferentially expands the CD8$^+$ DC subset and subsequently elicits Th2-type cytokine-mediated Ag-specific S-IgA Ab responses. Adenovirus expressing FL (Ad-FL) or a combination of CpG ODN and pFL induces a more balanced Th1- and Th2-type immune response. Ad-FL activates CD11b$^+$CD11c$^+$ DCs, whereas a combined nasal CpG ODN and pFL stimulates both CD8$^+$ DCs and pDCs for the induction of CMI and S-IgA Ab responses. Abs: Antibodies; Ag: Antigen; CMI: Common mucosal immune; CPG ODN: CpG oligodeoxynucleotides; DC: Dendritic cell; pDC: Plasmacytoid dendritic cell; pFL: Plasmid-expressing Flt3 ligand; S-IgA: Surface IgA.

FL is a growth factor that binds to the fms-like tyrosine kinase receptor Flt3/Flk2. *In vivo* FL treatment markedly upregulates the number of DCs but not their activation [63,64]. Mouse FL has been cloned and shown to be a key player in the proliferation and differentiation of early hematopoietic precursor stem cells [63,65–68]. Furthermore, it has been reported that FL could mobilize and stimulate not only DCs [64] but also natural killer cells and B cells [69]. Of interest, it was first reported that systemic FL injection facilitated oral tolerance induction because of its ability to result in significant increases in the number of DCs in several lymphoid tissues, including the intestinal lamina propria, PPs, MLNs, and spleen [70,71]. In contrast to tolerance induction, others showed that FL treatment also upregulated immune responses when delivered via mucosal [71], systemic [72], or cutaneous [73] routes. It has also been reported that when plasmid DNA encoding FL (pFL) was coadministered with plasmids encoding protein Ags or linked to the Ag itself, effective immune responses were induced [74,75]. In this regard, it has been suggested that FL possesses adjuvanticity for both humoral and cell-mediated immune responses and that the FL cDNA system may be a potential alternative approach to using the FL protein system [76–79]. To this end, pFL has been used as a mucosal DC-targeting adjuvant for the induction of Ag-specific protective mucosal immune responses (Figure 2). Nasal administration of pFL as mucosal adjuvant facilitated expansion of $CD8^+$ DCs, which subsequently elicited IL-4-producing $CD4^+$ T-cell- and Ag-specific S-IgA Ab responses [80]. NALT has been the major site for sampling pFL and for producing the FL protein locally, which subsequently induced the expansion and activation of DCs [80]. In this regard, pFL did not show any potential to migrate into the CNS.

Other types of FL-based NALT-DC-targeting immune modulators, including an adenovirus serotype 5 vector expressing FL (Ad-FL), were found to elicit Th1- and Th2-type responses, thereby providing both Ag-specific S-IgA Ab and cell-mediated immune responses [81]. When mice were nasally immunized with ovalbumin (OVA) and Ad-FL, high levels of Ag-specific Ab responses were elicited in both mucosal and systemic compartments. Furthermore, significantly increased levels of Ag-specific IFN-γ and IL-4 production were noted in cervical lymph nodes and spleen [81]. Because of OVA-specific Th1-type cytokine responses, Ag-specific CTL responses were upregulated in mice administered with nasal OVA and Ad-FL. Interestingly, the number of $CD11b^+$ $CD11c^+$ DCs was preferentially increased. This DC subset expressed high levels of costimulatory molecules and migrated from the NALT to mucosal effector tissues [81]. These findings show that nasal administration of Ad-FL facilitated the induction of mature-type $CD11b^+$ $CD11c^+$ DCs and Th1- and Th2-type $CD4^+$ T cells in the NALT for Ag-specific Ab and CTL responses (Figure 2). Balanced Th1- and Th2-type responses have become key issues in mucosal vaccine development because this type of cytokine response would not only provide Ag-specific S-IgA Ab and CTL responses against viral and bacterial infections but also avoid induction of allergic (IgE) and inflammatory-type responses.

CpG ODN has been shown to induce polarized Th1-type cytokine responses in mice [62]. In contrast, pFL preferentially elicits coadministered Ag-specific Th2-type cytokine immunity [80]. To this end, one could hypothesize that an ideal but balanced Th1- and Th2-type cytokine response would be elicited by using a combination of pFL and CpG ODN as DC-targeting nasal adjuvants. Indeed, recent studies clearly showed that pFL and CpG ODN as a combined nasal adjuvant induced the activation and expansion of plasmacytoid DCs and $CD8^+$ DCs in the nasal cavity for the development of Th1- and Th2-type cytokine-producing $CD4^+$ T cells. Thus, these Ag-specific $CD4^+$ T cells successfully upregulated coadministered Ag-specific immunity in both the mucosal and systemic immune compartments (Figure 2) [82,83]. Increased frequencies of mature-type DCs in NALT correlated well with induction of Ag-specific immune responses. Of significance, nasal delivery of pFL and CpG ODN successfully elicited significant levels of Ag-specific S-IgA Ab responses in 2-year-old mice [82,83]. To this end, aged mice given nasal pneumococcal surface protein A and a combination of pFL and CpG ODN showed protective immunity against nasal *Streptococcus pneumoniae* colonization [83]. These results suggest that nasal administration of pFL and CpG ODN as mucosal adjuvants provides an attractive possibility for the development of a vaccine against *S. pneumoniae* in the elderly.

M cells

As discussed earlier, GALT, including PPs, is covered by a specialized follicle-associated epithelium, 10–20% of which is composed of M cells that show a unique topical morphology (microfold/membranous) and form pockets for the inclusion of lymphoid cells, including B and T cells, DCs, and macrophages [84–89]. M cells show significantly different features compared with intestinal epithelial cells. M cells possess relatively short microvilli, small cytoplasmic vesicles and few lysosomes. Thus, M cells are able to capture and transport lumenal Ags, including viruses, bacteria, small parasites, and microspheres [86,87,89,90]. It has been suggested that M cells may also play a role as APCs because M cells express MHC class II molecules and acidic endosomal–lysosomal compartments [91]. In this regard, activation and potential MHC class II expression by M cells may depend on the nature of endocytosed Ag. M cells serve not only for transport of lumenal Ags but also for provision of an entry way for pathogens to invade the host. In particular, it has been shown that invasive but not noninvasive strains of *Salmonella typhimurium* enter the host through PP M cells [92]. In addition to PPs, the ILFs and NALT also contain a lymphoepithelium with M cells. Thus, *Mycobacterium tuberculosis* uses NALT M cells for host entry [93]. In addition, it was reported that M cells are also detected in nonlymphoid follicle-associated epithelium that covers small intestinal villi [94]. Thus, villous M cells in the small intestine were present in several PP-deficient mouse strains, including *in utero* LT-βR-Ig-treated, LT-$\alpha^{-/-}$, TNF/LT-$\alpha^{-/-}$ and inhibition of differentiation 2 $(Id2)^{-/-}$ mice [94]. Importantly, these villous M cells functionally take up bacteria and induce bacterial Ag-specific immune responses [94]. Indeed,

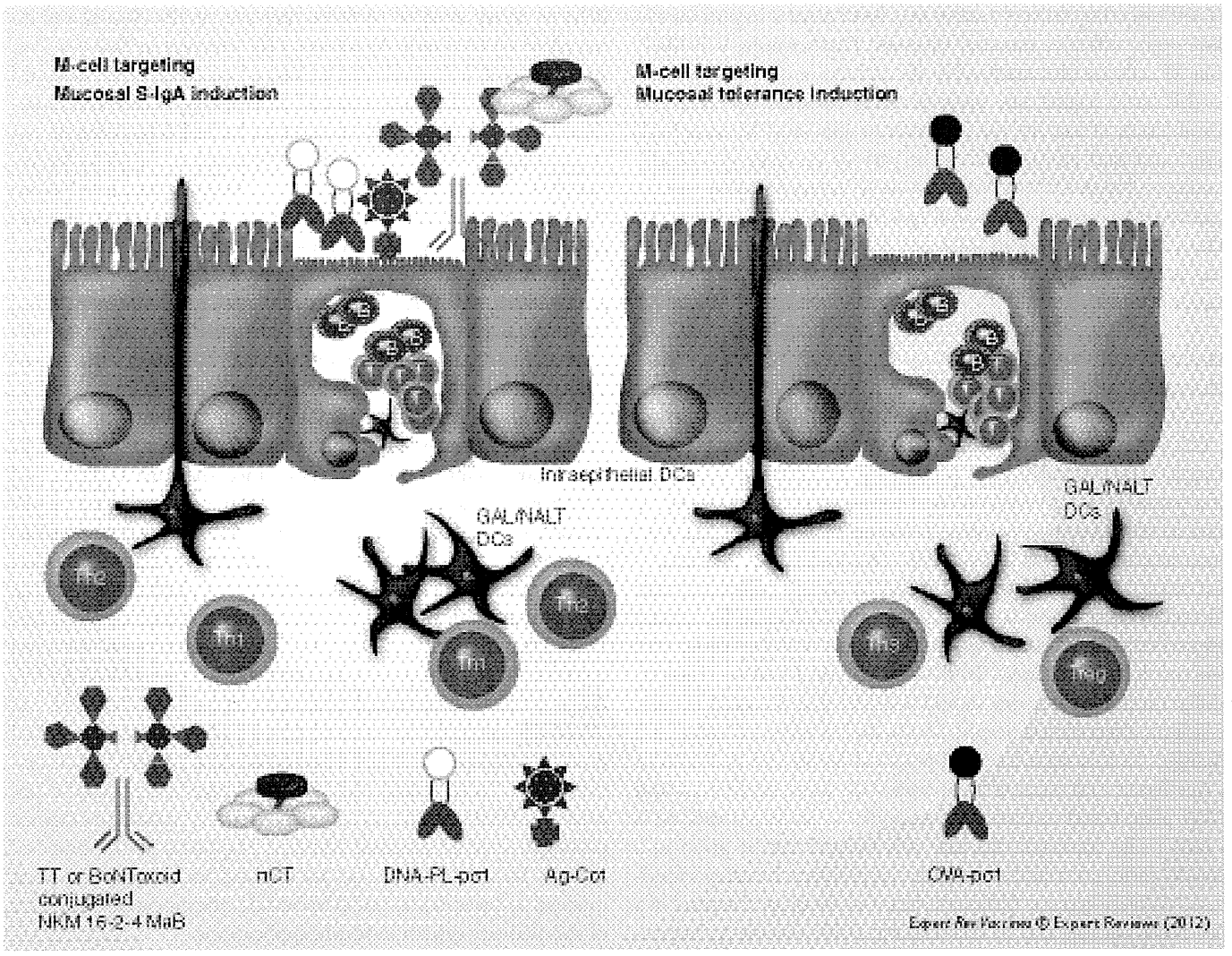

Figure 3. Potential for an M cell-targeting strategy: mucosal M-cell targeting by M cell-specific monoclonal antibody or surface proteins can facilitate Ag delivery for the induction of Ag-specific S-IgA antibody responses to provide effective immunity at the entry site of pathogens. M-cell targeting is achieved by using the protein sigma-1 (pσ1) from reovirus, the ligand for M cell-specific peptide (Co1) or M cell-specific mAb. However, mucosal administration of genetically conjugated OVA protein with pσ1 in the absence of an adjuvant elicits mucosal tolerance.
Ag: Antigen; DC: Dendritic cell; GAL: Gut-associated lymphoreticular; mAb: Monoclonal antibody; NALT: Nasopharyngeal-associated lymphoreticular tissue; S-IgA: Surface IgA; Th1: Type 1 helper CD4+ T cell; Th2: Type 2 helper CD4+ T cell; Th3: Type 3 helper CD4+ T cell; Treg: T regulatory cell.

the MLNs from PP-deficient mice play a key backup role as a mucosal inductive tissue [95]. It has been suggested that MHC class II+ sIgA+ B cells and lamina propria macrophages may be able to capture Ag through endocytic pathways and process and present peptides to CD4+ Th cells. These findings clearly suggest that the intestinal lamina propria–MLN axis performs a potent mucosal inductive function in addition to the PPs.

If one could identify the key molecules expressed by bacteria and viruses that are needed for their invasion or infection of M cells, it would be a great advantage for designing and constructing effective delivery systems for M-cell targeting of vaccines. Reoviruses initially infect the mouse through M cells [96], by using their surface protein sigma-1 (pσ1) [97,98]. In this regard, an M cell-targeting DNA vaccine complex consisting of plasmid DNA and the reovirus pσ1 covalently attached to poly-L-lysine induced significant mucosal S-IgA Ab responses and systemic immunity (Figure 3) [99]. Furthermore, a newly developed M cell-specific monoclonal Ab (NKM 16-2-4) was used as an M cell-targeting carrier for mucosal vaccines. Thus, oral administration of a chimeric vaccine consisting of NKM 16-2-4 and tetanus toxoid or botulinum neurotoxin type A toxoid (BoNToxoid/A), together with native cholera toxin, elicited increased levels of Ag-specific S-IgA and plasma IgG Ab responses (Figure 3) [100]. Importantly, oral immunization with BoNToxoid/A-NKM 16-2-4 provided protective immunity against lethal challenge with botulinum neurotoxin [100]. In addition, oral immunization of Ag fused with M cell-targeting peptide ligand (Co1) resulted in enhanced Ag-specific immune responses [101]. These studies show that an

M cell-targeting delivery system may be of central importance in developing effective mucosal vaccines. Furthermore, it is likely that M cells are also involved in the induction of oral tolerance. In this latter regard, one must carefully consider the nature of formulation of vaccine (or inclusion of adjuvant) because both nasal and oral administration of pσ1 of reovirus genetically conjugated with OVA (OVA-pσ1) alone induced systemic unresponsiveness instead of mucosal IgA immunity (Figure 3) [102,103]. Thus, mucosally induced tolerance was achieved with doses as low as 10–50 μg of OVA-pσ1 when given by the nasal or oral routes [102,103].

Mucosal delivery systems

MucoRice

In 1997, Curtiss and Cardineau successfully filed for and received a US patent (5686079) describing tobacco leaves expressing *Streptococcus mutans* surface protein Ag as an initial indication of a potential plant-based mucosal vaccine. Furthermore, others have developed edible plant-based vaccines by expressing Ags from enterotoxins, hepatitis B, Norwalk virus and respiratory syncytial virus expressed in tobacco leaves or potato tubers [104–111]. Although these plant-based vaccines exhibited some functional properties in experimental systems, their practical application still remains to be elucidated. To develop practical oral vaccines for global immunization, one should consider that the vaccine must maintain effectiveness despite *in vivo* and *ex vivo* environmental changes. In this regard, several practical merits can be found in a rice-based oral vaccine compared with most traditional and other plant-based vaccines. For example, a rice-based vaccine is a rather safe approach. Because this vaccine can be given in a powder form, one could avoid potential problems by using a food-based delivery system. Although the lot-to-lot quality control of a rice-based vaccine may be challenging, stable vaccine Ag expression could be achieved by the third generation of rice-based vaccine. Furthermore, a rice-based vaccine showed stability at room temperature for 2–3 years [112,113]. Oral administration of this rice-based vaccine did not lose activity when exposed to digestive enzymes and subsequently induced protective, Ag-specific Ab responses in mice and non-human primates [112–115]. Recent studies have provided direct evidence that oral MucoRice-cholera toxin B-subunit (CT-B) induced Ag-specific S-IgA Abs that played a critical role in protection against CT-induced diarrhea (Figure 4) [113]. Importantly, cold chain-free oral MucoRice-CT-B induced long-lasting cross-protective immunity against heat-labile enterotoxin-producing enterotoxigenic *Escherichia coli* in addition to CT-producing *Vibrio cholerae* [113]. These results demonstrate that oral administration of a rice-based vaccine provides a potent practical global strategy for the development of cold chain- and needle-free vaccines that protect from gastrointestinal infection.

Nanogels

The application of biomaterials, such as encapsulating Ags in polymer nanoparticles, microparticles, virosomes and liposomes, shows significant potential in the development of vaccines and immunotherapy [116–123]. Although use of liposomes can enhance Ag delivery across mucosal surfaces, they are rapidly cleared and do not allow for long-term Ag release at the mucosal surface [124–126]. In this regard, it is possible that using a bioadhesive gel one could upregulate the residence time and enhance Ag release and retention onto the epithelial cells themselves. Indeed, it has been shown that surface modifications or coadministration with bioadhesive materials, that is, chitosan, resulted in influenza-specific S-IgA Ab responses in nasal washes [127]. A nanometer-sized (<100 nm) bioadhesive polymer hydrogel (nanogel) system has been developed and used as an attractive drug delivery system [128]. Cholesteryl group-bearing pullulan (CHP) form self-assembly of associating polymers as physically crosslinked nanogels in water [129,130]. In general, hydrophobic interactions between CHP and various proteins revealed a CHP nanogel containing the protein inside. When CHP nanogels capture the proteins inside, they form a hydrated nanogel polymer network (nanomatrix) without aggregation. In this regard, trapped proteins maintained their native form and were slowly released [130]. On the basis of these advantages, a CHP nanogel strategy has been used for the development of adjuvant-free nasal vaccines. It was recently shown that nasal administration of a cationic type of CHP nanogel (cCHP nanogel) containing the C-terminus of the H chain (Hc) of botulinum neurotoxin-type A (BoNT/A; nanogel-Hc-BoNT/A) allowed adherence to the nasal epithelium for a longer period compared with naked Hc-BoNT/A (Figure 4) [131]. In this regard, gradually released Hc-BoNT/A was effectively taken up by mucosal APCs and subsequently elicited protective Ag-specific S-IgA Ab responses against BoNT/A intoxication [131]. In summary, this cCHP nanogel system could represent an ideal and effective mucosal delivery system to enhance pathogen-specific mucosal immune responses at the mucosal surface. Because vaccine Ags are retained for a longer period at mucosal surfaces, it is essential to consider the potential side effects of this delivery system in future applications.

Mucosal immunization routes

SL immunization

Oral and nasal routes have been the preferred ones to induce protective immunity in different mucosal compartments [1,5,21]. However, it has been demonstrated that rectal, vaginal or paramucosal (iliac and inguinal lymph nodes) immunization are also effective strategies for the induction of protective immunity against sexually transmitted infectious diseases, including HIV [132–135]. In addition to these mucosal immunization routes, SL administration of Ags has been used to treat allergic, autoimmune or infection-induced pathologic reactions [21,136], by taking advantage of the induction of oral tolerance [137–140]. It is well known that nasal immunization effectively elicits Ag-specific immunity in both mucosal and systemic compartments; however, one must consider that some nasal immunization strategies risk Ag trafficking into olfactory tissues and the CNS [141–145]. To obviate this potential problem, SL immunization may be an ideal mucosal Ag delivery system that avoids CNS involvement. SL administration is also a noninvasive route that has the advantage of requiring lower doses of Ag than the oral route because of the reduced exposure to proteolytic enzymes and lower pH of the stomach encountered