Q 麻疹の検査診断は，どのようにすればよいのでしょうか？

A 国立感染症研究所麻疹対策技術支援チームでは，フローチャート（p286）に示した検査診断の考え方を国立感染症研究所感染症情報センターのホームページ[5]に公開しました．また，前述した厚生労働省健康局結核感染症課長通知により，麻疹と臨床診断した場合は速やかに（発疹出現後7日以内に）保健所を通して全国の地方衛生研究所に血液（EDTA血），咽頭拭い液，尿の3点セット（できれば2点以上）を送付し，麻疹ウイルス遺伝子あるいはウイルスの直接検出法（RT-PCR法やウイルス分離など）による検査診断を求めています．

麻疹と臨床診断した場合は，麻疹ウイルスの直接検出による検査診断と，IgM抗体の測定（こちらは発疹出現後4～28日）を同時並行で行い，可能な限りペア血清による麻疹IgG抗体価の陽転あるいは有意上昇を確認することによって，確実な麻疹の診断を行ってほしいと考えています．

Q なぜ，麻疹は検査診断が必要なのでしょうか？

A 1．検査診断の重要性

麻疹はこれまで，経験の豊富な小児科医であれば臨床診断のみで診断できる疾患と考えられてきました．しかし，麻疹患者の減少に伴い，麻疹を診たことがない小児科医が増えていること，成人麻疹の増加により，これまで麻疹患者を診察する機会が少なかった内科を受診する麻疹患者が増えていることなどもあって，臨床症状のみでの麻疹の診断には慎重な対応が必要になってきています．

2．麻疹とまちがえられやすい疾患

また，発疹が通常の経過より重症である突発性発疹や風疹が麻疹と臨床診断されていたり，麻疹の初期の症状が発熱，咳，鼻汁，眼球結膜の充血といった非特異的な症状で始まるため，病初期に服用した様々な薬剤による薬疹と臨床診断されている場合が多くみられています．

3．麻疹IgM抗体の偽陽性

もう一つの問題としては，突発性発疹，伝染性紅斑，風疹，デング熱といったその他の発疹性ウイルス感染症の急性期に，麻疹のIgM抗体を測定すると，弱陽性になるという問題点が明らかとなり，麻疹の検査診断は麻疹ウイルスの直接証明による方法との併用が重要であることが明らかになってきました[3]．

4．麻疹IgM抗体の偽陰性

また，真の麻疹であっても発疹出現後3日以内の検体では麻疹IgM抗体が陽性にならない場合があるなど，麻疹の検査診断を1回のみのIgM抗体で行うことは誤診につながる危険性が指摘されるようになりました．

5．なぜ検査診断が必要か

以上のことから，麻疹の検査診断を確実に行うことは，公衆衛生上麻疹対策に重要という意味に加えて，麻疹患者本人にとっても大きなメリットがあります．もし麻疹ではない疾患で麻疹と診断されると，その後，麻疹風疹混合ワクチン（MRワクチン）の接種を受けそびれる可能性が高くなります．それは，すでに罹ったからワクチンは必要ないと思わ

れるからです．実際には麻疹ではなかった場合，麻疹の免疫は不十分となり，将来麻疹を発症してしまうリスクがあります．また，風疹のワクチンを同時に受けないことにつながる可能性も危惧され，風疹に対しても免疫が不十分となり，女性が妊娠出産年齢になってつらい思いをすることにつながることが心配されています．

＊　＊　＊

わが国のような先進国であっても，麻疹は死に至ることがある重症の感染症であるという認識をもっている人は，極めて少ないと思われます．死に至らなくても，麻疹脳炎を発症し重度の後遺症に苦しむ場合があることを知っている人も極めて限られています．さらに，空気感染する感染症であることから，体育館のような広い場所であっても同じ部屋にいるだけで感染してしまう極めて感染力の強い疾患であり，自分が罹って苦しむだけではなく，周りにいる多くの人々に麻疹をうつしてしまうこと，その中には，麻疹ワクチンを受けたくても受けられない基礎疾患をもつ人々がいることを，伝えてあげる必要があると考えています．「はしかにならない，はしかにさせない」というキャッチフレーズは[5]，そういう思いを同世代の人々にぜひ届けたいと，国立感染症研究所感染症情報センターに勤務していた20代の若者が考えました．

私たちは人々とともに暮らし，人々とともに成長しています．自分が罹ってつらいことは周りの多くの仲間にとってもつらいことです．自分を守るため，そして周りにいる多くの人々を麻疹から守るためにも，2回の予防接種を受けて欲しいと思います．

謝　辞

麻疹対策には，国立感染症研究所感染症情報センター・同ウイルス第三部・厚生労働省・文部科学省が国の麻疹対策技術支援チームとなって，全国の行政機関，研究機関，医療機関，教育機関，福祉機関とともに一丸となって取組んでいます．

本項で紹介した内容は，上記機関でともに麻疹対策に取組んでいる多くの関係者の皆様と一緒に作り上げてきたものですが，全員の方々のお名前をご紹介できないことを申し訳なく思います．

2012年の麻疹排除を目標に今後も一層の努力を続けて参りたいと考えておりますので，どうか引き続きご指導の程お願い申し上げます．

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総論

M 妊娠と予防接種

●はじめに

妊娠中に罹患すると妊婦本人が重症になる感染症や，胎児に影響を及ぼす感染症，あるいはその両者が認められる感染症があり，それぞれの感染症の特徴をよく理解して，妊娠中にできる限り感染症を発症しないように予防策を講じておくことは，非常に重要である。

感染源・感染経路を絶つことにより妊娠中の感染・発症を予防することはもちろんのこと，最も重要な対策の一つに予防接種が挙げられる。予防接種で予防可能な感染症については，できれば予防接種を受けて，妊娠中の発症あるいは重症化を予防することが大切である。

しかし，妊娠中は生ワクチンの接種を受けることができない。一方，不活化ワクチンについては，予防接種上の有益性が危険性を上回ると判断される場合にのみ接種することとなっており，主治医と接種時期について相談し，体調を勘案しながら接種を受けることで，重症化を予防したい感染症もある。

本項では，妊娠中には受けることができないために，妊娠前に受けておくことが勧められる予防接種と，妊娠中であっても受けることが可能な予防接種について概説する。

Ⅰ．日本で接種可能なワクチンの種類

2010年12月現在，日本で接種可能なワクチンの種類を表1に示す。生ワクチンが8種類あるが，黄熱ワクチンは通常の医療機関では接種できず，検疫所などでのみ接種が可能である。不活化ワクチンは16種類あるが，このうち，沈降新型インフルエンザワクチン（H5N1株）は通常の医療機関では接種できず，国家備蓄のワクチンである。

2009年10月に製造販売承認された小児用肺炎球菌ワクチン（7価結合型）と組換え沈降2価ヒトパピローマウイルス様粒子ワクチン（イラクサギンウワバ細胞由来）は，それぞれ2010年2月，2009年10月から国内での接種が始まっている。また，現在，国内臨床治験が継続中あるいは終了したワクチンもいくつかあり，今後の承認が待たれる。

Ⅱ．妊娠する前の接種が勧められるワクチン

国内の予防接種状況は上記の通りであるが，妊娠していることが明らかな場合，生ワクチンを接種することはできない。添付文書上，「接種してはならない」と記載されている。いわゆる，接種不適当者に該当する。

しかし，妊娠中に麻疹，風疹，流行性耳下腺炎，水痘に罹患すると，妊婦本人が重症になる可能性があるだけでなく，胎児に影響を及ぼす可能性がある（詳細は各論のそれぞれの疾患の項を参照）。

そのため，医学的な理由で予防接種を受けられない場合を除き，これらの4疾患に罹ったことがない女性は，妊娠する前に予防接種を受けて，免疫を獲得しておくことが望まれる。罹ったかどうかの記憶は曖昧な場合が多く，抗体検査で確認するという方法もあるが，検査を受けずに予防接種を受けても医学的には問題はない。不確かな場合は妊娠する前に，むしろ受けておいたほうが望ましい。

その場合，あらかじめ約1カ月間避妊した

表1　日本で接種が可能なワクチンの種類（2010年12月現在）

生ワクチン
乾燥BCGワクチン
経口生ポリオワクチン
乾燥弱毒生麻しん風しん混合ワクチン（MR）
乾燥弱毒生麻しんワクチン
乾燥弱毒生風しんワクチン
乾燥弱毒生おたふくかぜワクチン
乾燥弱毒生水痘ワクチン
黄熱ワクチン
不活化ワクチン
沈降精製百日せきジフテリア破傷風混合ワクチン（DPT）
沈降ジフテリア破傷風混合トキソイド（DT）
乾燥細胞培養日本脳炎ワクチン
インフルエンザHAワクチン
A型インフルエンザHAワクチン（H1N1株）：A型インフルエンザ（H1N1）2009に対するワクチン
沈降新型インフルエンザワクチン（H5N1株）：（国家備蓄）
組換え沈降B型肝炎ワクチン（酵母由来）
乾燥組織培養不活化A型肝炎ワクチン
沈降破傷風トキソイド
成人用沈降ジフテリアトキソイド
乾燥組織培養不活化狂犬病ワクチン
肺炎球菌ワクチン（23価多糖体，7価結合型）
インフルエンザ菌b型（Hib）ワクチン
組換え沈降2価ヒトパピローマウイルス様粒子（HPV）ワクチン（イラクサギンウワバ細胞由来）
ワイル病秋やみ混合ワクチン

※医師が個人輸入して接種しているワクチンは除く

後，予防接種を受け，接種後約2カ月間は妊娠を避けるように注意する必要がある。もし，不妊治療を考えている場合は，治療開始前にこれら4つの感染症に対する免疫をもっていることを確認しておくことが必要である。免疫がない場合は，予防接種を受けて免疫の獲得を確認してから治療を開始すると安心である。

麻疹と風疹は定期予防接種（以下，定期接種）の制度が変更となり，2006年6月から2回接種制度が始まった。また，2007年に発生した思春期から若年成人を中心とする大規模な国内麻疹流行を受けて，2008年4月から5年間の時限措置で，中学1年生と高校3年生相当年齢のものに2回目の接種が定期接種として実施されている。すなわち，平成2年4月2日以降に生まれたものは，2回接種の機会が法律に基づいて与えられたことになる。しかし，受けていない人も多く，妊娠して初めて免疫がないことに気付く場合も少なくない。予防接種を受けて，あらかじめ妊娠中の発症を予防しておくことは非常に重要である。

一方，水痘と流行性耳下腺炎は定期接種に導入されていないため，予防接種を受けている人は少なく，むしろ罹患済みの人のほうが多いという特徴がある。水痘は感染力も強いことから，小児期に罹患済みのことが多いが，未罹患の場合もある。流行性耳下腺炎は成人になるまで罹らずに過ごしている人も多く，また，定期接種に導入されていないことから接種率が低く，毎年子どもたちの間で大規模な流行を繰り返しているのが現状である。すなわち，いつどこで感染してもおかしくない状況にあるといえる。妊娠前に予防接種を受けて，免疫を獲得しておいてほしい感染症である。

妊娠中は，生ワクチンの接種が受けられないので，妊娠してから免疫がないことが判明した場合は，出産まで心配な日々を過ごすこ

とになる。このような状況を回避するのは，日本での流行を阻止する以外方法はない。「herd immunity」という言葉があるが，予防接種率が高くなって，周りの多くが免疫をもっていると，たとえその病原体が侵入してきても，大規模な流行は抑制されるといういわゆる集団免疫の考え方である。麻疹，風疹の2回目の接種率が目標の95%以上に達していないことはすでに報告されている[1]。水痘，流行性耳下腺炎の予防接種率は20～30%程度と推定されている。妊娠中にこれらの感染症に罹らないよう，女性自らが自分自身と胎児の健康を守ること，そして，家族や職場の同僚が，また，国民一人ひとりがまわりにいる予防接種を受けたくても受けられない人を自分が罹らないことで守ってあげることも必要であると考える。

もし，妊娠していることに気付かずにこれらのワクチンを受けてしまった場合，風疹を例に挙げると，ワクチンウイルスによる先天性風疹症候群（congenital rubella syndrome；CRS）の出生は報告されていない。万が一妊娠していることに気付かず，これらのいずれかのワクチンを受けてしまった場合であっても，そのことのみを理由に妊娠を中断する必要はないが，理論上のリスクを回避するために，妊婦はこれらのワクチンの接種不適当者であることに変わりはない。

ただし，生ワクチンの中で，ワクチン添付文書上，接種不適当者に含まれていないワクチンが2つある。BCGワクチンと黄熱ワクチンである。BCGワクチンは通常，乳児（一部，幼児）に接種されるワクチンであるため，妊娠中の接種が考慮されることはないと考えるが，黄熱ワクチンは受けていないと入国が許されない国があることから，検討が必要になる場合が想定される。

黄熱ワクチンの添付文書には，「妊娠又は妊娠している可能性のある婦人には接種しないことを原則とし，予防接種上の有益性が危険性を上回ると判断される場合にのみ接種すること（妊娠中の接種に関する安全性は確立していない。また，17Dワクチンウイルスは経胎盤感染する可能性が示唆されている）」と記載されている。予防接種上の有益性が危険性を上回ると判断される場合というのは，黄熱流行地域への渡航を中止あるいは延期できず，媒介蚊であるネッタイシマカに対する十分な対策が取れない場合のみと考えられるが，妊娠しているため接種が受けられないという医師の証明書をもらえば，検疫官が確認してくれる場合もある。あらかじめ旅行会社や検疫所でよく確かめ，できれば妊娠中の黄熱流行国への入国は避けるほうが無難と考えられる。ただし，以前接種を受けたことがある場合は10年間有効とされている。

Ⅲ．妊娠中に接種可能な予防接種

妊婦は，不活化ワクチンの接種不適当者には該当しない。生ワクチンとは異なり，基本的には妊娠中でも接種できる。ただし，添付文書上は，「妊娠中の接種に関する安全性は確立していないので，妊婦又は妊娠している可能性のある婦人には接種しないことを原則とし，予防接種上の有益性が危険性を上回ると判断される場合にのみ接種すること」と記載されている。

しかし，妊娠中，特に第3三半期にインフルエンザに罹患すると重症化のリスクが高くなることから[2]，2009年のA型インフルエンザ（H1N1）2009の世界的な流行を受けて，国はインフルエンザによる重症化予防の観点から，妊婦をA型インフルエンザHAワクチン（H1N1株）の優先接種対象者に位置付けた[3]。世界保健機関（WHO）も，副反応については注視していくべきであるとしているものの，妊婦をA型インフルエンザHAワクチ

ン（H1N1 株）の優先接種対象の一つに位置付けることを推奨した。

また，A 型インフルエンザ HA ワクチン（H1N1 株）の添付文書には，「小規模ながら，接種により先天異常の発生率は自然発生率より高くならないとする報告がある」との記載もなされている。さらに，チメロサールなどの保存剤が含まれていないワクチンも製造されているため，妊婦で希望する人には接種が可能となっている。

日本産科婦人科学会も，医療関係者対象 Q＆A で，「季節性インフルエンザワクチンに関しては安全性と有効性が証明されている[6]。季節性インフルエンザワクチンの安全性に関しては以下を参照して頂きたい（http://www.ncchd.go.jp/kusuri/index.html）」と述べている。新型インフルエンザワクチンの安全性については，WHO（2009 年 10 月 30 日）が以下「　」内声明を発表した。「新型インフルエンザワクチンの副作用について専門家らが検討したが，特に季節性インフルエンザワクチンの副作用と異なった点はなく，たいへん良好な結果であった。初期段階での結果は安心すべきものであったが，今後とも副作用については注視していくべきである」と記載している。

国内外での成人を対象とした検討結果から，1 回接種で国際的な評価基準を上回る十分な抗体価の上昇がみられたこと，抗体価の上昇について 1 回接種と 2 回接種に差がみられなかったことなどから，健康成人についての接種は 1 回接種となった。また，妊婦についても，健康成人を対象とした臨床試験の結果やこれまでの季節性インフルエンザでの知見，米国の妊婦に対する新型インフルエンザワクチンの臨床試験で健康成人と同様の反応が得られているとの情報などを踏まえて，健康成人と同様，1 回接種となった[4]。また妊婦は，インフルエンザワクチンの優先接種対象となった。季節性インフルエンザワクチンの接種についても，医師が必要と認めた場合，それぞれのワクチンを別の部位（例えば右上腕と左上腕など）に同時接種をすることが可能である。

インフルエンザ以外では，破傷風の予防を目的とした破傷風トキソイドの接種が挙げられる。新生児破傷風の予防のために，妊娠中の予防接種を行っている国もある。日本では，小児期に DPT ワクチンと DT トキソイドの接種が定期接種として接種されているために，妊娠出産年齢の女性の多くは破傷風抗毒素抗体を保有している。しかし，小児期の定期予防接種スケジュールに破傷風が導入されていなかった 40 代以上の女性では抗体保有率は低く，2008 年度感染症流行予測調査事業によると，20～30 代の女性の抗体保有率は 85％以上に維持されているが，40 代前半では約 50％，40 代後半では 25％程度となっている[5]。ワクチンの添付文書上，「妊婦又は妊娠している可能性のある婦人で，破傷風に感染するおそれがあり，本剤の接種による有益性が危険性を上回ると判断される場合は接種することができる。なお，新生児破傷風の予防のために接種を行う場合，予診等を慎重に行い妊娠 20～36 週頃に，0.5 mL ずつ 2 回 3～8 週間の間隔で皮下又は筋肉内に注射することが望ましい」とされている。

また，妊娠中の接種ではないが，B 型肝炎ウイルス母子感染予防として，HBs 抗原陽性の母親から産まれた児は，抗 HBs 人免疫グロブリンとの併用で，通常，生後 2，3，5 カ月に B 型肝炎ワクチンの接種をすることが求められている。これらは，いずれも健康保険で接種可能である。これらの接種を受けない場合，児のキャリア率が高いことから，HBs 抗原陽性の妊婦には，妊娠中から出生後の児への予防接種について指導をしておく必要がある。

おわりに

2009年はA型インフルエンザ(H1N1)2009の流行で，周産期医療は多忙を極めたが，日本では海外に比べて妊婦の重症化例は少なく，平成22年3月30日時点で，妊婦死亡例の報告はなかった。学会や国が海外での妊婦の重症化を受けて，国内での流行開始前から妊婦および医療機関に対して，度重なる情報提供と注意喚起を行い，妊婦に対する治療方針の早期決定や，これまでほとんど接種されていなかった妊婦に対するインフルエンザワクチン接種の方針が定められたこと，妊婦自身も感染予防に努めていることなどが功を奏しているものと考えられる。これを機に，妊娠と予防接種に関する情報が充実し，予防接種で予防可能疾患に対するいっそうの注目と対策の充実が期待される。

(多屋 馨子)

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Short communication

Immunization coverage and natural infection rates of vaccine-preventable diseases among children by questionnaire survey in 2005 in Japan

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ABSTRACT

We performed questionnaire survey in 2005, just before the introduction of the MR vaccine, concerning child vaccination and/or infection history for measles, mumps, rubella, varicella, influenza, diphtheria–pertussis–tetanus (DPT), BCG, and Japanese encephalitis. The vaccination rate against measles and rubella did not exceed 95% at any age levels. As a result, children who had contracted measles and/or rubella were observed at all age levels. The vaccination rate was 95% or higher only for BCG and DPT. The vaccination rates for influenza, mumps, and varicella, although vaccination against which diseases was being performed voluntarily, were low, and outbreaks of these diseases were expected to persist. The vaccination rates at a low level for these infectious diseases might be one of the most possible risk factors to the high prevalence of the diseases in nursery schools (daycare centers), kindergartens, and elementary schools all over Japan.

For infectious disease control by preventive vaccination, information on the vaccination rates and prevalence of infections in local populations is indispensable for the design of approaches for susceptible populations, along with accompanying information on infection sources and routes. We performed a questionnaire survey in 2005 concerning child vaccination history and infection history with the parents of nursery school (daycare center), kindergarten, and elementary school children with the cooperation of persons involved in local health care, welfare, and educational services. Measles, rubella, epidemic parotitis (mumps), varicella, influenza, pertussis (DPT), BCG, and Japanese encephalitis were selected as diseases (vaccines) of interest. The questionnaire consisted of questions concerning the children's histories of vaccination and infections (e.g., Did your child receive the vaccine within the last 1 year, or earlier? Did your child contract the disease within the last 1 year, or earlier?). These questions were designed to be answered by the children's parents, by selecting the correct responses from among the suggested answers and checking them off (√). In addition, attention was given to the distinguishing whether the vaccinations were performed routinely (during the period stipulated by the law) or voluntarily, and to collecting the data without individual identification. A document explaining the objective of the questionnaire survey and a letter of request for cooperation were submitted to the welfare sections, health promotion sections (preventive vaccination centers), child rearing support (child care) sections, boards of education (school hygiene, physical education, and school lunch sections), and nursery schools through the heads (general affairs sections) of cities, towns, and villages, requesting each to evaluate whether they would cooperate. To those sections that were willing to cooperate, copies of a letter of request to parents, copies of the questionnaire, and envelopes were sent in necessary numbers. The answers were then gathered by each section and mailed to us. Cooperation in the survey was requested from the local governments of the 43 cities, towns, and villages in Osaka Prefecture, and one local government in each of the 47 prefectures in Japan between April and June, 2005. As a result, during the survey period between June and November, 2005, a total of 20,000 responses were collected in 30 cites, towns, and villages (recovery rate, roughly selected 75%). Of those responses which were finally compiled, those obtained from 20 randomly selected cities, towns, and villages (17,816 responses) are analyzed in this report. The responses concerning the vaccination rates and incidence rates (cumulative, within 1 year) were classified according to the vaccines used, the diseases, and age (Figs. 1–3).

From the results shown in Fig. 1, the prevalence of diseases that can be prevented by vaccination appears to be inversely correlated with the corresponding vaccination rates, since outbreaks of measles, rubella, and pertussis are controlled by vaccination. However, the vaccination rates against mumps and varicella have not reached a level effective for the control of outbreaks. Regarding influenza, although a considerable percentage of children were vaccinated, outbreaks recurred annually, and the number of patients

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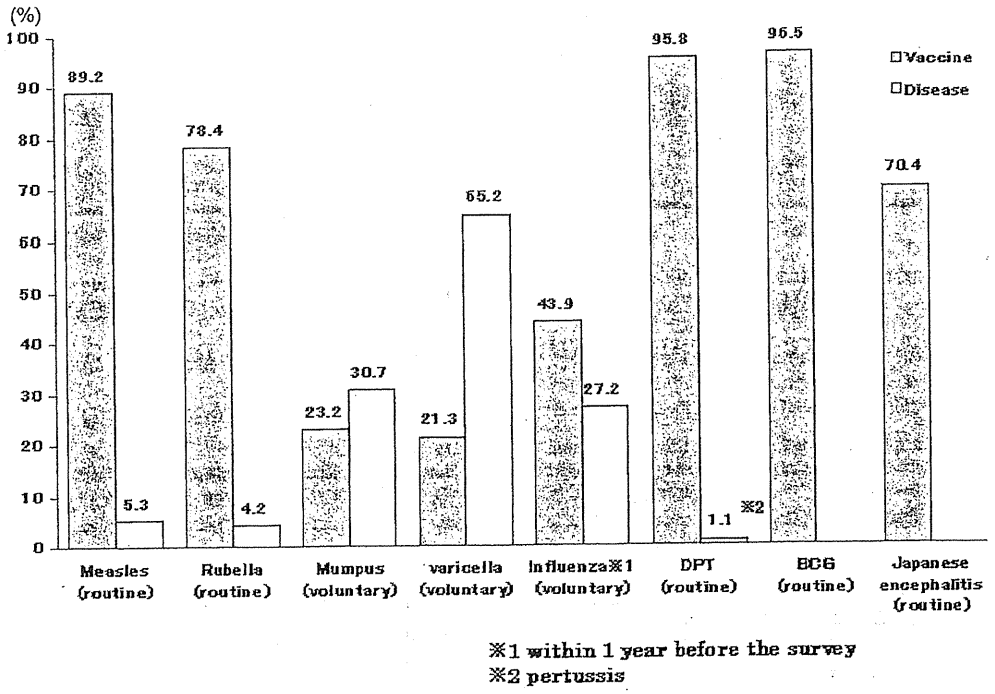
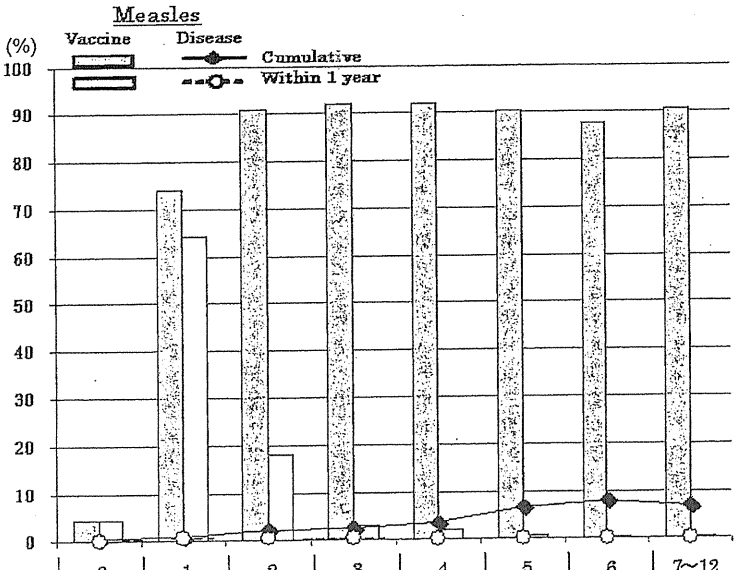


Fig. 1. Cumulative percentages of vaccination and natural infection (total number of 17,816 children aged 0–12 years in 20 cities, towns, and villages), in 2005 in Japan.

has not been reduced. The vaccination rate was 95% or higher only for BCG and DPT. The vaccination rates for other diseases, against which vaccination is performed routinely (Japanese encephalitis, measles, and rubella), were from 70 to 90%, although children will continue to contract these diseases given vaccination rates at this level. The vaccination rates for diseases against which vaccination is performed voluntarily (influenza, mumps, and varicella), are low, and outbreaks of these diseases are expected to persist at nursery schools (daycare centers), kindergartens, and elementary schools, probably explaining the high prevalence of these 3 diseases. Routine vaccination should be urgently instituted.

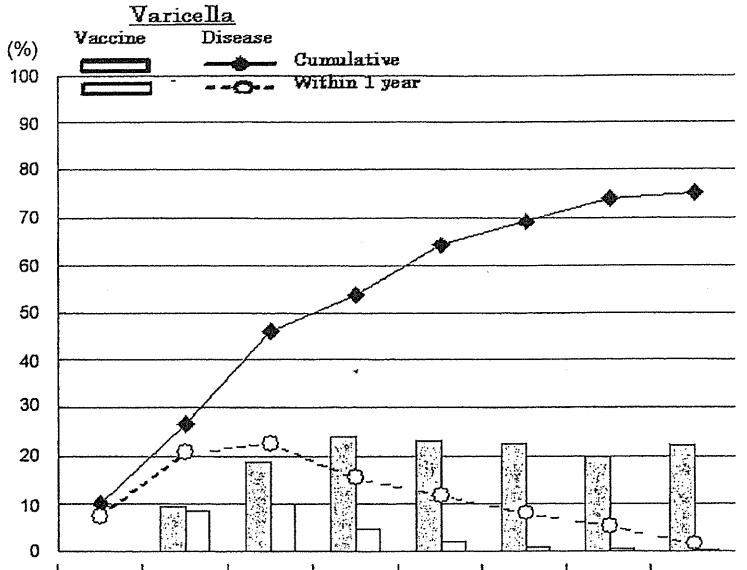
Vaccination rates against measles, along with its prevalence, prior to the introduction of the mixed measles–rubella (MR) vaccine, are shown in Fig. 2. The vaccination rate (cumulative) against measles did not exceed 90% until after the age of 2 years, and did not exceed 95% at any age level. As a result, children who had contracted measles were observed at all age levels. A rather higher percentage (64.2%) of children aged 1 year were vaccinated against



age	0	1	2	3	4	5	6	7~12
Vaccination rate	4.4(4.4)	73.9(64.2)	91.1(18.0)	92.1(3.1)	92.2(1.8)	90.4(0.6)	87.8(0.3)	90.8(0.3)
Incidence rate	0.5(0.0)	1.0(0.6)	2.3(0.4)	2.5(0.2)	3.5(0.0)	6.7(0.0)	8.0(0.1)	6.8(0.0)
Response number	181	798	1,091	2,359	2,330	2,675	1,593	6,599

cummulative (within 1 year)

Fig. 2. Vaccination rates against measles and its prevalence according to age (17,626 children aged 0–12 years in 20 cities, towns, and villages), in 2005 in Japan.



age	0	1	2	3	4	5	6	7~12
Vaccination rate	0.0(0.0)	9.2(8.5)	18.7(9.9)	23.9(4.6)	28.1(2.0)	22.7(0.8)	19.8(0.5)	22.1(0.2)
Incidence rate	9.9(7.2)	26.6(20.7)	46.1(22.6)	53.7(15.4)	64.2(11.7)	69.2(7.9)	74.2(5.3)	75.2(1.4)
Response number	180	802	1,096	2,377	2,334	2,683	1,615	6,655

cummulative (with in 1 year)

Fig. 3. Vaccination rates against varicella and its prevalence according to age (17,742 children aged 0–12 years in 20 cities, towns, and villages), in 2005 in Japan.

measles within 1 year before the survey, which means, however, that, more than 30% of children remained unvaccinated until they were 2–6 years old. It is important to strongly prompt parents to vaccinate their children with the MR vaccine as soon as they reach 1 year of age and to complete the vaccination before the age of two.

From the results on the vaccination rate against rubella and its prevalence, the vaccination rate (cumulative) against rubella did not exceed 80% until after 3 years of age, and remained at 85% or lower at all age levels. As a result, children who had contracted rubella were observed at all age levels. The percentage of children vaccinated against rubella within 1 year before the survey was highest, at 30.9% in 1-year-olds. Most children were vaccinated against this disease after the age of 2 years, and some were first vaccinated in elementary school. Parents should be strongly encouraged to vaccinate their children with the MR vaccine after the age of one, and before the age of 2 years.

From the results on the vaccination rate (cumulative) with routine DPT (1 time, 3 or more times) and the incidence rate (cumulative) of pertussis, the vaccination rate with DPT (1 time) was very high, reaching 95% or higher at the age of 2–3 years or above. The percentage of children who had received a DPT-3-or more times reached roughly 80% between the ages of 4–12 years. Consequently, the incidence rate of pertussis in these children, aged 4–12 years was lower (0.0–0.1%) than that (0.2–0.6%) in those aged 0–3 years, and was also lower than that (1.0–1.1%) in those aged 0–12 years who had received DPT one time only.

As shown in Fig. 3, the vaccination rate (cumulative) against varicella (voluntary) was very low, with only 1 of every 4–5 children having been vaccinated before entering elementary school. As a result, outbreaks of varicella recur every year at nursery schools (daycare centers), kindergartens, and elementary schools. Early implementation of free vaccination (routine) is recommended. A peak in prevalence of varicella within 1 year before the survey was observed at the age of 1–2 years. During this period, however, the vaccination rate (9.9% and 8.5% in 2-year-olds and 1-year-olds, respectively) was too low to prevent outbreaks of varicella. It is important to guide parents in having their children vaccinated as early as possible after the age of 1 year, so as to immunize younger children prior to exposure to epidemic varicella.

From the results on the vaccination rate against mumps and its prevalence, the vaccination rate (cumulative) against mumps was low (<25% among children 3–5 years of age), and only 1 of every 4–5 children had been vaccinated upon admission to elementary school. For this reason, outbreaks of mumps recur every year at nursery schools (daycare centers), kindergartens, and elementary schools. Early implementation of routine vaccination is recommended. The vaccination rate within 1 year before the survey was highest in 2-year-olds, at 9.1%. Since a peak in prevalence is observed between the ages of 3–6 years, routine vaccination of children at the lower ages of 1–2 years old is desirable as a measure to control mumps outbreaks in nursery schools (daycare centers) and kindergartens.

From the results on the vaccination rate against influenza and its prevalence in 2005 (within 1 year), was not low (50% among children 3–6 years of age), compared with that (36.9%) among elementary school children aged 7–12 years old, even though vaccination was being performed voluntarily. However, the incidence rate was higher (31%) in the younger children than in elementary school children (25%). The development of effective vaccines is urgently anticipated.

Regarding the vaccination attitude against measles, rubella, mumps, varicella, and influenza during the past 1 year before the survey, the frequency order of diseases against which 1-year-old children had been vaccinated was, measles (64.2% in Fig. 2), rubella (30.9%), influenza (25.3%), varicella (8.5% in Fig. 3), and mumps (7.4%). Two-year-old children were vaccinated in the frequency order: influenza (47.8%), rubella (23.1%), measles (18.0% in Fig. 2), varicella (9.9% in Fig. 3), and mumps (9.1%). The order of diseases against which children aged 3–6 years were vaccinated was: influenza (45.9–53.6%), rubella (1.3–5.6%), mumps (1.2–5.6%), varicella (0.5–4.6% in Fig. 3), and measles (0.3–3.1% in Fig. 2). The age at which the highest percentage of children were vaccinated was 1 year for measles (64.2% in Fig. 2), and rubella (30.9%), 2 years for

varicella (9.9% in Fig. 3), and mumps (9.1%), and 3 years for influenza (53.6%). The prevalence of the diseases in children aged 0–12 years during the past 1 year ranged in the order of influenza (27.2%), varicella (8.2% in Fig. 3), and mumps (8.0%), but was low for measles (0.1% in Fig. 2), and rubella (0.1%).

In order to achieve prevention of infectious diseases via elevation of the vaccination rate, the (1) diseases that show repeated outbreaks, and high-risk age groups, (2) effectiveness and safety of vaccines against the diseases, and (3) vaccination attitude of the susceptible population should be analyzed, and the obtained results should be used for (1) reevaluation of the vaccination strategy and (2) improvement in cooperation between the local government and physicians who perform vaccinations. In routine vaccinations, people who wish to be vaccinated visit their family doctors during the period stipulated by the law. The MR vaccine was newly introduced on April 1, 2006 due to an amendment of the preventive vaccination law. This amendment requires efforts to vaccinate 1-year-old children before reaching 2 years old with the intention of eradicating measles and rubella, by having most children (≧95%) vaccinated against these diseases while 1 year old. However in this questionnaire survey, the vaccination rate during the past 1 year was 64.2% for measles (Fig. 2) and 30.9% for rubella, both of which are low. In the campaign to elevate these vaccination rates to 95% or above by the introduction of the MR vaccine, there may be regional differences in the results depending on the promotional abilities of local governments, which are responsible for organizing vaccination activities. It is our wish that the valuable results of this survey, which could only be performed due to the understanding and voluntary cooperation of parents and local government officials, be used again for infectious disease control with the cooperation of physicians. Further attention to the vaccination rate of 1-year-old children with the MR vaccine in the years ahead is a focus of interest.

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Appendix A. Web sites that contain information about vaccines and vaccine preventable diseases

1. National Immunization Program (NIP) (www.cdc.gov/nip)
2. National Vaccine Program Office (NVPO) (www.cdc.gov/od/nvpo)
3. Children's Vaccine Program at PATH (www.childrensvaccine.org)
4. WHO's Vaccine Preventable Diseases Monitoring System (www.who.int/immunization_monitoring/en/globalsummary/countryprofileselect.cfm)
5. American Academy of Pediatrics (AAP) (www.cispimmunize.org)
6. National Foundation for Infectious Diseases (NFID) (www.nfid.org)
7. Varicella-Zoster Virus Research Foundation (VZVRF) (www.vzvfoundation.org)
8. National Institute of Infectious Diseases (NIID) (www.nih.go.jp)
9. Osaka Prefectural Institute of Public Health (www.iph.pref.osaka.jp)
10. The Research Foundation for Microbial Diseases of Osaka University (www.biken.or.jp/about/outline.html)

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A need for careful evaluation of endotoxin contents in acellular pertussis-based combination vaccines

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ABSTRACT

Two batches each of diphtheria-tetanus-acellular pertussis vaccine (DTaP) and that combined with inactivated polio vaccine purchased from foreign markets were tested by mouse body weight decreasing (BWD) toxicity test and Limulus amaebocyte lysate (LAL) test. Three out of the four imported vaccine batches showed the levels of BWD toxicity even comparable to that of DT-whole cell pertussis vaccine. BWD toxicity test is based on endotoxin dose-dependent weight loss of mice and has been used for controlling endotoxin in DTaP. Although of the strong BWD toxicity of the imported vaccines, there was no marked difference in LAL test results between the imported vaccines and Japanese DTaP. However, one imported DTaP batch showed very strong interference with LAL activity of spiked lipopolysaccharide (LPS). The batch interfered not only with LAL activity but also with pyrogenicity and prostaglandin E_2 induction activity. However, the pyrogenicity of the spiked LPS could be recovered from the precipitated fraction of the batch by treating with phosphate buffer to suggest the possibility of recovering *in vivo* toxicity. As an adequate *in vitro* test method could not be identified for controlling the safety of the interfering batch, an appropriate *in vivo* test would be required for testing such vaccines.

1. Introduction

Although diphtheria-tetanus-whole cell pertussis combination vaccine (DTwP) had been playing the role of effective prophylaxis against the diseases, immunization with DTwP was temporarily suspended in Japan after two cases of post vaccination death in the mid 1970s. To address the public concern over the safety of the vaccination, diphtheria-tetanus-acellular pertussis combination vaccine (DTaP) was developed and introduced for routine use in 1981 [1]. Development of Japanese DTaP targeted to reduce residual toxic activities detected in mice following immunization of DTwP to the level of less than one-tenth of average DTwP [2]. Quantitative laboratory test methods for assessing residual toxicities of acellular pertussis vaccine (aP) were developed [3] and implemented in Minimum Requirements for Biological Products of Japan (Minimum Requirements) in 1981 [4]. The mouse body weight decreasing (BWD) toxicity test is based on the linear dose–response relationship of mouse weight loss and log dose of whole cell pertussis vaccine (wP) or endotoxin [3]. Although the test could be affected by other constituents such as aluminium adjuvant, it was applied to effectively control endotoxin contents in DTaP batches. However, limitation in utility of the test became obvious due to its limited sensitivity and decreased endotoxin contents in the recent batches of Japanese DTaP. Thus the endotoxin test using Limulus amaebocyte lysate (LAL) was implemented for testing DTaP in 2004 [5]. The endotoxin test (LAL test) is based on highly sensitive clotting of LAL in the presence of endotoxin [6]. The conventional LAL reagent was reported to react not only with endotoxin but also with a cell wall component of fungi, (1-3)-β-D-glucan and some filter membrane components [7]. Methods and lysate reagents for the test have been remarkably improved to allow its practical application to a wide range of pharmaceuticals [8–10]. In particular, the specificity of lysate reagents to detect endotoxin was improved by the removal or suppression of factor G [11,12] in lysate reagents, which eliminated the reactivity to (1-3)-β-D-glucan and other non-pyrogenic substances.

DTaPs of various formulations were developed and implemented in Europe and the USA since the late 1990s and, accordingly, the World Health Organization (WHO) issued its first guidelines for the production and control of the acellular pertussis component of monovalent or combined vaccines in 1996 (WHO guidelines) [13]. The LAL test was prescribed in the WHO guidelines for testing residual endotoxin in DTaPs. However, it was reported previously that there were DTaP batches that strongly interfered with LAL activity of endotoxin without affecting pyrogenicity in rabbits [14]

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but that the interfering effect on LAL activity could be attenuated by treating with phosphate buffer (PB) [15]. We tested for endotoxin contents in DTaP and DTaP combined with inactivated polio vaccine (DTaP-IPV) purchased from European, the U.S.A and Asian markets in comparison with Japanese DTaP by the BWD toxicity test, LAL test, rabbit pyrogen test and an *in vitro* pyrogen test based on prostaglandin E_2 (PGE2) induction in rabbit peripheral blood [16].

The imported vaccines showed very strong BWD toxicity in spite of showing no significant difference in LAL test results from that of Japanese DTaP. Furthermore, one of the imported vaccine batches very strongly interfered with endotoxin activities. We made a detailed analysis on the characteristics of the strong interfering effect of the batch on endotoxin activities and its possible impact on the safety control for endotoxin in aP-based combination vaccines is discussed in the present paper.

2. Materials and methods

2.1. Reference pertussis vaccine for toxicity tests

Reference pertussis vaccine for toxicity tests Lot 2 (Reference vaccine), which is a lyophilized whole cell preparation of inactivated pertussis organisms being used for the National Control Tests of pertussis vaccines in Japan, was used [5]. Its assigned unit value of BWD toxicity was 1368 Body Weight Decreasing Units (BWDU) per vial. A vial of the vaccine was reconstituted in 12 ml of pyrogen-free physiological saline (Otsuka Pharmaceutical Co., Ltd., Tokyo) and was serially diluted at four fold intervals from 1 in 1 to 1 in 64 for use.

2.2. Vaccines

Two batches each of DTaP and DTaP-IPV used in the present study were those purchased from the European, the U.S.A. and Asian markets. Japanese DTaPs were those submitted for National Control Tests during 1999 and 2005. Formulations of the vaccines are shown in Table 1.

2.3. Toxicity test in mice

The BWD toxicity test was performed according to Minimum Requirements. In brief, groups of ten mice each were intraperitoneally injected with 0.5 mL of a test vaccine or a dilution of the Reference vaccine diluted serially at four fold intervals. Body weight change of the mice during 16 h after injection was measured for testing BWD toxicity [17]. BWD toxicity of a test vaccine was calculated relative to that of the Reference vaccine according to the parallel line assay method using logarithmic dose and the body weight change to express the results as BWDU/mL [18].

2.4. Endotoxin

Japanese Pharmacopoeia Reference Standard Endotoxin Lot 3 (RSE), which is a lyophilized preparation of Westphal endotoxin extracted from *Escherichia coli* UKT-B strain (13,000 endotoxin units (EU) per vial) [19], was used as the standard preparation in the LAL test.

Lipopolysaccharide (LPS) extracted and purified from *B. pertussis* phase I Tohama strain by Westphal's phenol water method [20] (Bp-LPS) was used for spiking to test vaccines in a suppression/enhancement test to examine an interfering effect of a vaccine. The EU value of Bp-LPS was measured relative to the activity of RSE. For the suppression/enhancement test, a test vaccine was spiked with Bp-LPS at a final concentration of 100 EU/mL and kept at 4 °C for one week before measurement if not otherwise stated.

2.5. The LAL test

Test samples, RSE and Bp-LPS were serially diluted at four-fold intervals with pyrogen-free distilled water (Otsuka Pharmaceutical Co., Ltd., Tokyo). A 50 μL-volume of a dilution was mixed with the equal volume of an endotoxin specific LAL regent (Endospecy; Seikagaku Biobusiness Corp., Tokyo) of which reactivity to (1-3)-β-D-glucan was eliminated [7]. LAL activity was measured as the rate of color development using a specially equipped microplate reader (Well Reader SK603; Seikagaku Biobusiness Corp., Tokyo) [11,21]. Endotoxin content of a test sample was calculated relative to that of RSE according to the parallel line assay method using logarithmic values of dose and rate of color development to express as EU/mL.

2.6. The rabbit pyrogen test

Test vaccines were mixed with one in ten volume of 1000 EU/mL of Bp-LPS. Bp-LPS in physiological saline (100 EU/mL) was served as the control endotoxin. Female rabbits of Japanese white strain (Kitayama Labes Co., Ltd., Nagano or Japan Laboratory Animals, Inc., Tokyo) weighing approximately 3 kg were housed in cages separately in an air-conditioned animal room. Three animals were allocated to each treatment. They were intravenously injected with 1 mL/kg of a test sample. Rectal temperature of the animals was monitored for 3 h using an electric thermometer (Scanner Unit X115 with High Accurate Data Logger K730, TECHNOL SEVEN, Kanagawa, Japan). The maximum rise in rectal temperature during

Table 1
Formulation of vaccines.

	Vaccines	PT[a] μg/dose	FHA[b] μg/dose	PRN[c] μg/dose	Fimbrie μg/dose	Dtd[d]	Ttd[e]	IPV			Aluminum salt	Al^{+++} mg/dose
								Type 1[f]	Type 2[f]	Type 3[f]		
A	DTaP	25	25	8		25Lf[g]	10Lf[g]				Hydroxide	0.5
B	DTaP	10	5	3	5	15Lf[g]	5Lf[g]				Phosphate	0.33
C	DTaP-IPV	25	25	8		25Lf[g]	10Lf[g]	40	8	32	Hydroxide	0.5
D	DTaP-IPV	25	25			≧30IU[h]	≧40IU[h]	40	8	32	Hydroxide	0.3
J	DTaP	23.4	23.4			≦15Lf[g]	≦2.5Lf[g]				Phosphate	0.08

[a] PT:pertussis toxin.
[b] FHA:filamentous haemagglutinin.
[c] PRN:pertactin.
[d] Dtd:diphtheria toxoid.
[e] Ttd:Tetanus toxoid.
[f] D-antigen units of poliovirus/dose.
[g] Lf:Limit of floculation units/dose.
[h] IU:International Units/dose.

3 h was taken as the pyrogenic response of each rabbit. Pyrogenicity of a test sample was expressed as mean maximum temperature rise of 3 rabbits during 3 h after injection.

2.7. In vitro PGE2 induction assay

The assay was carried out according to the method of Ochiai et al. [16,22]. Although blood from different rabbits show different responsiveness to endotoxin in PGE2 production, results are consistent and reproducible even in blood from different rabbits so far as assessing the activity of vaccine samples in relative to the response to the standard endotoxin using blood collected from a single rabbit. Therefore, each assay was performed with blood from one rabbit and replicated using blood from the indicated number of rabbits in tables and figure. Briefly, a 100 μL-volume of an appropriate dilution of a test sample or a dilution of serially diluted Bp-LPS was gently mixed with 150 μL of heparinized fresh blood of a rabbit (Kitayama Labes Co., Ltd., Nagano or Japan Laboratory Animals, Inc., Tokyo) in a pyrogen-free centrifuge tube (Assist Co., Ltd, Tokyo) containing 750 μL of pyrogen-free physiological saline. The mixture was incubated at 37 °C for 8 h. Supernatants of the mixtures were isolated by centrifuging at 500 × g for 2 min and stored at −20 °C until use. PGE2 concentration of the supernatants was assayed by a commercial enzyme-linked immunoassay (EIA) kit (Prostaglandin E_2 High Sensitivity Correlate-EIA, Assay Designs, Inc., MI., USA) [16]. The activity of a test sample was calculated relative to that of Bp-LPS according to the parallel line assay method using logarithmic values of dose and PGE2 concentration to express as EU-equivalent/mL.

2.8. Statistic analysis

Analysis of the data of parallel line assay was carried out according to Finney's method [18]. Significance and validity tests were made at a level of $P = 0.05$.

3. Results

3.1. Toxicity to mouse body weight gain (BWD toxicity)

BWD toxicity test is based on the linear dose-dependent weight loss of mice received serial dilutions of the Reference vaccine or endotoxin [4]. The vaccines were tested for BWD toxicity as described in Materials and methods and results were summarized in Table 2. In the table, BWD toxicity unit values of the imported vaccines are represented with their 95% confidence intervals and those of Japanese DTaP and DTwP were represented as geometric mean unit values of lots tested during 1999 and 2005 and before 1981, respectively. The imported vaccine batches showed strong BWD toxicity comparing to that of Japanese DTaPs. The level of BWD toxicity of batch D was slightly higher than that of average Japanese DTaP but other three batches (A, B and C) showed the levels of BWD toxicity comparable or even excess to that of Japanese DTwPs (Table 2).

Table 2
BWD toxicities of DTaP and DTwP vaccines.

Vaccines	BWD activity	
	BWDU/mL	95% C.I.[b]
Imported vaccine		
A (DTaP)	58.86	(31.11–111.36)
B (DTaP)	100.79	(39.92–254.47)
C (DTaP-IPV)	334.05	(66.98–1666.05)
D (DTaP-IPV)	19.63	(8.98–42.89)
Japanese vaccines		
Acellular(1999 ~ 2005) ($N = 158$)	9.06	
Whole cell(~ 1981) ($N = 176$)	56.4	
Control[a]	4.88	
Japanese Minimum Requirements for DTaP	≦10	

[a] Normal mice were measured as a control group.
[b] 95% confidence interval.

3.2. Endotoxin contents in vaccines

The BWD toxicity test was initially implemented for controlling mainly residual endotoxin in DTaP [3,23] but was possibly affected by various vaccine constituents such as adjuvant gel [24]. We tested the imported vaccine samples by the LAL test to compare their endotoxin contents with that of Japanese DTaP. Amounts of endotoxin detected for imported vaccine batches A, B, C and D were 0.05, 0.13, 0.04 and 0.25 EU/mL, respectively, and were not much higher comparing to the content in Japanese DTaP of which average endotoxin content in batches tested during 1999 and 2005 was 0.024 EU/mL.

For verification of the LAL test, the imported vaccine batches and a typical Japanese DTaP batch were spiked with LPS (RSE or Bp-LPS) and kept at 4 °C to monitor the change in detectable LAL activity. The LAL tests were carried out using several dilutions of imported batches spiked with RSE or Bp-LPS and endotoxin contents of test samples were calculated according to the parallel line assay method. As a representative experiment, Bp-LPS was spiked to undiluted vaccines at 100 EU/mL and kept at 4 °C to monitor the change in detectable LAL activity for 15 days and the results were summarized in Fig. 1. In the figure, weighted means of 5 batches from 5 Japanese manufacturers are shown as the results of Japanese DTaP batches. The imported vaccine batch A markedly interfered with LAL activity of Bp-LPS, while other imported vaccines and the Japanese vaccines did not show any significant effect on LAL activity of the spiked LPS. An obvious interfering effect of batch A on Bp-LPS was seen immediately after spiking and reached the maximum level of over 90% suppression by day 3 and no further change was seen until day 15.

We previously reported that Japanese DTaPs generally do not show interfering effect on endotoxin activities except for one

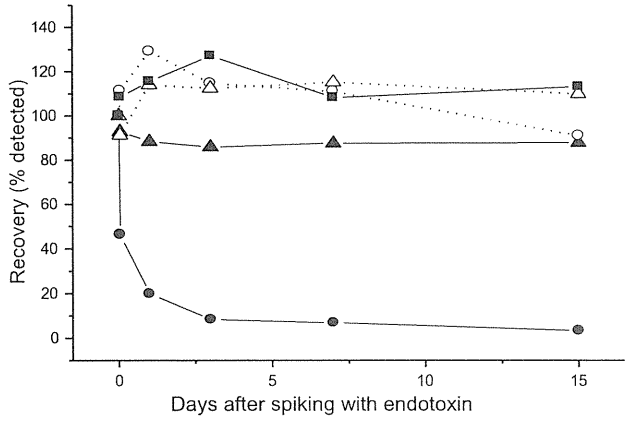

Fig. 1. Kinetics of interfering effect of acellular pertussis-based combination vaccines on LAL activity of Bp-LPS. DTaP batches A (●), B(▲) and Japanese DTaP batches (■), DTaP-IPV batches C(○) and D (△) were spiked with Bp-LPS at 100 EU/mL and stored to monitor the change in detectable LAL activity at 4 °C for 1 h, 1, 3, 7 and 15 days. The results of Japanese batches represent weighted means of 5 batches 5 five Japanese manufacturers. Batch A markedly interfered with LAL activity of Bp-LPS, while other batches showed no effect. Vertical bars represent 95% confidence intervals of recovery values.

DTaP product that interfered with LAL activity of spiked LPS without affecting pyrogenicity [14]. We, therefore, examined the effects of batch A on biological activities of endotoxin by the LAL test, pyrogen test and *in vitro* PGE2 induction assay to compare with a typical Japanese DTaP. Batch A and the Japanese DTaP were spiked with Bp-LPS at 100 EU/mL and kept at 4 °C for one week to monitor detectable LAL activity, *in vitro* PGE2 induction activity and pyrogenicity. Batch A strongly interfered with PGE2 induction activity and pyrogenicity as well as LAL activity of Bp-LPS while the Japanese DTaP did not show such interference with any of the activities (Table .3). As a result, detectable LAL and PGE2 induction activities of Bp-LPS spiked to batch A were suppressed to the level below 1.9% of the activities of the control Bp-LPS in saline, while those of Bp-LPS spiked to Japanese DTaP were not significantly suppressed but even seemed slightly enhanced as seen in Table 3.

3.3. Inhibitory factors in the vaccine

The possibility of attributing the strong interfering effect of DTaP batch A to strong adsorption of LPS to adjuvant gel was examined. DTaP batch A spiked with Bp-LPS at 100 EU/mL and kept at 4 °C for 3 or 4 days was centrifuged to separate supernatant and precipitate. When the supernatant was examined by LAL test, only 0.03 EU/mL of the activity was detected. However, without spiking with LPS, 0.04 EU/mL was detected for supernatant of batch A and, therefore, no change in LAL activity was seen for the supernatant even by spiking the vaccine with 100 EU/mL of LPS (Table 4). PGE2 induction activity was also measured for supernatant of batch A isolated after spiking with Bp-LPS at 100 EU/mL to detect only 2.20 EU-equivalent/mL (Table 4). When supernatant of batch A was isolated and then spiked with Bp-LPS at 100 EU/mL to examine the effects on the activities of LPS, LAL activity detected was only about 16 EU/mL even in the absence of aluminium gel, while pyrogenicity and PGE2 induction activity were not so much affected from the levels of their controls (Table 4).

As only trace LAL activity could be detected when directly assessed for batch A spiked with Bp-LPS at 100 EU/mL (Table 3), it was necessary to apply a special measure for attenuating the interfering effect to assess LAL activity of precipitate. Treating with 0.25 M PB could completely abolish the effect of Japanese interfering DTaP on LAL activity of spiked LPS [15]. Batch A was spiked with LPS and treated with 0.25 M PB directly but no marked effect of PB treatment was seen on the interference to LAL activity (Data not shown). For further characterization of the interfering effect, precipitate of batch A isolated after spiking with Bp-LPS at 100 EU/mL was treated with 0.25 M PB to examine the effect of PB treatment. In spite of the strong interfering effect of batch A, pyrogenicity could become detectable by treating the precipitate with 0.25 M PB, and some extents of LAL and PGE2 induction activities were also recovered by the treatment (Table 4). Other than the control Bp-LPS in saline we used also the control Bp-LPS in 0.25M PB to monitor the effect of saline and 0.25 M PB on Bp-LPS. Comparison of the two control Bp-LPS preparations indicates that although no difference in pyrogenicity and PGE2 induction activity, LAL activity of Bp-LPS in saline might have partially declined comparing to that of Bp-LPS in 0.25 M PB during one week of storage. In spite of the change in LAL activity of the Bp-LPS in saline, results of LAL test were not affected by the change so far as assessing in relative to RSE as in Materials and methods.

4. Discussion

Acellular pertussis-based combination vaccines are playing the essential role in immunization programs in many nations. When the first aP was developed and implemented in 1981, no LAL reagent that has sufficient specificity to LPS was available and the validation of the test method based on the correlation with pyrogen test was not feasible. BWD test based on endotoxin dose-dependent weight loss of mice was, therefore, implemented for controlling endotoxin in aP in Japan. The test contributed to reduce febrile response rate of DTaP vaccinees [3,25],

We tested for BWD toxicity of aP-based combination vaccines from the European, USA and Asian markets. Three out of the four imported vaccine batches showed the levels of BWD toxicity significantly excess over the limit value of 10 BWDU/mL and the levels of the toxicity were even comparable to that of Japanese DTwP (Table 2). Although BWD toxicity should basically correlate with endotoxin content [3,23], the test is not very specific for detecting endotoxin and might be affected by various vaccine constituents such as adjuvant gel and characteristics of antigens [24,26]. Therefore, higher concentrations of aluminum adjuvant in the imported vaccines could be a cause of the strong BWD toxicity but actual reason for the strong BWD toxicity could not be fully elucidated in the present study. Further investigation would be necessary for the causal mechanism of the strong toxicity to mouse weight and its possibility of relevance to reactogenicity.

Although of the contribution of BWD toxicity test in the control of residual endotoxin in Japanese DTaP batches in the 1980s [3,4], the test is not sensitive enough for evaluating recent batches of the vaccine due to marked reduction in residual endotoxin [27]. Specificity of LAL reagents to LPS was remarkably improved by eliminating or suppressing the reactivity to non-pyrogenic (1-3)-β-D-glucan in the 1980s [7] and the validation based on the correlation with pyrogen test became feasible. Accordingly, LAL test was implemented for testing DTaPs in Japan in 2004. In the course of the validation, we experienced a Japanese DTaP product that strongly interfered with LAL test without affecting pyrogenicity [14]. For such a product, LAL test is not relevant to the safety control. However, the interfering effect of the vaccine could be

Table 3
Effect of DTaP vaccines on the activities of LPS.

Samples	LAL test		Pyrogenicity		PGE_2 induction	
	EU/ml	95% C.I.[b]	Delta T (°C)[c]	95% C.I.[b]	EU-equivalent/ml[d]	95% C.I.[b]
Saline + LPS[a]	78.11	(67.41–90.50)	0.83	(0.48–1.18)	100	
Japanese DTaP	0.03	(0.02–0.03)	0.05	(−0.01–0.11)	0.95	(0.77–1.17)
Japanese DTaP + LPS[a]	141.79	(127.64–157.51)	1.32	(1.04–1.59)	153.03	(130.13–179.96)
DTaP batch A	0.05	(0.05–0.06)	0.24	(0.04–0.43)	1.87	(1.57–2.23)
DTaP batch A + LPS[a]	2.58	(2.31–2.87)	0.33	(0.14–0.52)	5.71	(5.04–6.47)

[a] Kept at 4 °C for 1 week after spiking with Bp-LPS at 100 EU/mL.
[b] 95% confidence interval.
[c] Mean of maximum rise in rectal temperature of 6 rabbits during 3 h.
[d] PGE_2 induction activity calculated in reference to that of Bp-LPS to express as EU-equivalent. The results represent weighted means of 7 independent measurements.

Table 4
Effect of DTaP batch A on the activities of LPS.

Samples	LAL test		Pyrogenicity		PGE_2 induction	
	EU/ml	95% C.I.[e]	Delta T (°C)[f]	95% C.I.[e]	EU-equivalent/ml[g]	95% C.I.[e]
Saline + LPS[a]	75.81	(67.57–85.06)	1.02	(0.28–1.76)	100	
0.25M PB + LPS[a]	115.57	(104.99–127.22)	1.04	(0.62–1.46)	118.70	(56.50–249.35)
(batch A) Sup	0.04	(0.02–0.07)	N.D		N.D	
(batch A + LPS) Sup[b]	0.03	(0.02–0.04)	N.D		2.20	(1.62–2.97)
(batch A) Sup + LPS[c]	15.95	(14.20–17.91)	0.88	(0.09–1.68)	82.76	(54.40–125.88)
(batch A + LPS) ppt + 0.25M PB[d]	38.52	(35.19–42.16)	0.77	(0.40–1.15)	17.05	(11.19–25.98)

[a] Kept at 4 °C for 1 week after spiking with Bp-LPS at 100 EU/mL.
[b] Kept at 4 °C for 3 or 4 days after spiking with Bp-LPS at 100 EU/mL to isolate supernatant by a centrigugation.
[c] Kept at 4 °C for 1 week before centrifuging and the supernatant was spiked with BP-LPS at 100 EU/mL to keep at 4 °C for another 1 week.
[d] Kept at 4 °C for 1 week after spiking with Bp-LPS at 100 EU/mL and the centrifuged precipitate was treated with phosphate buffer at 4 °C for 1 week.
[e] 95% confidence interval.
[f] Mean of maximum rise in rectal temperature of 3 rabbits during 3 h.
[g] PGE_2 induction activity calculated in reference to that of Bp-LPS to express as EU-equivalent. The results represent weighted means of 4 independent measurements.

eliminated by treating with 0.25 M PB and LAL test became applicable [15].

We attempted to validate LAL test for the imported aP-based combination vaccines. One of the vaccine batches purchased from the European market, batch A, very strongly interfered with LAL activity of Bp-LPS while no such interference was seen for other batches (Fig. 1). The strong interference to LAL activity was also seen when used LAL reagent for kinetic-turbidimetric and gelation assay from another manufacturer. Recoveries of LAL activity of Bp-LPS spiked at 100 EU/mL to batch A were 4.8% and only 0.01% after 1 h and 1 day storage, respectively (data not shown). Batch A suppressed not only LAL activity but also *in vitro* PGE2 induction activity and pyrogenicity of Bp-LPS (Table 3). Batch A showed significant interfering effect on LAL activity of Bp-LPS immediately after spiking and the effect reached the maximum level of over 90% suppression by day 3 (Fig. 1). When batch A was spiked with Bp-LPS at 100 EU/mL and supernatant was isolated after keeping at 4 °C for 3 or 4 days, only trace amounts of LAL activity could be detected in the supernatant and the level was equivalent to the activity of supernatant of the vaccine without spiking (Table 4). Furthermore, PGE2 induction activity of spiked Bp-LPS detected in the supernatant was only 2.2 EU-equivalent/mL.

When supernatant of batch A was isolated and spiked with Bp-LPS at 100 EU/mL, LAL activity detected was only about 16 EU/mL to suggest even without adjuvant gel, supernatant alone would suppress LAL activity. However, the supernatant did not significantly affect PGE2 induction activity and pyrogenicity (Table 4). These results indicate that the strong interference by batch A was mainly attributable to strong adsorption of LPS to adjuvant gel.

As cation was reported to impede the stability of LAL activity [28], aluminium ion, if exists, may interfere with LAL activity of LPS. However, when measured aluminium content in the supernatant of DTaP batch A using aluminium analysis kit [29], no aluminium ion was detected (data not shown). Furthermore, dispersion state of LPS was reported to influence activities of endotoxin [30]. The difference in the effect of supernatant on LAL and other activities might have reflected the difference in dispersion states of LPS in reaction mixtures for LAL test and other tests. The effect of dispersion state on LAL activity may also explain the slight but significant difference in LAL activities of the two LPS controls, Bp-LPS in saline and that in 0.25 M PB, in Table 4. However, the suppression of LAL activity by supernatant was much more extensive comparing to the difference of the Bp-LPS controls to suggest involvement of another mechanism.

Batch A had the similar constituents of DTaP antigens and aluminum to those of batch C differing in combination with IPV (Table 1). Although batch A and batch C contained the equal amount of aluminium hydroxide gel, the batches behaved in significantly different ways in interference with activities of LPS. This difference and the interfering effect of batch A supernatant particularly on LAL activity may be suggesting additional ingredients that influence dispersion state of LPS in batch A such as a higher concentration of detergent. However, actual cause of the difference in interfering effect of the batches on LPS activity could not be identified in the present study. Consequently, LAL test was suppressed not only by aluminium adjuvant but also by supernatant and, therefore, LAL test could not be relevant to safety control for vaccine products with the similar property as batch A.

Batch A was spiked with Bp-LPS at 100 EU/mL and precipitate was isolated for treating with 0.25 M PB. Although the PB treatment completely abolished interfering effect of Japanese interfering DTaP, interfering effect of batch A could be attenuated only partially by PB treatment to recover 38.5 EU/mL and 17.05 EU-equivalent/mL of LAL and PGE2 induction activities, respectively. On the other hand, of approximately 1.0 °C of pyrogenicity of the control Bp-LPS in saline, 0.77 °C of pyrogenicity was recovered by treating the batch A precipitate with 0.25 M PB (Table 4).

Consequently, batch A strongly suppressed activities of spiked LPS by the strong adsorption to adjuvant gel but *in vivo* pyrogenicity might readily be recovered to the most significant extent from the precipitate by treating with PB comparing to *in vitro* activities. This may suggest that the adsorption of LPS to adjuvant gel is stable without PB treatment, but the treatment with PB may facilitate to release when further injected intraveneously to rabbits. Therefore, endotoxin contaminated in such vaccines may readily lose *in vitro* activities but may still retain *in vivo* activities such as pyrogenicity. Monocyte activation test (MAT), which is a novel test method to detect or quantify pyrogens that activate human monocytes or monocytic cells to release endogenous mediators such as pro-inflammatory cytokines, has been implemented in the European Pharmacopoeia as a replacement for the pyrogen test [9]. Therefore, we examined if the MAT using human monocytoid cell line, 28SC cells [31] can be applied to measure endotoxin activity in batch A, but the cell line assay was found not applicable due to very strong cytotoxicity of the vaccine. An appropriate *in vitro* control measure for the safety of such a vaccine could not be identified in the present study and, therefore, an appropriate *in vivo* control test would be required, at least, where control tests on final products are mandatory for approving release of vaccine batches like batch A

until a suitable *in vitro* method could be developed for testing such vaccine products.

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Antigen-loaded dissolving microneedle array as a novel tool for percutaneous vaccination

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ABSTRACT

Antigen-loaded dissolving microneedle array (dMNA) patches were investigated as novel systems for vaccine delivery into the skin, where immuno-competent dendritic cells are densely distributed. We fabricated micron-scale needles arrayed on patches, using chondroitin sulfate mixed with a model antigen, ovalbumin. Insertion of dMNA effectively delivered substantial amounts of ovalbumin into the skin within 3 min and induced robust antigen-specific antibody responses in the sera of mice. The antibody dose–response relationship showed that the efficiency of dMNA patch immunization was comparable to that of conventional intradermal injections. Thus, Antigen-loaded dMNA patches are a promising antigen-delivery system for percutaneous vaccination.

1. Introduction

Skin is not only a physical but also an immunological barrier that protects the body against exogenous insults. A large number of professional antigen-presenting cells, including Langerhans cells and dermal dendritic cells, populate epidermal and dermal layers of the skin and sentinel exogenous pathogens [1,2]. Therefore, the skin has been considered as an ideal target for vaccine delivery. Many studies have reported that intradermal vaccine delivery could result in more superior immune responses quantitatively and/or qualitatively than would subcutaneous or intramuscular vaccine routes [3–7]. However, the majority of vaccines are commonly inoculated subcutaneously or intramuscularly using needles and syringes, since the so-called Mantoux technique, intradermal administration by a conventional needle and syringe, requires special skills for correct application.

In recent years, microneedles (MNs) have emerged as a novel tool for percutaneous drug delivery [7–12]. Progress in microelectronics technology has enabled generation of micron-scale needles that can penetrate the *stratum corneum* and reliably deliver drugs into the epidermal or dermal skin layers. In particular, MN array patches carrying antigens are attractive because they can be manufactured cost-effectively and potentially allow vaccine self-administration in a simple and painless manner. The titanium MN array, Macroflux®, coated with a model antigen, ovalbumin (OVA), showed significant dose sparing effect [8]. The polyvinylpyrrolidone based dissolving MN patches encapsulating inactivated influenza virus vaccine successfully induced protective immunity against the lethal challenge with homologous influenza virus [12]. Especially, dissolving MNs are attractive because they generate no sharp biohazardous waste.

We have developed a technology to produce MNs by using water-soluble, thread-forming biopolymers, chondroitin sulfate or dextrin. MNs are assembled into a 10×10 array on a patch called a dissolving microneedle array (dMNA) patch in this study [13,14]. We have succeeded in encapsulating various bioactive macromolecules, including peptides and proteins formulated as solid dispersions into MNs. The designed MNs are strong and sharp enough to penetrate the *stratum corneum* upon insertion into the skin and dissolve within few minutes, releasing the loaded macromolecules. Thus far, we have reported that dissolving MNs can successfully deliver various bioactive macromolecules, such

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as insulin, erythropoietin, interferon-α, human growth hormone, or low-molecular-weight heparin percutaneously in experimental animals [15–20].

In this study, we encapsulated a model antigen, ovalbumin, into dMNA patches and investigated the potency of these patches to induce antigen-specific antibody responses in mice.

2. Materials and methods

2.1. Mice

We used female BALB/c mice (Japan SLC Inc., Hamamatsu, Japan), aged 7–10 weeks at the time of the primary immunization.

Animals were housed in a specific pathogen-free facility and given free access to water and food. The use of animals and study protocols were approved by our institutional animal care and use committee.

2.2. Antigen and adjuvant

Ovalbumin (OVA) and cholera toxin (CT) were purchased from Sigma (St. Louis, Mo, USA).

2.3. Fabrication of antigen-loaded, two-layered dMNA patches

To make a concentrated antigen solution, "drug glue", a required quantity of OVA according to an intended dosage, and 0.5 mg Evans blue (EB; Nacalai Tesque, Kyoto, Japan) were added to 860 mg sodium chondroitin sulfate (Nacalai Tesque). Subsequently, 900 μl distilled water were added and the mixture kneaded at room temperature. The "drug glue" was then degassed under reduced pressure and dispensed into a mold containing 100 inverted, cone-shaped wells arrayed in a 1.0 cm^2 area. Each well was 500 μm deep and 300 μm across at its top. After the "drug glue" was poured into the wells, the mold was centrifuged for 5 min at 3000 rpm (Kubota 1700, Tokyo, Japan). After centrifugation, the "vehicle glue" comprising 1.0 g chondroitin sulfate and 1.0 ml distilled water was painted over the mold. It was then dried under the pressure of the stainless steel plate for 3 h. Thereafter, the plate was removed and the two-layered dMNA was detached using the supporting material, i.e. base (Fig. 1A and B).

2.4. Immunization

Mice were anaesthetized by intraperitoneal injection of a ketamine–xylazine mixture. The dorsa of mice were shaved using electric clippers followed by an electric razor. The application site on the bare dorsal skin of mice was gently swabbed with 70% ethanol and allowed to dry.

For the dMNA patch immunization, the dMNA patch was placed on the shaved dorsal skin and pressed by a thumb for 3 min to insert the MNs into the superficial dermal layer. The continuous pressing was required to keep insertion of all MNs into the skin, because the dMNA patch had size of 12 mm × 12 mm and did not fit the natural body shape of mouse, while it was easily applied human skin and fixed by adhesive tape.

Transcutaneous (TC) immunization was performed as previously described [21]. In brief, the dorsal skin of the mice was shaved carefully using a No. 40 clipper, and the mice were rested for 48 h. A 0.64-cm^2 square gauze patch with an adhesive lining (Shirojuhji, Tokyo, Japan) was soaked with 50 μl solution of antigen in physiological saline with or without adjuvant and fixed on the shaved dorsal skin using medical tape. The mouse was replaced into the cage. Eighteen hours later, the gauze patch was removed.

For intradermal (ID) administration, 50 μl antigen solution in physiological saline were injected into the shaved dorsal skin using a conventional needle and syringe according to the Mantoux technique.

2.5. ELISA for OVA-specific IgGs

OVA-specific IgG titers in the sera of mice were determined by ELISA. In brief, 96-well plates (MaxiSorp; Nunc, Roskilde, Denmark) were coated with OVA in 0.1 M carbonate–bicarbonate buffer, pH 9.0, and blocked with PBS containing 1% bovine serum albumin (Sigma). Thereafter, serially diluted serum samples were added to the OVA-coated plates which were incubated at room temperature for 1.5 h. The plates were washed 3 times with wash buffer (PBS containing 0.05% Tween 20) and peroxidase-labelled rabbit anti-mouse IgG (Zymed, San Francisco, CA, USA) was added. After a 1.5-h incubation at room temperature, the plates were washed 3 times with the wash buffer and *o*-phenylenediamine (Sigma) in phosphate–citrate buffer containing 0.03% H_2O_2 was added. The reaction was stopped 10 min later by adding 1 N H_2SO_4 and optical densities were measured at 492 nm. Endpoint titers were expressed as reciprocal log of the limiting dilutions with an optical density greater than 0.5. The titers not less than 4 were regarded as significant.

2.6. ELISA for OVA content

The OVA content in dMNA patches was determined using a sandwich ELISA kit (Morinaga, Tokyo, Japan) according to manufacturer's instructions. The OVA content of samples was extrapolated by a standard curve prepared using the reference OVA provided in the kit.

2.7. Statistical analysis

The data are presented as the geometric means of the values obtained from individual animals. Groups were compared using unpaired, two-tailed Student's t test and $p \leq 0.05$ was considered significant.

3. Results

3.1. Skin delivery of EB by dMNA patches

To visualize the effectiveness of skin delivery of molecules loaded in the dMNA patches, the indicator dye, EB, was encapsulated into dMNA patches (Fig. 1C). EB-loaded dMNA patches were put on the shaved dorsal skin of mice and pressed manually for 3 min. After removing the patches from the skin, it was observed that most of the MNs on the patches had dissolved and lost their shape, but some MN residuals were observed on the patch (Fig. 1D). On the site of dMNA-patch application, EB spots corresponding to all MNs of the patch were obvious (Fig. 1F). The EB spots gradually diffused in the tissue surrounding the puncture sites in several hours (Fig. 1G) and disappeared 24 h later (Fig. 1H), indicating that the dMNA successfully penetrated the *stratum corneum* and delivered the EB into the epidermal and/or dermal layer. Thus, we confirmed that the dissolving MNs composed of chondroitin sulfate were sharp and strong enough to pierce the skin of mice and dissolve within few minutes.

3.2. Comparison of serum antibody responses induced by dMNA-patches with those induced by TC patch immunization or ID injection

Serum antibody responses in mice induced by antigen-loaded dMNA patches were compared with those induced by TC patch immunization or ID injection. dMNA patches containing 10 or

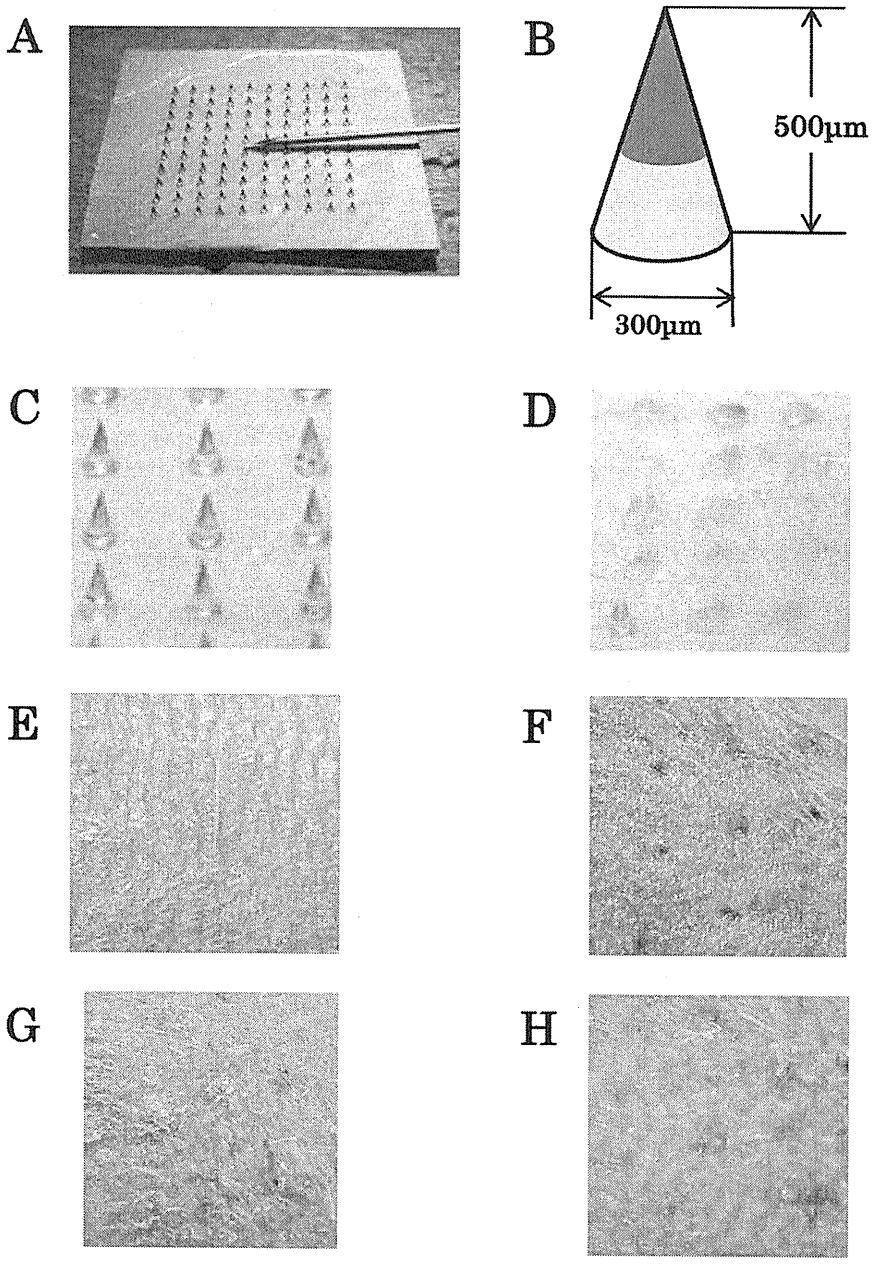

Fig. 1. An EB-loaded, two-layered dMNA patch. (A) A dMNA patch and a 26-G conventional needle. Dissolving MNs composed of chondroitin sulfate were arranged in 10 × 10 array on a patch. (B) Schematic view of the two-layered conical MN. (C–D) Close-up view of an EB-loaded, two-layered dMNA patch before (C) and after (D) skin insertion for 3 min. (E–H) Close-up view of dorsal shaved skin of a mouse before (E), immediately (F), 1 h (G), and 24 h (H) after application of an EB-loaded dMNA patch.

100 μg OVA with no adjuvant were applied in two groups of five mice. The other four groups of mice were immunized by TC route using gauze patches soaked with 50 μl solution containing 10 or 100 μg of OVA with or without 10 μg of CT as the adjuvant. The last group of mice were injected intradermally with 50 μl antigen solution containing 10 μg OVA. For boosting, all groups were re-immunized 2 weeks later and the OVA-specific IgG serum titers were determined 2 weeks after the first and the boost immunizations.

OVA-loaded dMNA patches induced substantial amounts of antigen-specific IgGs after the primary immunizations (Fig. 2A) but were boosted even more after the secondary immunizations (Fig. 2B). The dMNA containing 10 μg OVA induced comparable levels of antibody responses to those induced by ID injection of the same dose after both primary and secondary immunization. On the other hand, the TC patch immunization did not induce any significant antibody response after the primary immunization regardless of adjuvant co-application. Although the TC patches with 100 μg OVA induced significant antibody responses after the booster immunization, antibody titers were significantly less than those induced by dMNA patches. Thus, the antigen-loaded dMNA patch was far more effective than the TC patch immunization but comparably potent to ID injection in eliciting antibody responses in mice.