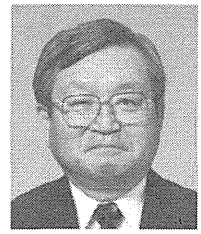
臨床疫学手法の重要性—北海道発インフルエンザ脳症と細菌性髄膜炎



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抄　録

平成６年（1994年）12月に経験した４歳男児に発症したインフルエンザ脳症をはじめて第27回日本小児感染症学会（平成７年，和歌山市）に報告した。当初は北海道の地方病との指摘を受けたが，やがて日本全国さらには世界各地に存在することが証明された。また北海道は医療圏が独立しており，約20分の１の人口を有する。全国に疾患が満遍なく発症しているとすると，全数を把握すれば20倍すると全国での発症数となる。本フォーラムでは北海道発のインフルエンザ脳症とワクチン普及前の小児細菌性髄膜炎の発症状況を紹介して臨床疫学手法の重要性を解説した。

キーワード：インフルエンザ脳症，サイトカインストーム，血管内皮細胞障害，細菌性髄膜炎，結合性ワクチン

はじめに

北海道は医療圏が独立しており全国の約20分の１の人口を占めている。したがってある時期にある疾患が全国で満遍なく発症しているとすると，北海道での発症者総数を知れば20倍することによって全国の発症数を推定することが可能となる。本稿では北海道から発したインフルエンザ脳症の存在の報告と，ワクチン普及前の北海道の細菌性髄膜炎の発症状況を紹介して臨床疫学手法の重要性を解説する。

１．インフルエンザ脳症

平成６年（1994年）４月１日付けで筆者は北大医学部小児科を離れ市立札幌病院小児科に移動した。その年の暮れの12月29日が筆者にとって初日直の勤務日だった。これまで全く健康に成長していた４歳の男児が前日12月28日午前３時から38.6℃の発熱とともに２～３分の全身痙攣を起こした。同日午前中に近所の小児科診療所を訪ね「熱性けいれん」と診断されて投薬を受けて帰宅した。翌29日午前０時30分，40℃の発熱とともに再度全身痙攣と意識混濁を起こし，午前３時救急車で市立札幌病院の救急部に搬送された。９時に対光反射消失，９時30分にCT室に移動中に心肺停止，AEDで蘇生されたが同日11時35分死亡した。発熱開始から死亡まで全経過わずか32時間であった。初日直だった筆者はCT室に移動中の児の心肺停止場面に遭遇したわけである。救急部に搬送された時に正常であった脳CT像は（**図１**）９時30分には全体浮腫，視床・脳幹の左右対称の低電位像（水口 雅氏の提唱した小児急性壊死性脳症のCT像に一致）（**図２**）。筆者の北大小児科在職期間中にはこのような発病から急速に病勢が進行して死の転帰をとるという症例を経験したことがなかった。

筆者はこの年の夏から市立札幌病院小児科外来を訪れる高熱を呈する小児の鼻咽頭スワブ材料を採取していた。ウイルスの分離同定材料採取を北海道衛生研究所から依頼されていたためである。この症例を経験した12月はインフルエンザウイルスA（H1N1）が分離同定されはじめた時期であり，この症例がインフルエンザウイルスに関連して発症したものと直感した。現在ならば迅速診断キットでいち早くインフルエンザAの診断が可能であったであろう。当時北海道には小児科医が常駐しており小児科の入院ベッドを持つ病院が59カ所あり，1994/95インフルエンザシーズンの終わりにこれらの病院の小児科部長に同様な症例の有無を

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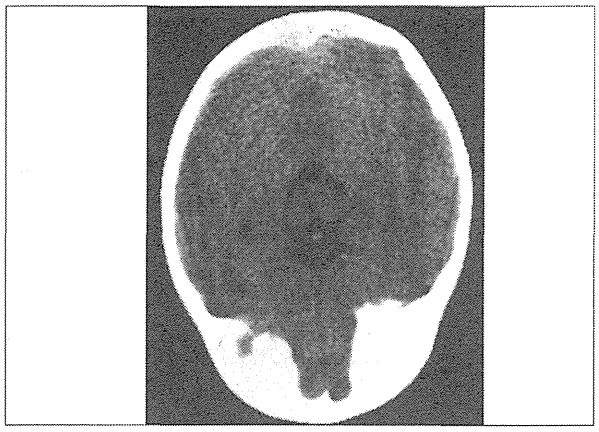

図1　4歳男児の脳CT像（ほぼ正常），12月29日午前3時撮影

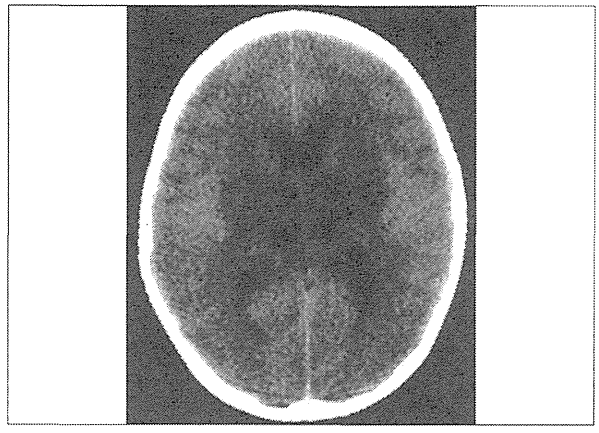

図2　同脳CT像，午前9：30撮影，視床の左右対称の低吸収域

たずねた。この結果このシーズンにトータル12例の発症（6例死亡，3例の神経学的後遺症）が確認された。そしてその発症のピークと北海道衛生研究所でのインフルエンザウイルスの分離ピーク，北海道のインフルエンザ様疾患の発症ピークが完全に一致した。すなわちこの疾患がインフルエンザウイルスの感染に関連して発症することが疫学的に推定されたわけである（**図3**）。

この疫学的手法は夕張地方で猖獗を極めたポリオの研究を始めた北大山田尚達名誉教授や札医大故中尾亨名誉教授に指導され，北大の故田中哲夫先生，檀上保先生，札医大の千葉峻三名誉教授に受け継がれた両大学のウイルス学研究の基礎をなすものであった。

この北海道で発症したインフルエンザ関連疾患の詳細を，平成7年（1995年）11月和歌山市で開催された第27回日本小児感染症学会（会長小池通夫教授）で報告した。また平成8年（1996年）に松薗嘉裕先生が日本小児科学会雑誌に[1]，平成9年（1997年）に筆者が日本臨床に「インフルエンザ流行中の小児期急性脳炎・脳症」として誌上報告した[2]。内外の小児科の教科書には脳炎あるいは脳症の原因ウイルスにインフルエンザウイルスが記載されてはいたものの，当時はこのような急激な経過をとるインフルエンザ関連疾患が多発するとの認識は一般的ではなかった。このためにこの疾患が北海道にのみ存在する地方病ではないかとの疑問が呈された。またライ症候群との関連で北海道ではまだインフルエンザに対してアスピリンを使用しているのかとの疑問も呈された（当然アスピリンの使用は皆無であった）。しかしながら2年，3年と症例を蓄積していくうちに日本全国にも同様の経過，症状を呈する症例が少なからず存在することがわかってきた。厚生省（当時）の医官であった葛西 健先生（現在WHOアドバイザーでハノイ在住）がいち早くこの疾患の重大性に気づかれ[3]，その肝いりで平成8年（1996年）厚生科学研究インフルエンザ脳症研究班（班長森島恒雄現岡山大学教授）が立ち上げられるに至った。国際的には1998年第22回国際小児科学会（Amsterdam），2000年第4回インフルエンザコントロールオプション会議（Crete），2001年第23回国際小児科学会（Beijing），2002年第1回ヨーロッパインフルエンザ会議（St.-Julians, Malta）に出席，発表してこの疾患の存在と重大さを強調したが当初は無視されがちであった。森島班長の国際誌への報告や欧米のインフルエンザ研究者への粘り強い説得が功を奏して，ようやく2002年のMaltaの会議あたりからインフルエンザ関連脳症として認められるに至った。

北海道の症例につきインフルエンザシーズンの終わりに毎年報告を求め，1994/95から2003/04シーズンの10シーズンの間に集積された症例は103例（年平均10.3例）となった。男児にやや多く（男女比1.3：1），平均年齢4.5±2.9歳，発熱—神経症状発現まで1.6±1.7日であり，予後は死亡34.0%，後遺症19.4%であった（**表1**，**図4**）。全国一律に発症していると仮定すると一年に平均200例の発症となり，研究班の成績もこれと一致した。

当時千歳市にいた武越靖郎先生と旭川市にいた長野奈緒子先生の粘り強い説得によって得られた貴重な2症例の剖検検体を，当時鳥取大学獣医学部におられた新矢恭子先生に病理学的，ウイルス学的検索をお願いした。2症例の剖検脳は，グリア細胞反応を伴う血管原性浮腫，血管壁の硝子変性，血漿成分の漏出，微小繊維素血栓と共通の特徴的所見がみられた（**図5，6**）[4]。さらに肺，小腸，脾臓など全身諸臓器にも病変が及んでいた。国立感染症研究所（当時）におられ

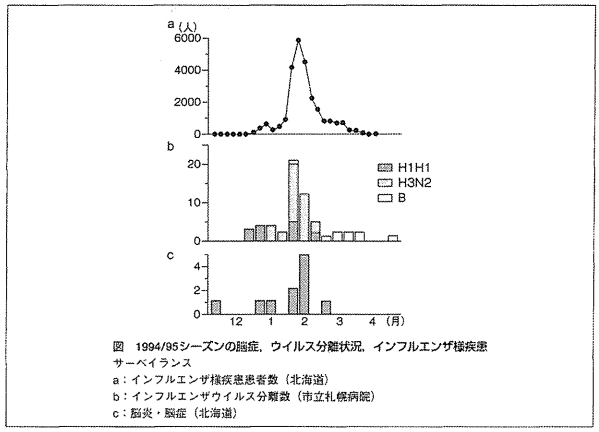

図 1994/95シーズンの脳症，ウイルス分離状況，インフルエンザ様疾患
サーベイランス
a：インフルエンザ様疾患患者数（北海道）
b：インフルエンザウイルス分離数（市立札幌病院）
c：脳炎・脳症（北海道）

図3　1994/95シーズンの脳症，ウイルス分離状況，インフルエンザ様疾患

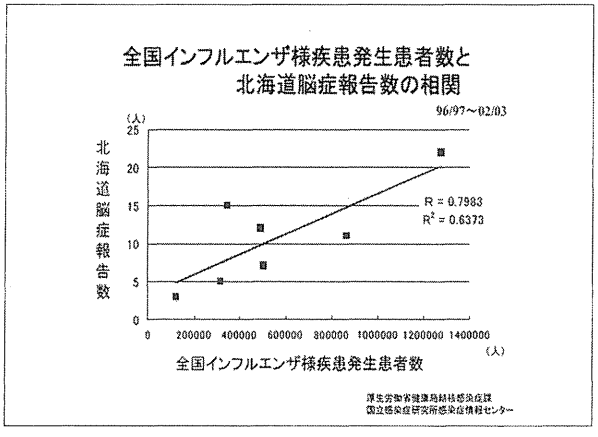

図4　北海道で発症したインフルエンザ脳症と全国のインフルエンザ様疾患の相関

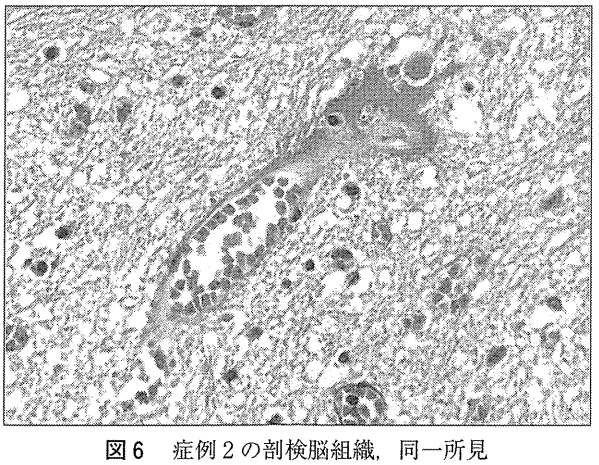

図6　症例2の剖検脳組織，同一所見

表1　北海道で発症したインフルエンザ脳症，10シーズンのまとめ

インフルエンザ脳症
（北海道　1994/95～2003/04　10ｼｰｽﾞﾝ）

	'94/95	'95/96	'96/97	'97/98	'98/99	'99/00	'00/01	'01/02	'02/03	'03/04	合計
北海道におけるインフルエンザの流行	A1 A3 B	A1 A3	A3 B	A3	A3 B	A3 A1	B A1 A3	A1 A3 B	A3 B	A3 B	
症例	12	14	5	22	11	7	3	15	12	2	103
性（男／女） （性比）	9/3	9/5	3/2	15/7	6/5	3/4	1/2	5/10	7/5	0/2	58/45 (1.3:1)
平均年齢 （歳）	3.1 (1-9)	3.9 (1-10)	5.0 (0-12)	4.5 (1-11)	2.5 (1-4)	3.9 (1-8)	4.9 (2-7)	5.1 (1-9)	5.0 (1-9)	6.5 (1-12)	4.5±2.9
発熱ー神経症状 （日）	2.5 (1-6)	2.9 (1-10)	3.2 (2-5)	1.2 (0-3)	0.8 (0-1)	1.1 (0-4)	1.0 (0-2)	1.6 (0-5)	0.5 (0-1)	0.0 (0)	1.6±1.7
転帰 死亡 後遺症 軽快	6 3 3	7 2 5	3 1 1	7 6 9	5 1 5	1 1 5	0 0 3	4 3 8	2 3 7	0 0 2	35(34.0%) 20(19.4%) 48(46.6%)

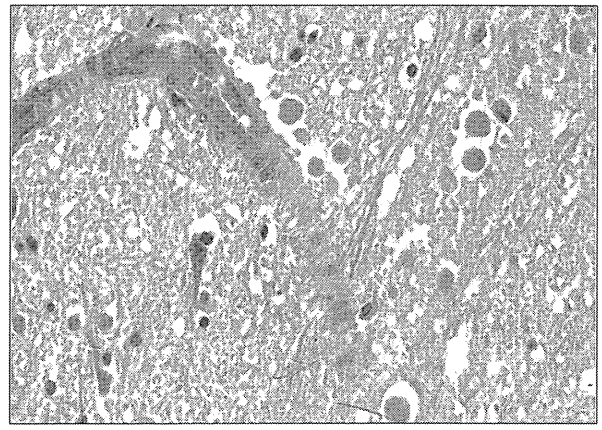

図5　症例1の剖検脳組織，血管原性浮腫，血漿成分の血管外漏出，血管内血栓

表2　各症例のIL-6，TNF-α，急速な経過をとって死亡した例できわめて高値をとった。

表　各症例の IL-6, TNF-α

	検体	採取年月日	IL-6 pg/mL	TNF-α pg/mL
症例 1	CSF*1	98.2.7	62,250	159
	血清	98.2.7	>700,000	1,059
症例 2	CSF	98.3.14	104,300	801
	血清	98.3.14	415,400	916
症例 3	CSF	96.2.26	134	10.2
	脳室液	96.3.3	1,058	11.4
	血清	96.3.3	86.9	51.3
症例 4	CSF	98.1.30	855.2	<7.5
対照				
症例 5*2	CSF	96.1.30	26.9	28.5
症例 6*3	CSF	97.8.2	10.2	<7.5
症例 7*4	CSF	98.2.10	16.2	<7.5

*1: cerebrospinal fluid
*2: 4 歳女，熱性けいれん
*3: 1 歳男，顔面神経麻痺
*4: 5 歳男，熱性けいれん，急速な経過をとって死亡した例で，極めて高値をとった．

た岩崎琢也先生により気管支上皮にインフルエンザAウイルスのNP抗原が証明されたが，剖検脳からはウイルス抗原は一切証明されなかった。これらの所見から「脳炎・脳症」と称していたものを「脳症」と統一した。脳脊髄液中のIL-6，TNF-αを当時北大小児科にいた成田光生，板倉 治先生に測定してもらったところ，急速な進行で死亡した2例で極めて高値であった（**表2**）[5]。このことからこの疾患は全身の血管内

表3　北海道で発症した細菌性髄膜炎の起因菌別発生数と予後，平成19－23年

細菌性髄膜炎：予後、起因菌

	症例数	インフルエンザ菌	肺炎球菌	GBS	大腸菌	その他
2007年	21	11	6 水頭症 1 高度難聴 1	2	1	1 水頭症 1
2008年	18	13 高度難聴 1	1 神経後遺症 1	2 神経後遺症 （尿崩症）1	1	1
2009年	19	12 高度難聴 2	4	1 神経後遺症 1	2	
2010年	18	13 死亡 1 神経後遺症 1	4	0	1	
2011年	16	9	5 難聴 1	1	1	
	92	58	20	6	6	2

北海道細菌性髄膜炎、平成19－23年[9]

表4　細菌性髄膜炎の起因菌別細菌学所見

細菌性髄膜炎：細菌学的検査所見

- インフルエンザ菌　b型　51/52, 98.1%
  - gBLNAR　30株
  - glowBLNAR　5株
  - gBLPAR　1株
  - gBLPACR-Ⅰ　3株
  - gBLPACR-Ⅱ　5株
  - gBLNAS　1株
- 肺炎球菌　PCV7(11/17)　PCV13(14/17)
  - 6A(gPISP,PCV13含有)　1株
  - 6B(gPRSP,PCV7含有)　4株
  - 6C(gPISP)　2株
  - 14(gPISP,PCV7含有)　2株
  - 19F(gPISP,PCV7含有)　2株
  - 19A(gPISP,PCV13含有)　2株
  - 23F(gPRSP,PCV7含有)　3株
  - 34(gPSSP)　1株
- GBS
  - Ⅰb,Ⅲ,Ⅳ,Ⅴ

北海道細菌性髄膜炎、平成19－23年 10

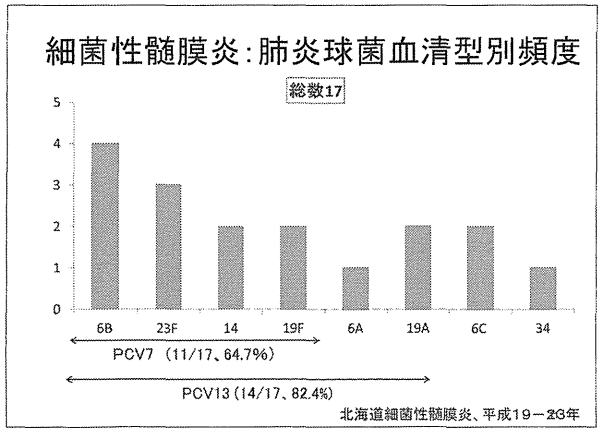

図7　肺炎球菌株の血清型

表5　肺炎球菌株血清型の年次推移

細菌性髄膜炎：肺炎球菌血清型年別発症数

		6B	23F	14	19F	6A	19A	6C	34
平成19年	4		1		1	1			1
平成20年	1	1							
平成21年	3	2			1				
平成22年	4			2			2		
平成23年	5	1	2					2	
	17	4	3	2	2	1	2	2	1

※平成23年肺炎球菌ワクチン2回接種後細菌性髄膜炎発症　血清型：6C
最終接種日23年1月27日　、平成23年5月12日発症

北海道細菌性髄膜炎、平成19-23年 12

皮細胞が障害されてサイトカインストームが惹起されて発症するものと想定されるが本態はまだ不明な点が多い。

2005年　のNew England J. Medicineに2003－2004シーズンの米国でインフルエンザに関連して死亡した18歳未満153例の報告が載った[6]。96例（63％）が5歳未満児であり，43例（31％）が病院に転送される前に死亡，45例（29％）がインフルエンザ発症3日以内の死亡と報告されている。わが国の「インフルエンザ脳症」との比較検討が待たれる。

2．北海道の細菌性髄膜炎

筆者はHibワクチン（アクトヒブ®）[7]，7価肺炎球菌結合型ワクチン（プレベナー®）[8]の国内治験責任医師として両ワクチンのわが国への導入に協力した。諸外国には両ワクチン導入前後のHib，肺炎球菌による侵襲性感染症の疫学データが存在しており，ワクチン導入効果を知ることができた。しかしわが国にはこれらのデータはほとんど皆無に等しかった。そこで平成18年暮れにインフルエンザ脳症発症調査に協力いただいた，北海道の小児科施設の小児科部長に細菌性髄膜炎の発症状況と起因菌の蒐集をお願いした。蒐集した細菌の細菌学的検索は北里大学の生方公子先生にお願いした。

この結果5年間の細菌性髄膜炎は平成19年21例，20年18例，21年19例，22年18例，23年18例の合計94例（男48，女46例，年平均18.8例）発症したとの報告を受けた（**表3**）。このうちインフルエンザ菌によるものは各年それぞれ11，13，12，13，11例（合計60例，年平均12.0例），肺炎球菌によるものが各年それぞれ6，1，4，4，5例（合計20例，年平均4.0例）であった。その他GBS 6例，大腸菌6例，リステリア菌1例，髄膜炎菌1例であった。この5年間を平均すると5歳未満の人口10万人あたりインフルエンザ菌による髄膜炎は5.7人，肺炎球菌による髄膜炎は1.7人となった。インフルエンザ菌の莢膜型は検査された54検体中53検体（98.1％）がb型であり，アンピシリン感受性で分類すると46株中45株（97.8％）が耐性株であり感受性

株はわずか1株にすぎなかった。肺炎球菌の血清型は17株で検索され血清型6A（gPISP，PCV13含有）1株，6B（gPRSP，PCV7含有）4株，6C（gPISP）2株，14（gPISP，PCV7含有）2株，19A（gPISP，PCV13含有）2株，19F（gPISP，PCV7含有）2株，23F（gPRSP，PCV7含有）3株，34（gPSSP）1株であった（**表4**）。従って7価結合型肺炎球菌ワクチン（PCV7）含有株は11株（64.7%）であった。現在国内治験中の13価結合型肺炎球菌ワクチン（PCV13）含有株は14株（82.4%）であった（**図7**）。患者から得られた肺炎球菌株の年次変化を表示したが（**表5**），平成22年に19Aが2株，23年に6Cが1株得られた。この期間中該当ワクチン被接種者からの髄膜炎発症者は血清型6Cによる肺炎球菌髄膜炎の1例のみであった。Hibワクチンは平成20年12月，7価結合型肺炎球菌ワクチンは平成22年2月から市販されたが，任意接種で開始されたため接種率は低迷していた。しかしながら平成23年4月から両ワクチンの公費助成が始まって接種率が急速に高まり現在に至っている（平成23年末の札幌市の5歳未満児でHibワクチン44.8%，PCV7ワクチン54.2%の接種率，7カ月未満児でそれぞれ94.5，92.1%の接種率である）。北海道では平成23年10月25日発症のHib髄膜炎男児，5月29日発症の肺炎球菌髄膜炎女児以降，インフルエンザ菌，肺炎球菌を起因菌とする細菌性髄膜炎の発症報告は無い（平成24年10月現在）。

おわりに

北海道から提唱したインフルエンザ脳症との出会いと北海道で発症した細菌性髄膜炎の5年間の集積を報告して，臨床疫学手法の重要性を強調した。

この論文の要旨は平成24年6月9日（土）第23回日本小児科医会総会フォーラムで会頭講演として報告した。

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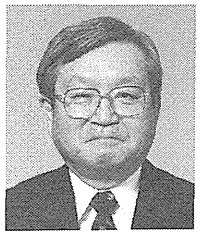
Hibワクチン

Hib vaccine



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◎Hib(インフルエンザ菌b型)ワクチンが2008年12月に市販された．市販当初から任意接種とされ，複数回(標準4回)接種が必要なため接種率が低迷していた．しかし，2011年度から“子宮頸がん等ワクチン接種緊急促進事業”としてHPVワクチン，PCV7(7価肺炎球菌結合型ワクチン)とともに公費補助の対象となり，急速に接種率が上昇した．このワクチンの予防対象疾患はHibの菌血症によって起こる細菌性髄膜炎であり，その発症時期は生後2～3カ月から2歳までの乳幼児期に集中している．このワクチンが定期接種に取り入れられた国々ではHibによる髄膜炎の発症が激減している．Hib感染症の防御は，菌の莢膜多糖体(PRP)に対する抗体が担う．18カ月未満の幼小児はPRPのみを接種しても十分な抗体を産生せず，PRPにキャリア蛋白を結合したワクチンが開発され，2カ月児でも効率的に抗体が産生され，接種が可能となった．わが国で認可されているワクチンはフランスのサノフィパスツール社で開発されたPRP-T(キャリア蛋白として破傷風トキソイドを使用，アクトヒブ®)である．生後2カ月から4週ごとに3回接種(初回接種)して，生後12カ月を過ぎて追加接種するのが標準的である．この初回接種の時期は，PCV7，DPT-IPV(百日咳・ジフテリア・破傷風・不活化ポリオワクチン)やロタウイルスワクチンの接種時期と重なっている．日本小児科学会はこれらのワクチンの同時接種を基本とした学会が推奨する接種スケジュールを公表している．PCV7とともに定期接種対象ワクチンに採用される日が待たれる．

Key word：Hibワクチン，小児細菌性髄膜炎，結合型ワクチン，同時接種

2008年12月，Hib(インフルエンザ菌b型)ワクチンが市販された．フランス・サノフィパスツール社のアクトヒブ®(ActHIB®)である．このワクチンが定期接種に取り入れられた国々ではHibによる細菌性髄膜炎が激減している．わが国では導入当初から任意接種とされたことから，接種率が低迷していた．しかし，2010年秋に国により“子宮頸癌などワクチン接種緊急促進事業”(補正予算)としてHPV(ヒトパピローマウイルス)ワクチン，PCV7(7価肺炎球菌結合型ワクチン)とともにHibワクチンも公費補助の対象となって接種率が急速に上昇した．このため乳幼児期に発症するHibによる細菌性髄膜炎をはじめとする全身感染症が激減している．

本稿ではHibワクチン導入前のわが国のHibによる全身感染症と，Hibワクチンの現状を概説する．

細菌性髄膜炎

細菌性髄膜炎は小児にとって最重症な感染症であり，罹患すると時に死に至ったり重篤な神経系の後遺症を遺す．そして，その起因菌はインフルエンザ菌(*Haemophilus influenzae*)が第1位，第2位を肺炎球菌(*Streptococcus pneumoniae*)が占めている．インフルエンザ菌は莢膜の有無によって莢膜型と無莢膜型に分類され，莢膜型はa～fの6種類の血清型に分類される．中耳炎などの局所感染症を引き起こす菌が無莢膜型であるのに対して，髄膜炎，肺炎，喉頭蓋炎など菌血症を伴う全身感染症を引き起こす菌はおもに莢膜型で，その

ほとんどがb型(Hib)である.

臨床症状は，高熱，頭痛，悪心・嘔吐，不きげん，痙攣などであるが，病初期は風邪などの症状と似ており，早期診断が難しい.

1996～1997年に6都道府県で実施された前向き調査によると[1]，インフルエンザ菌髄膜炎の罹患率は年8.6人(5歳未満人口10万人対，以下同じ)であり，年間患者数は500人以上となる．全身感染症の頻度に関して石和田らは千葉県の調査で，2007年6.4，2008年13.5，2009年11.2と報告し[2]，西村らは2003年から4年間の調査で髄膜炎が30.9と報告している[3]．厚生労働科学研究による全国10道県による班研究(神谷・庵原班)によると，2008年8.3，2009年7.1，2010年7.8と報告されており，人口から計算して2010年の全国の発症数を412例と算出している[4].

北海道では2007～2011年の5年間で58例の報告があり(5歳未満；表1)，年平均の発症数は5歳未満人口10万人当り5.7となった[5]．発症年齢では0歳代は26例(44.8%)，1歳代は15例(25.9%)と，両者合わせた2歳未満が41例(70.7%)を占めた(図1)．予後は死亡1例(1.7%)，重症神経後遺症1例(1.7%)，高度難聴3例(5.2%)であったが，ほかは後遺症なく治癒した．起因菌の莢膜血清型別ではb型が51/52(98.1%)を占め，アンピシリン感受性検査では30/45(66.7%)がβラクタマーゼ非産生アンピシリン耐性(BLNAR)株であり，アンピシリン感受性株は1/45(2.2%)であった(表2).

表1　2007～2011年インフルエンザ菌性髄膜炎の発症状況
(北海道，5歳未満)

	インフルエンザ菌	総数
2007年	11	19
2008年	12	16
2009年	12	19
2010年	12	16
2011年	11	18
合計	58	88

Hibワクチンとその効果

Hib感染症の防御は，Hibがもつ莢膜多糖体(polyribosyl ribitol phosphate：PRP)に対する抗体が担う．Hibワクチンは当初PRPワクチンがアメリカで開発され，1985年認可された．ところが，PRP単独では18カ月未満児で十分な免疫原性を発揮できなかった．そこでPRPにキャリア蛋白を結合させたHib結合体ワクチンが開発され，

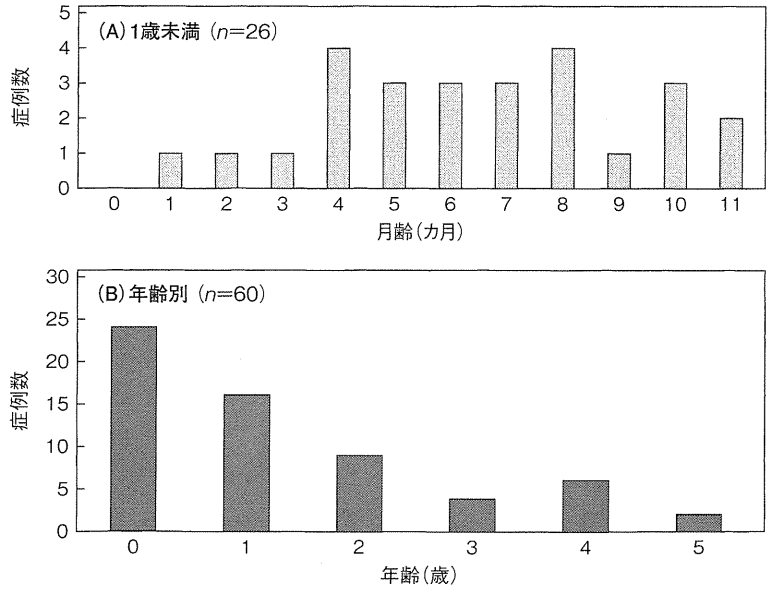

図1　2007～2011年インフルエンザ菌性髄膜炎の発症数(北海道)
A：月齢別，B：年齢別.

表 2　2007～2011年インフルエンザ菌性髄膜炎患者から分離された細菌の血清型と遺伝子型(北海道)

インフルエンザ菌 b 型：53/54，98.1%	
gBLNAR	30株(βラクタマーゼ非産生アンピシリン耐性菌)
glowBLNAR	6株(βラクタマーゼ非産生アンピシリン軽度耐性菌)
gBLPAR	1株(βラクタマーゼ産生アンピシリン耐性菌)
gBLPACR-Ⅰ	3株(βラクタマーゼ産生アモキシシリン/クラブラン酸耐性菌Ⅰ型)
gBLPACR-Ⅱ	5株(βラクタマーゼ産生アモキシシリン/クラブラン酸耐性菌Ⅱ型)
gBLNAS	1株(βラクタマーゼ非産生アンピシリン感受性菌)

1990年に2カ月以上の児に接種可能となった．このワクチンが広く接種された結果，アメリカのインフルエンザ菌髄膜炎の罹患率は1984年24.0人(5歳未満児10万人対)から1991年3.7人へと激減した．

Hib結合体ワクチンはすでに90カ国以上で小児の定期予防接種プログラムに組み込まれている．WHOは1998年にこのワクチンを定期予防接種プログラムに組み込むことを推奨した．

著者らは2000年2月～2002年6月の間に全国19の施設の小児科で，2～6カ月齢の健康小児を対象に，Hibワクチンの安全性と有効性(免疫原性)試験を行った[6]．用いたワクチンは，フランスのサノフィパスツール社で開発・製造されたPRP-T(キャリア蛋白として破傷風トキソイドを使用，ActHIB®，日本名アクトヒブ®)ワクチンである．1バイアル中にHibPRP多糖体10 μgを含む凍結乾燥製剤で，使用前添付溶剤0.5 mLで溶解して皮下接種した．初回接種として3回(3～8週間隔)，その1年後に追加接種した．初回接種および追加接種のそれぞれ前後の4回採血し，抗PRP抗体価を測定した．安全性の確認のため，接種医師は接種30分以内の有害事象を観察し，接種当日に保護者に健康調査日誌を手渡しその記入を依頼した．日誌には接種7日後までの体温や接種部位の発赤腫脹の有無・程度の記入欄があり，次回来院時に回収した．

19施設で122例に接種され，抗体価測定は119例で行った．119例中初回接種前後の1 μg/mL以上(長期保護レベル)の抗体保有率はそれぞれ2.5%(3/119)，92.4%(110/119)であり，0.15 μg/mL以上(保護レベル)の抗体保有率はそれぞれ13.4%(16/119)，99.2%(118/119)であった．抗体価の幾何平均値は，接種前0.06 μg/mL，初回接種後9.68 μg/mLであった．また，116例が追加接種を完了した．追加接種4週後には1 μg/mL以上の抗体保有率が100%(116/116)となり，GMTが117 μg/mLであった(図2)．

副反応

臨床試験で用いられたHibワクチンは，フランスでは1992年，アメリカでは1993年に承認され，現在100カ国以上で発売されている．すでに安全

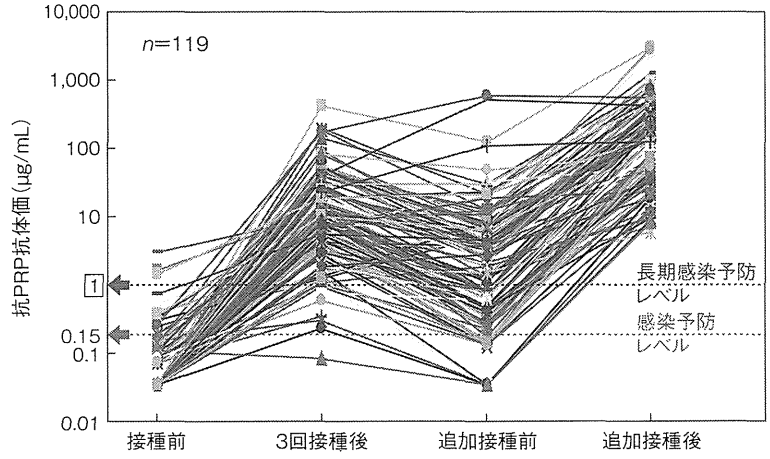

図 2　Hibワクチン初回接種(3回)と追加接種(1回)接種前後の抗PRP抗体価の推移

性と有効性は約111,000例の臨床試験で検討されており，副反応は全般的に軽微かつ一過性であった．わが国で行われた臨床試験でも副反応はおもに局所反応であり，注射部位の発赤(最高発現率45.9%)，腫脹(同23.1%)，硬結(同21.5%)，および疼痛(同9.1%)であったが，いずれも一過性であった．全身反応は発熱(最高発現率4.1%)，不きげん(同23.0%)，食欲不振(同13.2%)，下痢(同10.7%)，嘔吐(同8.3%)などが認められたが，外国臨床試験ですでに報告されている症状と類似していた．

同時接種

前述のように細菌性髄膜炎の発症時期は2歳未満(70.7%)，とくに1歳未満の乳児(44.8%)に集中していた．したがって，このワクチンは生後2～3カ月齢に初回接種(0.5 mL，皮下接種)し，1～2カ月間隔で3回接種，その後1年を過ぎて1回追加接種するのが標準的である．この接種時期はPCV7(プレベナー®)の接種時期と一致する．また，百日咳・ジフテリア・破傷風・不活化ポリオ(セービン株)ワクチン(テトラビック®，クアトロバック®)が3カ月齢から1～2カ月間隔で3回接種，その後1年を過ぎて1回追加接種する時期とも一致する．したがって，この生後2～6カ月の間は複数のワクチンを同時に接種することが必然となる．さらには，あらたに登場した内服用ヒトロタウイルスワクチン(ロタリックス®，ロタテック®)の接種時期とも重なっている．

著者らはインフルエンザ菌b型ワクチン(アクトヒブ®)と国産沈降精製百日咳・ジフテリア・破傷風混合ワクチン(DPT)を同時接種した際の免疫原性および安全性を，アクトヒブ®の製造販売後試験として検討した．その結果，同時接種群とDPT単独群とも，免疫原性も副反応発現率いずれも同等であった[7]．

日本小児科学会は，生後2カ月からスタートし，同時接種を基本とした学会が推奨する予防接種スケジュールを公表して，ホームページと学会雑誌に掲載した[8]．ワクチンの種類が増加したり接種回数が変更された場合には適宜内容が更新される．

おわりに

Hibワクチンについて現状を概説した．このワクチンは“子宮頸がん等ワクチン接種緊急促進事業”の接種対象ワクチンとして，1年ごとの補正予算により市町村への地方交付税の形で補助されている．市町村によっては補助が受けられない自治体もあり，1年ごとの更新などという不安定な状況は改められなければならない．ワクチン接種で予防することができる疾患(vaccine preventable diseases：VPD)は，すべての国民が無料で予防できる環境づくりが急がれる．これには予防接種法の全面改正が急務である．

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J Infect Chemother (2012) 18:272–275
DOI 10.1007/s10156-011-0312-4

NOTE

Bactericidal activity of topical antiseptics and their gargles against *Bordetella pertussis*

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Received: 4 February 2011 / Accepted: 14 September 2011 / Published online: 4 October 2011

Abstract *Bordetella pertussis* is the etiological agent of whooping cough, a common cause of respiratory illness in both children and adults. In the present study, we investigated the bactericidal activity of four antiseptics—povidone–iodine (PVP-I), benzethonium chloride (BEC), chlorhexidine gluconate (CHG) and benzalkonium chloride (BAC)—against *B. pertussis* ATCC9797 and clinical isolates. Among the topical antiseptics, PVP-I, BEC, and BAC, PVP-I and BAC in particular, showed high bactericidal activity, whereas CHG had low activity. PVP-I gargle also showed high bactericidal activity, similar to topical PVP-I. However, BEC gargle had low bactericidal activity. Our results indicate that topical PVP-I and BAC, and PVP-I gargle would be useful as effective antiseptics against *B. pertussis*.

Keywords *Bordetella pertussis* · Antiseptics · Bactericidal activity · Povidone–iodine · Benzalkonium chloride

Pertussis is an acute respiratory infection caused by the gram-negative coccobacillus *Bordetella pertussis* [1]. This disease is highly communicable, with a second attack rate of up to 90% among unvaccinated household contacts. *B. pertussis* is transmitted from an infected person to susceptible persons, primarily through aerosol droplets of respiratory secretions and secondarily through direct contact with the respiratory secretions. In Japan, the incidence of pertussis has been successfully decreased through the introduction of pertussis vaccines; however, there has been an increase in adult patients with pertussis since 2002 [2]. To prevent healthcare-associated bacterial pneumonia including pertussis, hand hygiene and disinfection of medical apparatus are strongly recommended for prevention of transmission of microorganisms [3]. In addition, oropharyngeal cleaning and decontamination with antiseptics are effective ways to prevent nosocomial respiratory infection [3, 4]. Although hand hygiene and oral rinse are recommended, no reports on the bactericidal activity of antiseptics against *B. pertussis* have been published. In the present study, therefore, the bactericidal activity of commercial topical antiseptics and their gargles against *B. pertussis* ATCC and clinical strains was determined.

Ten *B. pertussis* clinical isolates, collected from 2004 to 2008 in Japan, were investigated. The isolates were selected from the National Institute of Infectious Diseases (NIID) strain collections, according to their genotype (multilocus sequence type, MLST): five isolates, MLST-1; three isolates, MLST-2; one isolate, MLST-3; one isolate, MLST-4. The MLST-1 and MLST-2 strains were commonly isolated during the past two decades in Japan, but MLST-3 and -4 strains were not [2]. *B. pertussis* ATCC9797 was used as a laboratory strain. The *B. pertussis* clinical and ATCC9797 strains were cultured on Bordet–Gengou agar containing 15% defibrinated sheep blood for 48 h at 35°C, followed by subculture for 48 h. The following topical antiseptics and gargles were tested in

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this study. The topical antiseptics were povidone–iodine (PVP-I; Isodine solution 10%; Meiji Seika Kaisha), benzethonium chloride (BEC; Hyamine solution 10%; Daiichi-Sankyo), chlorhexidine gluconate (CHG; Hibitane 20%; Dainippon Sumitomo Pharma), and benzalkonium chloride (BAC; Osvan S; Takeda Pharmaceutical). The gargle antiseptics were PVP-I (Isodine gargle solution 7%; Meiji Seika Kaisha) and BEC (Neostelin green 0.2% mouthwash solution; Nippon Shika Yakuhin).

Topical PVP-I, BEC, CHG, and BAC were diluted with sterile water at two or three concentrations according to the package insert instructions: PVP-I, 0.05–0.5%; BEC, 0.005–0.2%; CHG, 0.05–0.5%; BAC, 0.05% and 0.2%. The bacterial inoculum suspension and each antiseptic solution were mixed at 1:25 and incubated. After 0.25, 0.5, 1 and 3 min, 0.1 ml of the mixture was inoculated into 0.9 ml of neutralizer containing Tween 80, soybean lecithin, and sodium thiosulfate. Tenfold serial dilutions of each mixture were prepared, and 0.1 ml dilute solution was plated on Bordet–Gengou agar and incubated for 72 h at 35°C. The number of colonies was counted, and the number of colony-forming units (CFUs) in the mixture was calculated from the dilution rate [5].

Table 1 shows the bactericidal activity of the topical antiseptics against *B. pertussis* ATCC9797. A 0.25-min treatment with 0.05% PVP-I was found to successfully reduce the viable cells by more than 1×10^5 CFU/ml. A 0.25-min treatment with 0.05% BEC reduced the viable cells by more than 1×10^5 CFU/ml, whereas with 0.005% BEC, a 3-min treatment was required to achieve the same effect. A 0.25-min treatment with 0.05% BAC reduced the viable cells by more than 1×10^5 CFU/ml. With 0.05% or 0.2% CHG, even a 3-min treatment could not reduce the viable cells enough. To reduce the viable cells by more than 1×10^5 CFU/ml, treatment with 0.5% CHG for 3 min was required. This finding indicates that topical CHG has a lower bactericidal activity than topical PVP-I, BEC, and BAC against *B. pertussis* ATCC9797.

The bactericidal activity of topical PVP-I, BEC, and CHG against *B. pertussis* isolates was also investigated. The level of bactericidal activity is shown in Fig. 1. All isolates that received a 0.25-min treatment with 0.2% or 0.05% PVP-I had viable cells reduced by more than 1×10^5 CFU/ml. In contrast, with 0.005% BEC, a 3-min treatment was required to achieve the same effect. On the other hand, a sufficient decrease could not be achieved even when a 3-min treatment with 0.2% CHG was performed. With all the antiseptics, no marked differences were seen in terms of MLST and bactericidal effect. Table 2 shows the bactericidal activity of PVP-I and BEC gargles against *B. pertussis* ATCC9797. A 0.25-min treatment with 0.05% PVP-I gargle was found to reduce the viable cells by more than 1×10^5 CFU/ml, indicating that the PVP-I gargle had the same bactericidal activity as topical PVP-I. In contrast, with 0.2% BEC gargle, no significant decreases in viable cells were observed with 3-min treatment, although topical BEC has high bactericidal activity (Table 1).

To our knowledge, this is the first report on the bactericidal activity of antiseptics, especially PVP-I, against *B. pertussis*. Here, we show that topical PVP-I and BAC, and PVP-I gargle have high bactericidal activity compared with BEC and CHG. CHG is classified as a low-level antiseptic according to Spaulding's classification and shows variable bactericidal activity depending on the bacterial species. Furthermore, it was reported that a

Table 1 Bactericidal activity of topical povidone–iodine (PVP-I), benzethonium chloride (BEC), chlorhexidine gluconate (CHG), and benzalkonium chloride (BAC) against *Bordetella pertussis* ATCC9797

Antiseptic	Concentration (%)	Viable cells (CFU/ml)			
		0.25 min	0.5 min	1 min	3 min
PVP-I	0.5	–	–	–	–
	0.2	–	–	–	–
	0.05	–	–	–	–
BEC	0.2	–	–	–	–
	0.05	–	–	–	–
	0.005	8.5×10^6	1.3×10^6	3.0×10^3	–
CHG	0.5	1.8×10^7	1.1×10^7	1.4×10^6	–
	0.2	1.8×10^7	1.8×10^7	8.1×10^6	6.0×10^4
	0.05	3.0×10^7	2.5×10^7	1.9×10^7	1.9×10^6
BAC	0.2	–	–	–	–
	0.05	–	–	–	–

–, not detected ($<1 \times 10^2$ CFU/ml)

Initial cell concentration was $2.9–5.5 \times 10^7$ CFU/ml

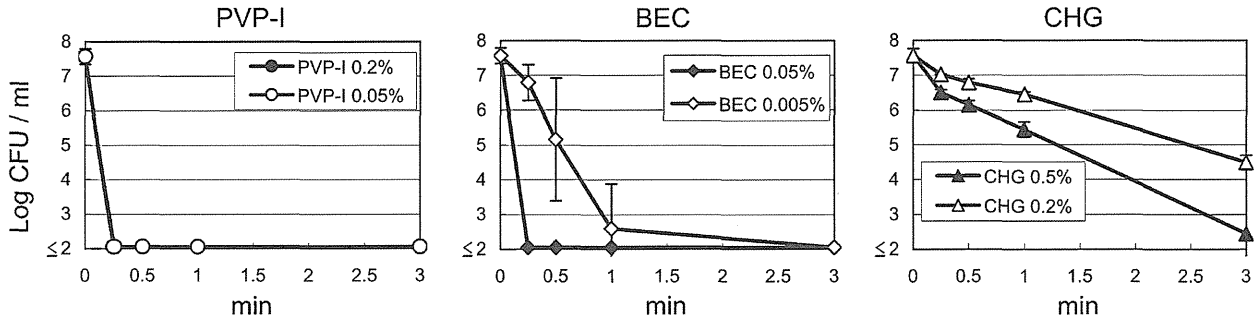

Fig. 1 Bactericidal activity of topical povidone–iodine (PVP-I), benzethonium chloride (BEC), and chlorhexidine gluconate (CHG) against *Bordetella pertussis* clinical isolates. Ten isolates [$\sim 5 \times 10^7$ colony-forming units (CFU)] were individually mixed with the topical antiseptic solution at different concentrations: PVP-I, 0.05% and 0.2%; BEC, 0.005% and 0.05%; CHG, 0.2% and 0.5%. After 0.25, 0.5, 1, and 3 min, the mixture was inoculated into a neutralizer. The number of viable cells was determined using plate count methods, and the number of CFUs in the mixture was calculated from the dilution rate. Detection limit was 1×10^2 CFU/ml

Table 2 Bactericidal activity of PVP-I and BEC gargles against *Bordetella pertussis* ATCC9797

Antiseptic	Concentration (%)	Viable cells (CFU/ml)			
		0.25 min	0.5 min	1 min	3 min
PVP-I	0.5	–	–	–	–
	0.2	–	–	–	–
	0.05	–	–	–	–
BEC	0.2	6.8×10^7	6.2×10^7	8.2×10^7	6.6×10^7
	0.05	8.5×10^7	8.9×10^7	7.5×10^7	4.4×10^7
	0.005	8.7×10^7	7.9×10^7	6.3×10^7	3.8×10^7

–, not detected ($<1 \times 10^2$ CFU/ml)

Initial cell concentration was $2.8–5.5 \times 10^7$ CFU/ml

relatively longer drug contact period is necessary for some bacterial species [6]. It is therefore not surprising that similar results were seen in this study with *B. pertussis*. It is not clear why the BEC gargle shows weak bactericidal activity. Similar findings were obtained in methicillin-resistant *Staphylococcus aureus* (MRSA) (data not shown); thus, the difference in bactericidal effect between topical BEC and BEC gargle is not considered to be an issue specific to *B. pertussis*. The BEC gargle contains several additive agents, e.g., polysorbate 80, mentha oil, spearmint oil, saccharin sodium, thymol, and sodium copper chlorophyllin. The additive agent(s) might be the cause of the low bactericidal activity against *B. pertussis*. Our findings indicate that equal effects of an active ingredient should not be expected when administered in different forms.

PVP-I is known to have potent broad-spectrum activity against bacteria, mycobacteria, fungi, and viruses [7], whereas BAC has no bactericidal activity against mycobacteria [8, 9]. Topical PVP-I and BAC have become widely used as antiseptic and disinfectant in hospitals, and PVP-I gargle is generally used for oral disinfection in Japan, especially at the time of year when the common cold and influenza are prevalent. *B. pertussis* frequently causes hospital and intrafamilial infections transmitted via aerosol droplets as well as the common cold and influenza. In light of this, gargling with PVP-I would be important among household members and hospital patients.

Adolescents and adults are assumed to be the primary reservoir of *B. pertussis* and play a crucial role in the transmission of the microbe to infants and unvaccinated children [10–12]. Macrolide antibiotics, such as erythromycin, are widely used for treatment of patients with pertussis and are currently recommended for prophylaxis in the United States as well. However, erythromycin resistance in *B. pertussis* has been reported in the United States, with an occurrence rate of <1% [13]. Fluoroquinolones are also widely used to treat respiratory tract infections in adults. These antibiotics have excellent in vitro activity against *B. pertussis*; however, several quinolone-resistant strains of *B. pertussis* were recently found in Japan [14]. Considering the mechanism of antiseptics, disinfection and gargling with PVP-I may be an effective way to eliminate *B. pertussis* regardless of drug resistance.

In conclusion, topical PVP-I and BAC, and PVP-I gargle, have high bactericidal activity against *B. pertussis*. To prevent the spread of pertussis infections, PVP-I and BAC would be useful as effective antiseptics against *B. pertussis*.

Acknowledgments The authors thank Dr. Yoshichika Arakawa, National Institute of Infectious Diseases, for his considerable support in conducting this study.

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Vaccine 30 (2012) 3885–3890

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Alum-adjuvanted H5N1 whole virion inactivated vaccine (WIV) enhanced inflammatory cytokine productions

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ARTICLE INFO

Article history:
Received 11 October 2011
Received in revised form 29 March 2012
Accepted 1 April 2012
Available online 13 April 2012

Keywords:
H5N1
Whole virion inactivated influenza vaccine
Aluminum adjuvant
IFN-α
IL-1β
IL-6
Inflammasome

ABSTRACT

Alum-adjuvanted H5 whole virion inactivated vaccine (WIV) was licensed for adults in Japan but induced marked febrile reactions with significantly stronger antibody responses in children. In this study, the mechanisms behind the different responses were investigated. Lymphocytes were obtained from 25 healthy subjects who were not immunized with H5 vaccine, to examine the innate immune impact of the various vaccine formulations, analyzing the cytokine production profile stimulated with alum adjuvant alone, alum-adjuvanted H5 WIIV, plain H5 WIV, and H5 split vaccine. Alum adjuvant did not induce cytokine production, but H5 split induced IFN-γ and TNF-α. H5 WIV induced IL-6, IL-17, TNF-α, MCP-1, IFN-γ, and IFN-α. An extremely low level of IL-1β was produced in response to H5 WIV, and alum-adjuvanted H5 WIV enhanced IL-1β production, with similar levels of other cytokines stimulated with H5 WIV. Enhanced production of cytokines induced by alum-adjuvanted H5 WIV may be related to the higher incidence of febrile reactions with stronger immune responses in children but it should be further investigated why efficient immune responses with febrile illness were observed only in young children.

1. Introduction

In 2009, swine H1N1 influenza virus caused rapid global human-to-human transmission and was initially suspected as a new pandemic strain [1]. However, it actually emerged from swine influenza virus, which was first isolated in North America, genetically combined with human, swine, and avian genome compartments [2,3]. In this sense, pandemic A/H1N1 2009 was not a new pandemic strain [4,5]. Pre-existing antibody levels were reportedly low in young generations and most patients were young adults and children, not elderly [6]. A 2009 pandemic H1N1 vaccine seed was obtained after adaptation to egg, but the virus yield was poor in comparison with seasonal seeds. In Japan, egg-derived pandemic split vaccine was produced and introduced just after the peak of the outbreak. This pandemic raised several pressing issues: vaccine development, prompt supply and distribution, antigen saving, and vaccine efficacy to prepare for the unknown forthcoming pandemic.

In the 20th century, three pandemics of influenza occurred. The most devastating pandemic dated back to 1918, known as Spanish flu, caused by a highly pathogenic H1N1 influenza virus transmitted through some animals from avian pathogenic virus, estimated to have killed 40–50 million people [7]. In 1957, Asian influenza A/H2N2 caused the second pandemic, and Hong Kong influenza A/H3N2 appeared as the third pandemic in 1968. Seasonal influenza outbreaks or epidemics are caused by an antigenic drift of A/H1N1 or A/H3N2, whereas the pandemics appeared as antigenic shift, leading to new strains which are thought to be recombination with non-preexisting features of hemagglutinin (HA) and neuraminidase (NA) in human influenza viruses. After the 1968 pandemic of A/H3N2, several cases and small local outbreaks were reported, caused by new strains, H5, H7, or H9, and they were considered to be from poultry, and H5 is very close to human as a target for vaccine development [8–13]. There was a regional outbreak of H5 in Hong Kong in 1997, and six of 18 patients died, causing an H5 pandemic threat [9]. Sporadic H5 transmission on

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0264-410X/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.vaccine.2012.04.004

poultry farms and in migratory birds has spread across Asia to the EU and Africa, and approximately 550 cases of human H5 infection have been reported since 2004, showing a high mortality rate of approximately 60%. Most cases have involved close and direct contact with poultry, with no definite case of human-to-human transmission [14]. There are several barriers to human-to-human transmission: receptor usage of HA protein, cleavage efficiency by cellular protease, and host factors. Now, H5 is very close to the human, and the primary strategy to prevent and control influenza pandemics is the development of an effective and safe vaccine to mitigate the uneasiness, uncertainty, and pandemic threat.

Split vaccine has been used for more than 40 years and H5 is known to be poorly immunogenic. A two-dose schedule of 90 μg split vaccine of H5/Vietnam/1203/2004 induced 57% seropositivity of HI ≥1:40, and 53% seropositivity of NT ≥1:40 without adjuvant [15]. The addition of alum adjuvant improved the immunogenicity and could reduce the antigen usage to 30 μg with a similar immunogenicity to plain split, 90 μg [16].

In Japan, alum-adjuvanted H5N1 whole inactivated virion (WIV) (alum concentration: 300 μg/ml) was developed using a genetically engineered reassortant, the NIBRG-14 strain, originated from H5/A/Vietnam/1194/2004. In a clinical phase II trial in healthy adults, alum-adjuvanted 15 μg HA protein of WIV led to favorable immunogenicity (>70% sero-conversion rate in NT test) without demonstrating any serious systemic illnesses [17]. Whereas, when it was administered to young infants and children with a reduction in antigen doses, 7.5 or 3 μg, a high fever ≥37.5 °C was observed in over 60% of the recipients at less than six years of age, but, unexpectedly, NT antibody titers were higher than those observed in a clinical trial in adults. Recent detailed insights into the mechanisms of adjuvant effect on innate immunity and inflammasome have led to the better understanding of immunogenicity and immunotoxicity [18–20]. In this study, cytokine and chemokine responses were investigated to analyze the reason why a high incidence of febrile reactions was observed after the administration of alum-adjuvanted whole inactivated H5 vaccine to children.

2. Materials and methods

2.1. Study design and subjects

Twenty-five healthy subjects were enrolled in this study, aged 3 months to 59 years, who were not immunized with H5 vaccine. Among them, 20 subjects were under 20 years of age. The study design and protocol were discussed and approved by the ethical committee of Tokyo Medical University. Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation through Ficoll-Paque™ Plus (GE Healthcare Bio-science, Uppssala, Sweden). They were adjusted to 1×10^6 cells in a 24-well plate in 1 ml of RPMI 1640 medium supplemented with 4% FBS and adequate antibiotics. They were stimulated with 100 μl of vaccine preparations or alum adjuvant alone.

2.2. Vaccine antigens

The NIBRG-14 strain, a genetically reassortant vaccine seed strain, originated from H5/A/Vietnam/1194/2004 and PR-8, was grown in MDCK and purified through zonal ultracentrifugation. Purified virus particles were inactivated by formalin treatment and used as whole inactivated vaccine (WIV). Alum-adjuvanted WIV was produced by adding alum adjuvant (1:1 mixture of Al phosphate and hydroxide) at a final alum concentration of 300 μg/ml. Purified virus particles were split by treatment with ether and Tween 80 and inactivated with formalin, and used as split vaccine material. Other strains were employed to compare the immunological responses: seasonal A/Brisbane/H1N1 and 2009 pandemic A/California/07/2009, produced by Kitasato Institute for Biologicals, Saitama. All vaccine materials were adjusted to 30 μg/ml HA protein concentration.

H5 WIV pandemic vaccine for clinical trial was produced from egg-derived WIV materials by Kitasato Institute for Biologicals, Saitama and Biken Intitutes, Kannonji.

2.3. Cytokine assay

Culture supernatants were harvested at 24 hr after stimulation with influenza vaccine materials and subjected to Bio-Plex Pro™ Human Cytokine Assay 17-plex, using Bio-Plex 200 (Bio-Rad, USA). The concentration of IFN-α was measured using an EIA kit (Verikine™ Human IFN-Alpha Serum Sample ELISA kit, pbl interferon, USA) and IL-1β and IL-6 were also measured using Quantikine Human IL-1β and Quantikine IL-6, respectively (R&D Systems, USA), following the instruction manual.

3. Results

3.1. Summary of alum-adjuvanted vaccine trial in children

An alum-adjuvanted H5N1 WIV clinical study was conducting involving 337 subjects aged 20–59 years. Two doses were given at 21–28 day intervals, and HI and NT antibodies were examined before immunization, just before the second dose, and one month after the second dose. NT antibodies became sero-converted in 260/337 (77%) in the15 μg group. No serious systemic adverse reaction was observed: febrile reaction ≥37.5 °C was reported in 3%. Alum-adjuvanted H5N1 WIV was licensed for stockpiling to prepare for a pandemic.

Using the same vaccine, a clinical trial was performed involving 374 subjects aged 6 months to 19 years. 0.1 ml was given to those less than one year, 0.25 ml for those 1–6 years, and 0.5 ml for those over six years of age. Febrile illness ≥37.5 °C was observed in 203/374 (54%) after the first dose, but decreased to 33/367 (9.0%) after the second dose. Unexpectedly, a high incidence of febrile reaction ≥38.0 °C was demonstrated in recipients aged less than 6 years and the incidence of febrile reaction (≥38 °C) after vaccination reduced by age: 5/5 (100%) in those less than one year, 52/92 (57%) in those 1–3 years, 48/90 (53%) in those 4–6 years, 39/134 (29%) in those 7–12 years, and 3/53 (6%) in those 13–19 years (Table 1).

NT titers after two-dose vaccination were compared in subjects who had a febrile reaction and those without febrile illness. The mean NT titer was $10 \times 2^{3.56 \pm 1.30}$ in those with febrile illness, being significantly higher than those without febrile illness, $10 \times 2^{2.76 \pm 1.26}$ ($p < 0.01$). Higher NT antibody titers seemed to be induced in those with a higher body temperature after vaccination (Table 2).

3.2. Cytokine induction by alum adjuvant

Alum adjuvant was prepared at the same concentration of 300 μg/ml. PBMCs were stimulated with 3 μg or 30 μg of

Table 1
Incidence of febrile reactions in different age groups.

	n	Fever+	≥38.0 °C
<1 year	5	5 (100%)	5 (100%)
1–3 years	92	68 (74%)	52 (57%)
4–6 years	90	57 (63%)	48 (53%)
7–12 years	134	63 (47%)	39 (29%)
≥13 years	53	10 (19%)	3 (6%)
Total	374	203 (54%)	147 (39%)

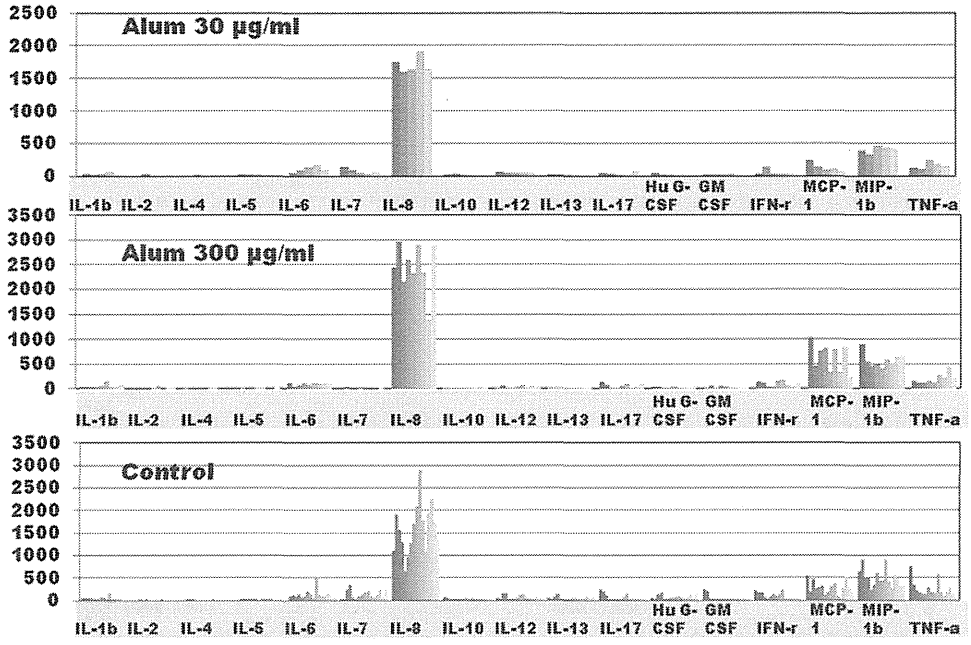

Fig. 1. Cytokine profile in PBMC cultures stimulated with aluminum solution. PBMC were stimulated with 0.1 ml of Alum adjuvants of 300 μg/ml (similar concentration as alum-adjuvanted H5 vaccine) and 30 μg/ml (1:10 dilution).

aluminum, and the results of cytokine profiles are shown in Fig. 1. Culture fluids were assayed using human 17plex. In control cultures of 25 subjects, IL-6, IL-7, IL-8, IFN-γ, MCP-1, MIP-1β, and TNF-α were produced at the baseline without any stimuli, and no additionally enhanced cytokine production was noted when stimulated with 30 μg alum adjuvant.

3.3. Cytokine production in response to different formulations of H5 influenza vaccines

H5 split materials were prepared and cytokine production profile was compared to those in response to the seasonal A/H1N1/Brisbane and A/H1N1/California/04/2009. IFN-γ was produced when stimulated with each split antigen, showing different levels of IFN-γ (Fig. 2). There was no significant difference in the other cytokine profiles among three split materials.

Alum-adjuvanted WIV, plain WIV, and the split formulation of the H5 vaccine antigen were adjusted to 30 μg/ml HA protein concentration. PBMC were stimulated with 3 μg of HA antigen. Through the analysis of 17 cytokines and chemokines, the productions of IL-1β, IL-6, IL-17, IFN-γ, TNF-α, and MCP-1 showed different profiles from control culture or when stimulated with aluminum alone. Results of cytokine profiles are shown in Table 3. IFN-γ and TNF-α were produced when stimulated with H5 split material. H5 WIV induced the higher production of IL-6, IL-17, TNF-α, and MCP-1 than control culture or those stimulated with Alum or H5 split materials. There was no increase in IL-1β production when stimulated with aluminium alone and H5 split antigen, but slightly higher levels of IL-1β production were observed in response to plain WIV. When stimulated with alum-adjuvanted WIV, the enhanced production of IL-1β was demonstrated and the other cytokines were produced similar to the stimulation with H5 WIV.

Table 2
Relationship between acute febrile reactions and antibody response.

	N	Mean ± SD[d]	95% C.I.	
Fever−	170	2.76 ± 1.26	2.58–2.95	
Fever+	200	3.56 ± 1.30	3.38–3.74	[a]
37.5–<38.0 °C	56	3.11 ± 1.27	2.77–3.45	
38.0–<39.0 °C	79	3.53 ± 1.32	3.24–3.82	[b]
≥39.0 °C	65	3.98 ± 1.17	3.70–4.27	[c]

[a] Mean NT titers were significantly different between subjects with febrile reactions after immunization and those without febrile reactions ($p < 0.01$).
[b] Significant difference was noted between NT titers in subjects with high body temperature ≥37.5–38.0 °C and in those with 38.0–39.0 °C ($p < 0.05$).
[c] Significant difference was noted between NT titers in subjects ≥37.5–38.0 °C and in those with ≥39 °C ($p < 0.01$).
[d] Mean titer of NT antibody expressed as 10×2^n.

The 17-plex human cytokine assay demonstrates the cytokine profile and does not reflect the actual concentrations of the cytokines. As shown in Table 3, enhanced production of IL-1β was noted but IFN-α is not assayed in 17-plex kits. IL-1β, IL-6, and IFN-α were evaluated using EIA, and the results are shown in Fig. 3. IFN-α was produced when stimulated with WIV, and higher levels of IFN-α were demonstrated in subject numbers 21–25. In younger subjects less than one year of age (subject numbers 1–5), the enhanced production of IFN-α was shown in response to alum-adjuvanted WIV. A very low level of IL-1β was produced in response to WIV, and IL-1β production was enhanced when stimulated with alum-adjuvanted WIV. IL-6 was also produced in response to both WIV and alum-adjuvanted WIV, and alum-adjuvanted WIV enhanced the production of IFN-α, IL-1β, and IL-6. The production pattern of IFN-α in different age groups was similar to that of IL-6. IL-1β production profile was different from the others. Production of these cytokines seemed to be prominent in young infants at less than one year of age (subject Numbers 1–5) and adults (subject Numbers 21–25). Cytokine productions seemed to be different in each individual.

4. Discussion

High-level immunogenicity is primarily required for a highly pathogenic pandemic, such as H5N1. Current split H5 was poor immunogenic and the WIV vaccine formulation has been reconsidered to have renewed merits concerning immunogenicity and cross-reaction [21–25]. Besides alum adjuvant, squalene oil emulsion adjuvants (MF59 and AS03) were used in H5 pandemic investigational split vaccines and induced

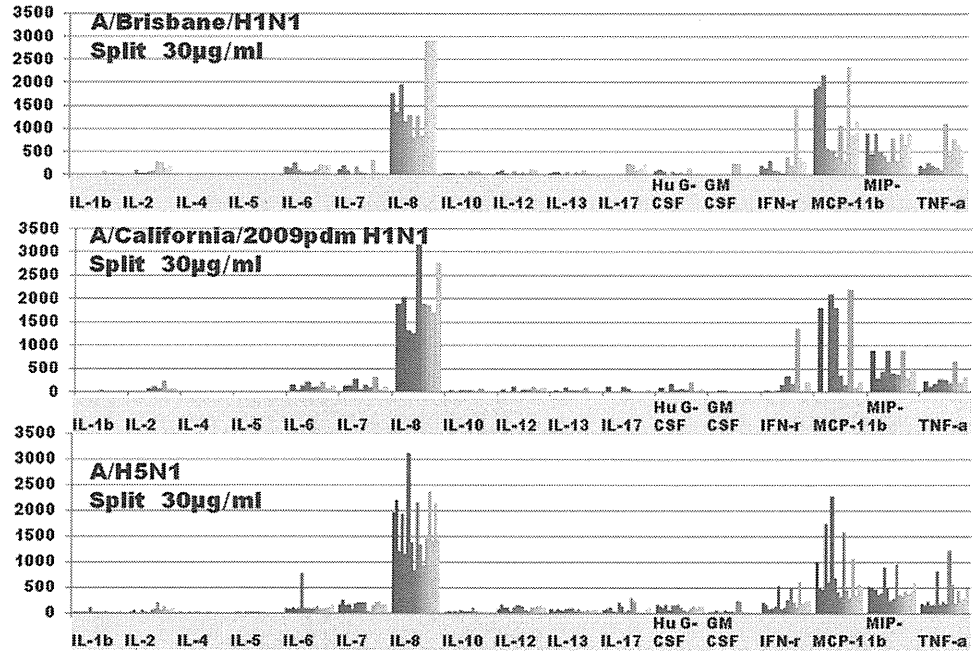

Fig. 2. Cytokine profile of PBMC cultures stimulated with split influenza vaccines. Split vaccine materials were used: H5N1 pandemic NIBRG-14 strain, originated from H5/A/Vietnam/1194/2004, A/H1N1/Brisbane/2007, and 2009 pandemic A/California/07/2009. Each antigen was prepared at the concentration of 30 μg/ml of HA antigen, and PBMC were stimulated with 0.1 ml (3 μg/test).

high-level immunogenicity with allowing for antigen saving, along with cross protective broad antibody responses [26,27]. This type of adjuvant was also applied for the 2009 pandemic vaccines, and resulted in efficient immunogenicity [23,24,28].

WIV was originally considered to induce high-level reactogenicity, and it was replaced by a split formulation in the 1960s [29–31]. H5 split vaccine was poorly immunogenic, and most European companies used oil emulsion adjuvants such as MF59 or AS03. Waddington et al. [25] reported the immunogenicity and reactogenicity of H1N1 pandemic vaccine comprising different formulations of AS03 oil-in-water emulsion adjuvanted and WIV in children at 6 months to 12 years of age. Seroconversion rates were nearly 98–99% in the AS03-adjuvanted vaccine group, but 80.6% at <5 years, and 95.9% at 5–12 years after immunization with WIV. An important finding was that WIV showed a strong age-dependent response in terms of immunogenicity, probably influenced by a past history of influenza infection. As for systemic adverse illness, febrile reaction was observed in approximately 10% of recipients aged <5 years, and in 3% of those aged 5–12 years after the administration of WIV. Wu et al. [21] reported that 5–15 μg of alum-adjuvanted H5 split vaccines were tolerated by children aged 3–11 years and 5–30 μg alum-adjuvanted split and 5 μg WIV vaccines were also tolerated by those aged 12–17 years. 10–15 μg of alum-adjuvanted split vaccine induced a 55% seroconversion and seroprotection rate in those aged 3–11 years, and 5 μg of alum-adjuvanted WIV induced a higher immunogenicity than 10 μg of adjuvanted split vaccine. When alum-adjuvanted WIV was used in young infants, a high incidence of febrile reactions (50–60%) was reported in a study in China although the number of recipients was very small [21].

In Japan, alum-adjuvanted WIV was licensed for adults but not for children. In a clinical trial of alum-adjuvanted WIV in a pediatric group, the incidence of febrile reactions (≥38 °C) after vaccination reduced by age: 100% in those less than one year, 50–60% in those 1–6 years, 29% in those 7–12 years, and 6% in those 13–19 years. The cytokine response was investigated in lymphocyte cultures stimulated with different H5 vaccine formulations to identify the reason for the immunogenicity and immunotoxicity of alum-adjuvanted H5 WIV. Cytokine production by PBMC was higher in young infants, but some teenagers and adults demonstrated a high-level cytokine response.

Many kinds of adjuvant have been developed, and they cause adverse reactions at the inoculation site or systemic reactions. Alum-based adjuvant was first approved for human use and continues to be widely used in many vaccines as an immuno-potentiator [29–31]. Two potential mechanisms are basically considered: (a) the formation of a depot from which the antigen is gradually released; (b) soluble antigen is converted to a particle form easily phagocytosed by antigen presenting cells (APC) such as dendritic cells or macrophages [31].

Recently, the stimulation on the innate immunity has been found to modulate the development of an acquired immune response through the production of cytokines [19,20]. The innate immune system consists of Toll-like receptors (TLRs), retinoic

Table 3
Production of IL-1β, IL-6, IL-17, IFN-γ, TNF-α, and MCP-1 when stimulated with Alum, H5 split, H5WIV and Alum adjuvanted H5 WIV.

	IL-1β	IL-6	IL-17	IFN-Y	TNF-α	MCP-1
Control	26.8 (13.3–40.3)	86.9 (46.4–127.3)	26.4 (13.3–39.5)	73.5 (45.7–101.3)	224.1 (148.4–299.9)	194.1 (120.8–267.4)
Alum	36.3 (21.6–51.0)	71.8 (50.7–92.9)	40.3 (26.1–54.5)	75.1 (56.6–93.7)	151.4 (114.4–188.4)	294.8 (154.5–435.0)
H5 split	21.6 (12.3–30.8)	145.4 (88.3–202.5)	69.3 (38.0–100.6)	182.3 (118.8–245.7)	328.5 (226.9–430.2)	544.3 (299.9–788.6)
H5WIV	50.1 (38.1–62.2)	503.6 (370.8–636.3)	180.0 (154.8–215.3)	354.4 (226.2–482.5)	843.4 (681.4–1005.4)	1452.5 (927.2–1977.8)
H5WIV + Alum	142.7 (63.0–22.4)	467.6 (306.3–628.8)	159.2 (133.5–185.0)	274.8 (169.0–380.5)	624.0 (424.3–823.7)	1023.2 (576.5–1469.9)

Lymphocytes were obtained from 25 healthy individuals who were not immunized with H5 vaccine. Mean values (pg/ml) are shown and ranges of 95% CI are in the parenthesis.

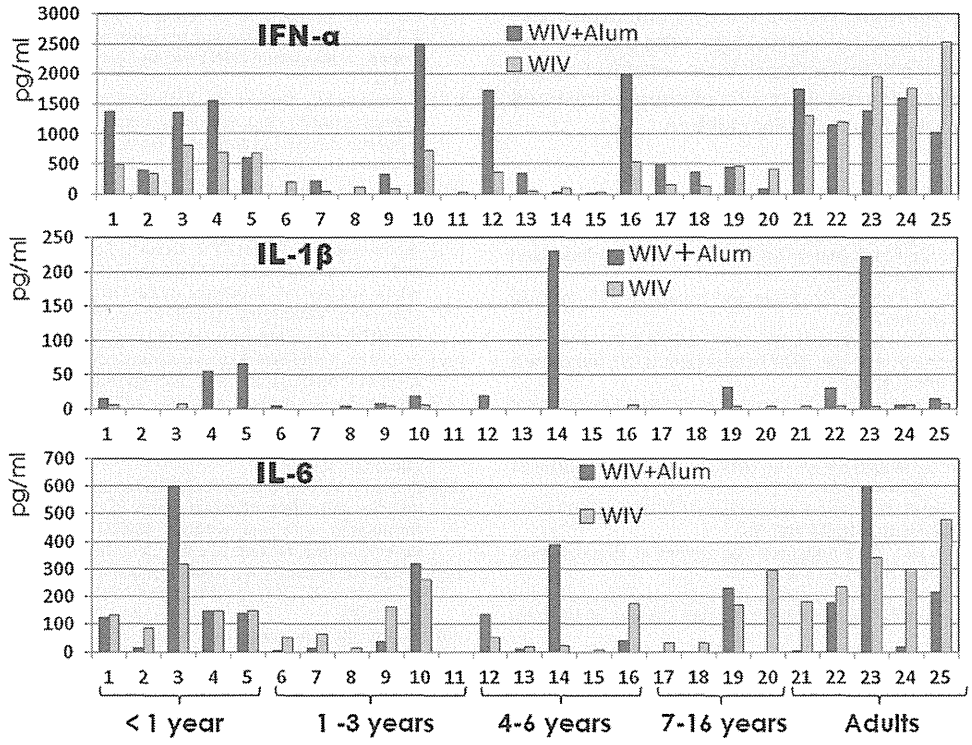

Fig. 3. IFN-α, IL-1β, and IL-6 production. IFN-α, IL-1β, and IL-6 were measured by EIA in PBMC cultures. PBMC were stimulated with H5 WIV and alum-adjuvanted WIV vaccine materials. Samples 1–5 were obtained from healthy individuals less than one year, those 6–11 from 1 to 3 years of age, those 12–16 from 4 to 6 years, those 17–20 from 7 to 16 years, and those 21–25 from adults. Black columns are cytokine productions stimulated with adjuvanted H5WI, and grey columns show those stimulated with H5 WIV.

acid inducible gene-based (RIG)-like receptors, and nucleotide oligomerization domain (NOD)-like receptors (NLRs), known as inflammasome [20,32–34]. Inflammasome consists of NLRP3, apoptosis-associated speck-like protein (ASC), which is thought to be an adaptor molecule of NLRP-3, resulting in the recruitment of caspase. It stimulates the production of inflammatory cytokines, IL-1β, IL-6, and IL-18 from proinflammatory molecules through the enzymatic activity of caspase [34]. Alum adjuvant induced cellular lysosomal damage or tissue damage and stimulated NLRP3 inflammasome through increased levels of uric acid caused by tissue damage [35,36]. The mechanisms of immunogenicity induced by Alum adjuvant have remained poorly understood regarding whether the stimulation of NLRP3 inflammasome is dispensable or not [37–39].

The activation of innate immunity increased antigen-specific adaptive immunity through TLRs induced by influenza vaccine without influencing NLRP3 inflammasome [40]. WIV influenza virus induced antigen-specific antibodies through the production of type I IFN involving the activation of TLR7 in mice [32,41]. Kuroda et al. [42] reported that alum induced LPS-primed macrophages to produce prostaglandin E2 (PGE2) and IL-1β. PGE_2 production was independent of NLRP3, ASC, and the caspase-1 inflammasome complex, and PGE_2 expression depended on cyclooxygenese (COX) and PGE synthase, regulated by spleen tryrosin kinase (Syk) and p38 MAP kinase in macrophages. PGE_2 was found to suppress Th1 responses with a reduced production of IL-2 and IFN-γ, but facilitated the differentiation of Th1 cells in the presence of IL-12 and, thus, cytokine species and their balance regulated PGE2 function on antibody production [18,42,43]. WIV and alum-adjuvanted WIV induced the production of the endogenous cytokines IL-1β, IFN-α, IL-6, and TNF-α, and they induced PGE2 in circumventricular organs through capillary fenestration, which is a well-known pyrogen [20,44].

WIV has genomic RNA that is recognized by TLR-7, inducing IFN-α [40]. In the clinical trial of alum-adjuvanted WIV, the incidence of febrile reactions (>38 °C) after vaccination reduced by age: 100% at less than one year, 50–60% at 1–6 years, 29% at 7–12 years, and 6% at 13–19 years. However, there was no comparative control group who received non-adjuvanted H5 plain WIV to discuss the incidence of febrile reactions. Cytokine production by PBMC was higher in young infants, some teenagers and adults in response to WIV. Enhanced productions of IFN-α, IL-1β, and IL-6 were demonstrated in very young subjects, and were suggested to be associated with a higher incidence of febrile reactions (immunotoxicity) and high immunogenicity (adjuvantogenicity). Cytokine profiles should be checked in serum from those who had high fever after immunization with alum-adjuvanted H5 WIV to observe the direct relationship between the enhanced cytokine level and febrile illness. Lymphocytes from adults also produced high levels of cytokines in response to alum-adjuvanted H5 WIV. Even though, sufficient immune responses were not observed in adults with lower incidence of febrile illness. It should be further investigated to clarify the different responsiveness to cytokines by aging.

Acknowledgements

This study was supported by Research on Regulatory Science of Pharmaceuticals and Medical Devices Grants, and funding for Research on the Accumulation of Evidence for Effective Vaccine Use and Vaccine Policy, from the Ministry of Health, Labour, and Welfare.

The clinical trial of H5N1 pandemic influenza vaccine for children was performed under the chief investigator, Dr. Hitoshi Kamiya, and was supported by a clinical trial promotion project of Japan Medical Association, which was funded by a Health Labour Sciences Research Grant from the Ministry of Health, Labour, and Welfare.

We dedicate this paper to Dr. Hitoshi Kamiya, Emeritus President of the Japanese Association of Vaccinology, who passed away in 2011, for his organization of this research team, and his desire to know the reasons for the unfavorable results of alum-adjuvanted H5N1 whole virion inactivated vaccine for children.

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Vaccine 30 (2012) 7662–7666

Contents lists available at SciVerse ScienceDirect

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Review

Alum-adjuvanted H5N1 whole virion inactivated vaccine (WIV) induced IgG1 and IgG4 antibody responses in young children

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ARTICLE INFO

Article history:
Available online 24 October 2012

Keywords:
H5N1
Whole virion inactivated influenza vaccine
IgG subclass antibody
Th1 and Th2 balance

ABSTRACT

IgG subclass antibody responses are not fully understood. Alum-adjuvanted H5N1whole virion inactivated vaccine (WIV), a genetically reassortant vaccine seed strain originating from H5N1/A/Vietnam/1194/2004 and PR-8, induced significantly stronger antibody responses in neutralizing antibodies in children. In this report, IgG subclass antibody responses were investigated, and most serum samples were positive for IgG1 antibody before immunization. A significant response (more than 4-fold increase) of IgG1 antibody was observed in 67/193 (34.7%) and that of gG4 antibodies in 42/193(21.8%). Children <4 years of age showed a significant increase in IgG subclass antibodies but those ≥4 years showed lower responses. Alum- adjuvanted H5N1WIV induced an efficient immune response in young children especially <4 years.

Contents

1. Introduction

The 20th century saw three pandemics of influenza. The most devastating pandemic dated back to 1918, known as Spanish flu, and killed an estimated 40–50 million people, caused by H1N1 influenza virus transmitted through some animals not directly from an avian influenza virus [1]. Asian influenza A/H2N2 caused the second pandemic in 1957, and Hong Kong influenza A/H3N2 the third in 1968. After the 1968 pandemic, small local outbreaks were reported. Caused by H5N1, H7N7, or H9N2, they were considered to be from poultry. There was a regional outbreak of H5N1 in Hong Kong in 1997, and six of 18 patients died, causing a pandemic threat [2]. H5N1 is considered to be a target for pandemic vaccine, and WHO addressed sharing viruses and sequence information for a future pandemic vaccine development [3–5], and the development of an effective and safe vaccine is expected to mitigate the threat of a pandemic.

In Japan, alum-adjuvanted H5N1 whole virion inactivated vaccine (WIV) (alum concentration: 300 μg/ml) was developed using a genetically engineered reassortant, the NIBRG-14 strain, originating from H5N1/A/Vietnam/1194/2004. In a clinical phase II/III trial in healthy adults, alum-adjuvanted WIV (HA protein: 15 μg) led to favorable immunogenicity (>70% sero-conversion rate in NT antibodies) without causing any serious systemic illnesses [6]. However, when it was administered to young infants and children at a reduced dose, 7.5 or 3 μg, a high body temperature (≥38.0 °C) was observed in >60% of recipients <7 years of age, but, unexpectedly, NT antibody titers were higher than those observed in the clinical trial in adults.

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0264-410X/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.vaccine.2012.10.006