インフルエンザワクチン
——その特徴と効果
Influenza vaccine：Its features and effectiveness

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◎現在，季節性インフルエンザ対策に使われているワクチンの剤型はスプリットワクチンであり，プライミング効果は劣るものの，優れたブースティング効果が認められている．注射で接種したワクチンで誘導される免疫の主体は血中IgG抗体であり，粘膜に滲み出ることで感染防御に働いている．赤血球凝集抑制抗体40倍は50%の成人の発症を予防する抗体価である．IgG抗体は変異したインフルエンザウイルスに対する反応がIgA抗体や細胞性免疫よりも劣る欠点がある．インフルエンザワクチンの効果が低いのは小児と高齢者である．今シーズン(2011/12)から小児への免疫原性を高めるために，接種量が世界標準量に増加した．アメリカでは高齢者の免疫原性を高めるために，成人接種量の4倍量のヘマアグルチニンを含むインフルエンザワクチンが使用されている．インフルエンザ発症予防のためには，高い血中抗体価を誘導しておくことが大切である．

Key word：インフルエンザ，インフルエンザワクチン，スプリットワクチン，免疫原性，パンデミック

インフルエンザウイルスは，核蛋白の抗原性からA型，B型，C型に分類される．A型インフルエンザウイルスの自然宿主はカモであり，多くの鳥類や哺乳類に感染するが，B型とC型はヒトのウイルスである．突然の高熱，頭痛，筋肉痛，関節痛などのインフルエンザ様症状(influenza like illness：ILI)を示すのはA型とB型である．B型はヤマガタ系とビクトリア系に大別される．

A型インフルエンザウイルスにはエンベロープ上に2種類の構造蛋白〔ヘマグルチニン(HA)とノイラミニダーゼ(NA)〕が存在する．HAは16種類，NAは9種類あるため，A型インフルエンザウイルスは理論上144種類の亜型が存在する．現在ヒトの間で流行している亜型はA(H1N1)とA(H3N2)である．2009年にパンデミックを起こしたウイルスはA旧ソ連型と免疫原性が大きく異なるA(H1N1)亜型である．毎年ヒトの間では規模の大小はあるもののA(H1N1)，A(H3N2)，B型が流行してILIを発症させるため，毎年のインフルエンザワクチン接種が勧められている．本稿では，インフルエンザワクチンの特徴と効果について解説する．

インフルエンザワクチンの剤型(表1)

インフルエンザワクチンには毎年のインフルエンザ流行に備える季節性インフルエンザワクチンと，現在ヒトの間で流行しているA型インフルエンザウイルス亜型と異なる亜型が出現したときに備えて準備しているプロトタイプワクチンとがある．わが国で現在使用している季節性インフルエンザワクチンの剤型は発育鶏卵で増殖したインフルエンザウイルス全粒子を精製，不活化した後，接種局所の副反応や発熱に関与しているエンベロープをエーテル処理で取り除いたスプリットワクチンである．感染防御抗原(「サイドメモ1」参照)であるHAを分離精製していることからHAワクチンともよばれている．皮下注射で接種する．

スプリットワクチンは全粒子ワクチンに比べプライミング効果は劣るが，ブースティング効果は認められている(「サイドメモ2」参照)．現在カイ

表 1　インフルエンザワクチンの剤型と免疫効果

剤型	各インフルエンザウイルスの HA 量(μg/dose)	免疫効果	
		プライミング	ブースティング
季節性インフルエンザワクチン			
不活化ワクチン			
全粒子ワクチン	15	＋	＋
スプリットワクチン			
通常量ワクチン	15	±	＋
高用量ワクチン*	60	±	＋＋
皮内接種用ワクチン*	9	±	＋
サブユニットワクチン†	開発中	－？	＋
ビロゾーマルワクチン†	15	＋	＋
生ワクチン	15	＋＋	＋
プロトタイプワクチン(不活化ワクチン)			
全粒子ワクチン	15	＋	＋
アルミアジュバント加全粒子ワクチン	15	＋＋	＋
スクワレン系アジュバント加スプリットワクチン	15/7.5‡	＋＋＋	＋＋

HA 量：インフルエンザワクチンに含まれる各コンポーネントの HA(ヘマグルチニン)量.
*：日本では認可されていないが，アメリカで認可されている.
†：サブユニットワクチンは培養細胞で HA 蛋白を増幅させて製造されたワクチン，ビロゾーマルワクチンはビロゾームに HA とノイラミニダーゼ(NA)を付着させたワクチンでヨーロッパで使用.
‡：MF59 を用いているノバルティスの HA 量は 15μg/dose，AS03 を用いているグラクソスミスクラインの HA 量は 7.5μg/dose.

コ由来細胞に HA 遺伝子を挿入して増生させた HA を精製したサブユニットワクチンの開発が行われている[1]．サブユニットワクチンのプライミング効果も不十分である.

皮内接種は少ない抗原量で皮下接種や筋肉接種と同等の免疫原性が認められている．アメリカでは 2011/12 シーズンから，スプリットワクチンを用いた皮内接種用インフルエンザワクチンが認可された[2]．接種抗原量は 9μg である．0.1 mL 皮内接種する.

アメリカやロシアでは経鼻接種するインフルエンザ生ワクチンが使用されている．3 種類の温度変異株(「サイドメモ 3」参照)を親株とし，HA と NA をそのシーズンのワクチン株に組み換えて製造する．インフルエンザの免疫がない小児に接種すると感冒様症状が出現するリスクが高く，成人

サイドメモ 1　感染防御抗原

感染防御抗原とは，ウイルスが細胞に感染するときに中心的役割を果たすウイルス蛋白のことであり，これらの蛋白に対する抗体が感染防御の中心的役割を担っている．インフルエンザウイルスのヘマグルチニン(HA)，B 型肝炎ウイルスの HBs 抗原，麻疹ウイルスの H 蛋白と F 蛋白などが代表である.

サイドメモ 2　プライミングとブースティング

免疫に関与する細胞群として抗原提示細胞，免疫未熟細胞，免疫記憶細胞，免疫実行細胞がある．抗原提示細胞は生体に入った異物を認識し，免疫未熟細胞に情報を提示する細胞群で，樹状細胞，Langerhans 細胞などのマクロファージ系の細胞である．免疫情報の提示を受けた免疫未熟細胞(Tho 細胞，Bo 細胞)は，成熟して免疫記憶細胞(Th_1 細胞，Th_2 細胞，B 細胞)になる．免疫記憶細胞は免疫実行細胞(形質細胞，キラー T 細胞)を誘導し抗体を産生させる．免疫記憶細胞をおよび免疫実行細胞を誘導することをプライミングといい，誘導された免疫実行細胞の数を増加させ，免疫を高めることをブースティングという．生ワクチンでは一度に大量の免疫実行細胞を誘導することができるが，不活化ワクチンでは 2 回以上接種してまずプライミングし，4～6 カ月後以降にブースティングする．一度誘導された免疫記憶細胞は消失しないので，免疫記憶細胞が誘導されていると 4～6 カ月後以降ならばいつでもブースティングが認められる．なお，キラー T 細胞は生きたウイルスでないと誘導できない.

表 2　インフルエンザワクチンと麻疹ワクチンの比較

項目	インフルエンザ	麻疹
発症させるウイルス	A(H1N1)，A(H3N2)，B	1種類
ウイルスの変異	連続変異しやすい	ゆっくりと変異
	不連続変異あり	不連続変異なし
感染症の病態	局所性ウイルス感染症	全身性ウイルス感染症
感染予防	sIgA 抗体，血中抗体，CMI	血中抗体，CMI*
発症予防抗体価		
50%予防	HI 抗体 40 倍	
90%予防	HI 抗体 160 倍	120 mIU/mL†
感染予防抗体価	発症予防抗体価と同じ	800 mIU/mL†
ワクチン		
剤型	スプリットワクチン(HA)‡	生ワクチン
抗体価の半減期	半年	約3年
接種後の発症予防抗体価	≧70%**	≧95%
接種回数	毎年1回	生涯2回

sIgA：分泌型 IgA，CMI：細胞性免疫，HI：赤血球凝集抑制，HA：ヘマグルチニン．
*：麻疹では，CMI は感染からの回復に重要な役割を果たしている．
†：国際単位，≧120 mIU/mL は中和抗体で≧4 倍，≧750 mIU/mL は中和抗体で≧32 倍．
‡：エーテル処理によりウイルスの立体構造をこわし，HA を分離精製している．
**：ヨーロッパ医薬品庁が定めるインフルエンザワクチン評価基準における抗体陽性率，HI 抗体 40 倍以上の割合．

ではスプリットワクチンと比べてブースティング効果が劣っている．なお，生ワクチンでは分泌型 IgA(sIgA)抗体と細胞性免疫が賦活されるため，ウイルスの変異に対する対応力が優れている[3,4]．アメリカでは 2〜49 歳が接種対象者である．

多くの人が免疫をもたない新型インフルエンザウイルス対策用にわが国で準備されているプロトタイプワクチンの剤型は，発育鶏卵で増殖させたウイルス全粒子をアルミアジュバントと反応させたワクチンである(アルミアジュバント加全粒子ワクチン)[5]．現在培養細胞で増殖させたウイルスを用いたプロトタイプワクチンの開発が行われている．

一方，ヨーロッパではスプリットワクチンにスクワレン系アジュバントを加えたインフルエンザワクチンを，アメリカでは Vero 細胞で増殖させたインフルエンザウイルスを用いた全粒子ワクチンをプロトタイプワクチンとして準備している．いずれのワクチンもプライミング効果とブースティング効果，さらにブースティングによる交差免疫の誘導が認められている．

インフルエンザの病態と発症予防(表 2)

インフルエンザは気道にウイルスが感染して症状が出現する局所性ウイルス感染症であり，ウイルス血症は認められない．局所性ウイルス感染症でワクチンが開発されているのはインフルエンザだけである．

インフルエンザの発症予防および回復には，sIgA 抗体，血中 IgG 抗体，細胞性免疫が関与している．血中 IgG 抗体はほぼ同じ濃度が気道粘膜に滲み出る．血中赤血球凝集抑制(hemagglutination inhibition：HI)抗体が高いほど，発症予防効果が優れている．全身性ウイルス感染症の発症予防レベルは 90%以上のヒトの発症を予防する抗

サイドメモ 3

温度変異株(ts mutant)

一般にヒトに感染するウイルスは 37℃で効率よく増殖し，高温(39℃)でも比較的よく増殖する．温度変異株とは野生株と比べて高温での増殖が低下した株であり，麻疹ワクチン株である AIK-C 株が代表である．多くの生ワクチン株は温度変異性をもっている．インフルエンザ生ワクチンの製造に使用される 3 種類の親株(ワクチン製造の鋳型になる株)はいずれも上気道の温度である 33℃で増殖効率がよく，37℃では増殖効率が劣る温度変異株である．

表 3　不活化インフルエンザワクチンの有効率[8)]

年齢群	診断基準	有効率(%)	
		日本	欧米
小児　6歳未満	ウイルス学的		58
	ILI	22～25	28
6歳以上	ウイルス学的		65～78
	ILI	24～40	28
成人	ILI		70～90
	入院回避		90
高齢者	ILI	34～55	30～40
	入院回避		50～60
	死亡回避	≧80	80

ILI：インフルエンザ様疾患，ウイルス学的診断：ウイルス分離，血清診断などを用いた実験室診断．

体価であるが，インフルエンザの発症予防抗体価であるHI抗体40倍は50%の成人の発症を予防する抗体価である[6)]．

インフルエンザウイルスは変異が早いウイルスであり，シーズンごとに接種される3種類のワクチン株のいずれかが通常毎年更新されること，一度接種したとしてもインフルエンザワクチン後の抗体陽性率(HI抗体≧40倍)は麻疹と比べて低率であり，しかも抗体価は半年で1/2に低下することから，毎年流行前に1回の接種が勧められている．

インフルエンザワクチンの効果(表3)

インフルエンザワクチンの効果は主として，流行時のインフルエンザ発症予防で評価される．インフルエンザ流行中にILIを発症したとしても，すべての原因はインフルエンザウイルスとは限らないため，インフルエンザの診断を臨床診断で行うと，ウイルス学的に診断したときと比べインフルエンザワクチンの有効率が低下する[7)]．また，流行株とワクチン株の抗原性が一致すると有効率が高く，抗原性が変異すると有効率は低下する．HAの抗原性が4倍以上変異したとき，ワクチン株と流行株に変異があったと診断する．ILIを指標としたときの成人のインフルエンザワクチンの有効率は70～90%であり，ワクチン株と流行株の抗原性が異なると成人の有効率は60%程度に低下する[3)]．

インフルエンザワクチンの有効率が低いのは，乳幼児と高齢者である．高齢者では加齢により免疫応答が低下するためである．高齢者の免疫応答を高めるためには，3～4週間隔で2回接種するよりも1回に多量接種する方が優れている．アメリカでは高齢者用に成人に接種する4倍量のHAが含まれたインフルエンザワクチンが使用されている[2)]．

乳幼児でインフルエンザワクチンの効果が劣る原因として，1歳未満の乳児は1歳以上の子どもよりも抗体反応が低いことや，細胞性免疫が弱いために発症予防には成人よりも高い抗体価が必要なことが示唆されている[8,9)]．2011/12シーズンから小児の抗体反応を高めるために，わが国小児のインフルエンザワクチン接種量がWHO推奨量に増量された．WHO推奨量で接種したときの抗体反応を表4に示した[10)]．1歳未満児ではいずれの型に対しても抗体陽性率が低かったが(ヨーロッパ医薬品庁(EMA)基準≧70%)，A/H1N1およびA/H3N2に対しては抗体陽転率(EMA基準≧40%)および幾何平均抗体価(GMT)上昇率(EMA基準≧2.5倍)ともにEMAの基準を満たしていた．

小児におけるインフルエンザワクチン接種回数に関しては，B型インフルエンザウイルスに対する抗体反応を期待するならば，13歳未満は2回接種が勧められる．なお，今回接種量を増量させたのは2回接種によるプライミング時の抗体反応，または1回追加接種によるブースティング効果を高めるためであり，接種量が増加してもプライミングが必要な人は2回接種が必要である．

インフルエンザワクチンの集団免疫効果

インフルエンザはヒトからヒトに感染する感染症であり，基本再生産数は1.5～2.4，集団免疫率50%程度である(「サイドメモ4」参照)．インフルエンザワクチンを高齢者施設や障害者施設の職員に接種すると，接種率が低い施設と比べて接種率が高い施設では入所者のインフルエンザ発症率やインフルエンザ流行期間中の死亡率が低下する[11)]．また，小児にインフルエンザワクチンを接種すると同居している高齢者のインフルエンザ発症が61%低下するなど[12)]，インフルエンザワクチンの集団免疫効果が示されている．

表 4　小児におけるインフルエンザワクチンの免疫原性(阪大微生物病研究会)[10]

年齢(人数)	ワクチン	A/H1N1			A/H3N2			B		
		陽転率	GMT増加率	陽性率	陽転率	GMT増加率	陽性率	陽転率	GMT増加率	陽性率
6カ月<1歳	1回後	5.9	1.6	5.9	11.8	2.1	11.8	0.0	1.0	0.0
(17)	2回後	41.2	3.8	41.2	58.8	6.0	58.8	23.5	2.4	23.5
1歳<3歳	1回後	47.1	5.3	52.9	64.7	9.4	64.7	52.9	4.3	52.9
(17)	2回後	76.5	7.7	76.5	94.1	13.6	94.1	64.7	6.5	64.7
3歳<6歳	1回後	61.1	6.6	66.7	88.9	6.3	94.4	66.7	5.2	77.8
(18)	2回後	72.2	7.1	72.2	94.4	7.4	94.4	77.8	5.9	83.3
6歳<13歳	1回後	87.5	9.1	87.5	81.3	7.3	100	18.8	5.5	37.5
(16)	2回後	87.5	9.1	87.5	81.3	7.3	100	31.3	3.4	50.0

EMA の基準：抗体陽転率(≧40%)，GMT 増加率(≧2.5 倍)，抗体陽性(HI 抗体≧40 倍)率(≧70%)．

基礎疾患のある人への接種

糖尿病，肝硬変，慢性腎不全などの慢性基礎疾患をもつ人は，インフルエンザに罹患すると重症化するリスクが高い人である．基礎疾患のある人は高齢者と同様にインフルエンザワクチンに対する免疫応答が低下した人である．免疫低下者の抗体反応を高めるためには，理論上高齢者と同様に接種する抗原量を高める必要がある．

妊婦がインフルエンザを発症すると肺炎を合併する頻度が高いため，妊娠期間中がインフルエンザ流行と重なる妊婦にはインフルエンザワクチン接種が勧められている．インフルエンザワクチンは妊娠時期にかかわらず接種が推奨されている．母乳を与えている母親へのインフルエンザワクチン接種も安全性が確認されている．

第三三半期の妊婦にインフルエンザワクチンを接種すると，妊婦が発熱性呼吸器疾患に罹患する率が29%減少し，生まれた生後6カ月未満の子どもも発熱性呼吸器疾患を発症する率が36%減少する[13]．インフルエンザ抗体は3種類ともほぼ同じ濃度で児に移行する[14]．生後6カ月未満の子どもをインフルエンザから予防するために，妊婦にインフルエンザワクチンを接種する対策が検討されている．

サイドメモ4　基本再生産数(R_0)と集団免疫率(H_0)

基本再生産数(R_0)とはひとりの感染者が周囲の免疫のない人に感染させる数で，この数字が高いほど感染力が強いことを示している．R_0は感染症ごとに異なっており，一番感染力が強い感染症は麻疹と百日咳で16～21である．

集団免疫率(H_0)とは，ある集団でヒトからヒトに感染する感染症の流行を阻止するために必要な免疫率のことである．$H_0=(1-1/R_0)\times100$の関係がある．この率も感染症ごとに異なっており，麻疹の集団免疫率は90～95%である．

インフルエンザワクチンの副反応と卵アレルギー児への接種

インフルエンザワクチン接種後約30%に注射部位の紅斑や疼痛が認められる．発熱はまれである．1976年のブタインフルエンザ騒動時に用いられたインフルエンザワクチンではGuillain-Barr症候群(GBS)の出現率が高かったが，近年用いられているインフルエンザワクチンではGBSの有意な増加は認められていない[3]．GBS既往者はインフルエンザワクチンの接種不適当者である．

現行のインフルエンザワクチンは発育鶏卵を用いて製造されるため，欧米では卵を食べてアナフィラキシーを起こす人は接種不適当者とされている．インフルエンザワクチン接種によりアナフィラキシーを引き起こすオボアルブミン量は600～700 ng/dose以上である[2,15]．一方，わが国のインフルエンザワクチンに含まれるオボアルブミン濃度は1 ng/mL程度である[16]．わが国のワクチンを卵アレルギー児に接種したとしても，理論上オボアルブミンによるアナフィラキシーは起こらないと判断されている．

プロトタイプワクチンの製造と接種計画

WHOは2009年のパンデミック後も多くの人が免疫をもたないインフルエンザウイルス(新型インフルエンザウイルス)の流行を危惧している．新型インフルエンザウイルスの出現を予測して準備するワクチンがプロトタイプワクチンであり，新型インフルエンザウイルスがパンデミックを起こしたとき，パンデミック株を用いて製造するのがパンデミックワクチンである．パンデミックワクチンの剤型は出現した亜型により異なってくる．2009年のパンデミックではスプリットワクチンが用いられた．H1，H2，H3以外の亜型が出現した場合はプロトタイプワクチンの剤型が用いられる．

わが国ではA/H5N1亜型の出現を危惧して毎年1,000万人分のプロトタイプワクチンの備蓄を行っている．現在インフルエンザウイルスの増殖に発育鶏卵を用いているが，高病原性A/H5N1がパンデミックを起こしたときは発育鶏卵でのインフルエンザウイルス増殖が不可能であること，発育鶏卵の数に制限があり，急いでワクチンを製造することが困難であることなどの理由で，培養細胞を用いたインフルエンザワクチンの開発が進んでいる[1)]．

現在のところ，新型インフルエンザウイルスとして予測されているのは，①現在流行しているA/H3N2香港型およびA/H1N1 pdm 09と大きく抗原性が異なるA/H3N2亜型またはA/H1N1亜型の出現②，A/H2N2亜型の再燃，③H1，H2，H3以外のHA亜型をもつインフルエンザウイルスの出現の3パターンである．2009年パンデミック時の経験から，現在流行しているA(H3N2)やA(H1N1)と免疫原性が大きく異なる同じ亜型のインフルエンザウイルスが出現した場合，乳幼児を除く多くの人は抗体が陰性でもこれらのウイルスに対して免疫記憶をもっているので，1回の接種で十分である．A(H2N2)が出現した場合は，1968年以前に生まれた人は免疫記憶を有しているので1回接種，それ以降の人は2回接種が必要である．これらの亜型以外のHA亜型が出現した場合は，全員2回接種が必要である．

おわりに

インフルエンザワクチンの剤型および有効性について解説した．現在流行しているインフルエンザウイルスと抗原性が異なるインフルエンザウイルスが出現したとしてもインフルエンザウイルスがヒトに感染して発症する臨床像はILIである．しかし，WHOは高病原性A/H5N1によるパンデミックのリスクをいぜん考えている．このためわが国では，季節性インフルエンザ対策と新型インフルエンザウイルスによるパンデミック対策を考えたインフルエンザワクチン製造および開発を行っている．

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ORIGINAL ARTICLE

Survey of Japanese infants younger than 3 months who were treated with oseltamivir for influenza: Safety of oseltamivir treatment

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Abstract

Background: Young infants with influenza virus infection are frequently hospitalized, and are at risk of serious complications including death. With the emergence of pandemic influenza A/H1N1 2009, oseltamivir was approved for use in Europe and the USA, including use in infants aged < 3 months. However, few data are available regarding the safety of oseltamivir treatment for influenza in infants aged < 3 months. *Methods:* The clinical data from Japanese infants aged < 3 months with laboratory-confirmed influenza virus infections, who were treated with oseltamivir between October 2009 and April 2011, were collected and analyzed. *Results:* Forty-four infants were included in the study. The median age was 1 month (range 4 days to 2 months) and median body weight was 4.5 kg (range 2.6–7.6 kg). Thirty-eight infants (86%) had no underlying diseases. The most common presenting symptom was fever (42 infants, 95%). There were no cases of influenza-associated encephalopathy or myocarditis. The median time between the onset of influenza symptoms and initiation of oseltamivir treatment was 0 days (range 0–7 days), with treatment initiated within 1 day in 40 infants (91%). The oseltamivir dose was 1.5–2 mg/kg twice daily in 98% of infants. No serious adverse events were identified during treatment. All infants recovered completely. *Conclusions:* Treatment of influenza with oseltamivir 1.5–2 mg/kg twice daily may be safe in infants aged < 3 months.

Keywords: *Infant, influenza, oseltamivir, outcome, safety*

Introduction

In the USA, young children with pandemic influenza A/H1N1 2009 virus infection are 2- to 3-times more likely to be hospitalized than older children [1]. Infants aged < 6 months are at risk of death from both seasonal influenza virus and pandemic influenza A/H1N1 2009 virus infection [2,3]. Although vaccination provides effective protection against influenza, it is not recommended for infants aged < 6 months because a low rate of immunity after vaccination has been observed in this age group [4]. It is therefore essential to establish effective and safe treatment strategies for infants with influenza, especially infants aged < 6 months.

Oseltamivir is widely used for the treatment of influenza in children aged > 1 y. The recent influenza A/H1N1 2009 pandemic led to emergency approval of oseltamivir for the treatment of infants aged < 1 y by the US Food and Drug Administration and the European Medicines Agency. Some reports have

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shown that oseltamivir appears to be as safe in infants aged <1 y as in older children [5–9]. The response to influenza virus infection and to oseltamivir treatment may be different in young infants, such as those aged <3 months, than in older infants and children, but few data are available for this vulnerable age group. Furthermore, the outcome of Japanese infants aged <3 months who have influenza and are treated with oseltamivir is unknown. The Committee for the Control and Prevention of Influenza of the Japan Pediatric Society (JPS) therefore organized a survey to investigate the symptoms and outcomes of Japanese infants aged <3 months with influenza virus infection who were treated with oseltamivir. The safety of oseltamivir treatment was evaluated.

Patients and methods

The survey targeted Japanese infants aged <3 months with influenza virus infection who were treated with oseltamivir between October 2009 and April 2011, to evaluate clinical presentations, short-term outcomes, and safety of treatment. Infants with influenza were routinely diagnosed at baseline using a rapid antigen detection kit (most commonly: Quick Chaser Flu A, B: Mizuho Medy Co., Ltd; Espline Influenza A & B-N: Fujirebio Inc.; Quick Ex-Flu Seiken: Denka Seiken Co., Ltd; BD Flu Examan: Nippon Becton Dickinson; Poctem Influenza A/B: Otsuka Pharmaceutical Co., Ltd and Sysmex Co., Ltd; Quick Vue Rapid SP influ: DS Pharma Biomedical Co., Ltd; Capilia Flu A + B: Alfresa Pharma Co., Ltd; or Rapid Testa FLU stick: Daiichi Pure Chemicals Kagaku Co., Ltd, Japan). In some patients with negative rapid antigen detection kit results, reverse transcription polymerase chain reaction was used to detect pandemic influenza A/H1N1 2009 virus in clinical specimens including nasal swabs, because detection kit test sensitivities are low to detect pandemic influenza A/H1N1 2009 virus infection [10]. For patients with an influenza virus infection, blood tests including complete blood count and serum aspartate aminotransferase, alanine aminotransferase, and C-reactive protein levels were performed at the same time. In patients with an abnormal white blood cell count and C-reactive protein level, bacterial cultures of blood, urine, bronchoalveolar lavage fluid, cerebrospinal fluid, and/or nasopharyngeal swabs were performed to determine if there was bacterial co-infection.

The parents of patients with influenza were offered off-label treatment with oseltamivir based on the guidelines of the JPS, the American Academy of Pediatrics, and the World Health Organization [11–14], and patients were given oseltamivir if the parents consented. Infants aged <3 months with an influenza virus infection who were treated with oseltamivir were generally hospitalized, and their clinical symptoms and any adverse effects were carefully scored by pediatricians and pediatric nurses. If infants were not hospitalized, they were assessed daily by a pediatrician at an outpatient office until their condition improved. Pediatricians considered those symptoms that occurred for the first time after the initiation of oseltamivir treatment to be the side effects of oseltamivir.

Patient information including age and body weight, underlying diseases, date of onset of symptoms, date of initiation of oseltamivir treatment, type of influenza, clinical symptoms at presentation, clinical course, dose and duration of oseltamivir treatment, adverse clinical effects during or after treatment, and outcome were e-mailed or faxed to the JPS. Data were collected anonymously. This study was approved by the board of directors of the JPS.

Results

Patient characteristics

During the study period, a total of 44 patients were included in the analysis (Table I), of whom 43 (98%) were hospitalized. The median age at presentation was 1 month and the median weight was 4.5 kg. Most of the patients had no underlying diseases. All patients were diagnosed with type A influenza, except for 1 patient with type B.

Clinical symptoms

The clinical symptoms at presentation are shown in Table II. The most common symptom was fever, and other non-specific symptoms such as poor feeding, listlessness, liver dysfunction, and apnea were also observed. No gastrointestinal symptoms were detected, and there were no cases with

Table I. Patient characteristics ($N = 44$).

Characteristic	
Age, median (range)	1 month (4 days to 2 months)
Weight (kg), median (range)	4.5 (2.6–7.6)
Underlying disease, *n* (%)	
None	38 (86%)
LBW	5 (11%)
LBW with VSD	1 (2%)
Type of influenza, *n* (%)	
A	43 (98%)
B	1 (2%)

LBW, low birth weight; VSD, ventricular septal defect.

Table II. Clinical symptoms and signs at presentation ($N = 44$).

Symptoms and signs	Number of infants (%)
Fever	42 (95%)
Rhinitis	10 (23%)
Poor feeding	6 (14%)
Cough	4 (9%)
Listlessness	4 (9%)
Liver dysfunction	3 (7%)
Apnea	1 (2%)

influenza-associated encephalopathy or myocarditis. Two infants (5%) had bacterial co-infections (Streptococcus pneumoniae or Escherichia coli). The infant with Streptococcus pneumoniae co-infection developed severe pneumonia and was mechanically ventilated.

Oseltamivir treatment and outcome after treatment

The median time between the onset of influenza symptoms and the initiation of oseltamivir treatment was 0 days (range 0–7 days), with 40 infants (91%) starting oseltamivir within 1 day. The oseltamivir dose was 1.5 or 2 mg/kg twice daily for 3, 4, or 5 days in most of the infants. Forty-one infants (93%) did not have any adverse side effects during treatment, 1 infant developed mild diarrhea, and 1 infant was irritable. Adverse effects resolved completely with discontinuation of oseltamivir. Most importantly, all infants recovered completely without sequelae (Table III).

Table III. Oseltamivir treatment details and outcomes ($N = 44$).

Treatment and outcome variables	Number of infants (%)
Time between the onset of symptoms and oseltamivir treatment	
0 days	26 (59%)
1 days	14 (32%)
2 days	2 (5%)
6 days	1 (2%)
7 days	1 (2%)
Dosage of oseltamivir	
2 mg/kg twice daily	35 (80%)
1.5 mg/kg twice daily	8 (18%)
Other	1 (2%)
Duration of oseltamivir treatment	
5 days	38 (86%)
4 days	2 (5%)
3 days	3 (7%)
Other	1 (2%)
Adverse effects	
No	41 (93%)
Yes	2 (5%)
Unknown	1 (2%)
Treatment outcome	
Complete recovery	44 (100%)
Sequelae	0 (0%)
Death	0 (0%)

Discussion

During the influenza A/H1N1 2009 pandemic, affected infants were observed to be more frequently hospitalized than older children and adults [1], and infants aged <6 months were found to be at risk of death from both A/H1N1 2009 and seasonal influenza virus infections [2,3]. With the emergence of the pandemic influenza A/H1N1 2009 virus, oseltamivir was approved for use in Europe and the USA, including use in infants aged <3 months. Based on these approvals, the JPS proposed that oseltamivir could be used to treat newborn patients with influenza or with signs suspicious of influenza virus infection [12]. Because there are no data regarding young infants with influenza in Japan, the JPS organized a survey of infants aged <3 months with influenza who were treated with oseltamivir, to evaluate their clinical presentations and the short-term outcomes and safety of oseltamivir treatment.

Although there are 2 brief retrospective reports describing the treatment of influenza with oseltamivir in Japanese infants with a median age of 7 months [5,7], our study is the first to investigate Japanese infants aged <3 months. Most of the surveyed infants had no underlying diseases, indicating that influenza can even develop in healthy infants aged <3 months. Most of the surveyed infants had a type A influenza virus infection, because influenza A/H1N1 2009 was the prevalent subtype in Japan during the 2009–10 and 2010–11 influenza seasons [15].

It is generally difficult for parents of infants with influenza to detect the clinical symptoms of infection and the adverse effects of oseltamivir treatment. In Japan, unwell infants aged <3 months with fever >38°C are usually diagnosed and treated in hospital. In this survey, all patients except for 1 were hospitalized, and were assessed by pediatricians and pediatric nurses.

A previous study by Silvennoinen et al. [16] showed no differences in signs and symptoms between children with influenza A and B virus infections in any age groups. We therefore evaluated the symptoms of all patients in this survey. The most common symptom at presentation in this study was fever, which is similar to findings in older infants and children with influenza [6,16,17]. However, we found that coughing and rhinorrhea were less common in infants aged <3 months than in older infants and children [6,16,17], and we did not detect any gastrointestinal symptoms. Furthermore, none of the infants developed encephalopathy or myocarditis, which are serious complications of influenza and have a high mortality rate [18,19]. This pattern of clinical presentation may be a distinctive feature of infants aged <3 months. Further studies using a larger group of infants with influenza aged <3 months are required

to confirm these results. In this study, 5% of infants had a bacterial co-infection. Pediatricians should be aware that bacterial co-infections may occasionally occur in these infants, because previous reports have shown that secondary bacterial pneumonia can cause severe illness or death in influenza patients [20,21].

In this study, 1.5–2 mg/kg oseltamivir was administered twice daily for 3, 4, or 5 days. This dose is consistent with the JPS guidelines, which recommend 2 mg/kg twice daily for 5 days in newborn patients [12,13]. We did not detect any serious adverse effects, consistent with the results of previous studies of infants aged < 1 y, including newborn patients [5–7,9]. There have been some reports of gastrointestinal symptoms such as diarrhea and vomiting in infants aged < 1 y who were treated with oseltamivir, but the symptoms were mild and did not require medical intervention [6,7,9]. In our study, only 1 patient developed mild diarrhea, which resolved after oseltamivir was discontinued.

It is very important to consider the possibility of neurotoxicity associated with oseltamivir treatment in infants. Administration of a high dose of oseltamivir to juvenile rats (approximately 250-times the dose administered in the current survey) resulted in neurotoxicity and death due to an immature blood–brain barrier [22]. A recent study showed that neurological events are not more common with oseltamivir than with adamantanes in infants aged < 1 y [8]. Other studies did not find any signs of encephalopathy during oseltamivir treatment in infants aged < 1 y, including newborns [5,6,9]. In our study only 1 patient was irritable, and this patient recovered after oseltamivir was discontinued. Neurotoxicity may be infrequent in infants aged < 1 y, including infants aged < 3 months, who are treated with 2 mg/kg oseltamivir twice daily.

In the USA, mortality rates due to seasonal influenza have been reported to be more than 8-times higher in infants aged < 6 months than in older children [2]. Furthermore, children aged < 2 y were at high risk of death during the influenza A/H1N1 2009 pandemic [23]. The Centers for Disease Control and Prevention reported that approximately 80% of children who died in the USA during the influenza A/H1N1 2009 pandemic either did not receive antiviral treatment or did not receive treatment until more than 2 days after the onset of symptoms [23]. Most of the infants in our study were treated with oseltamivir within 2 days after the onset of symptoms, and all the infants recovered completely. These results are consistent with those of a retrospective study of term and preterm newborns with influenza A/H1N1 2009 virus infections or signs suspicious of infection, who all recovered completely after early initiation of oseltamivir treatment [9].

This study has some limitations, as follows: (1) this was a retrospective survey with a small number of patients and no control group; (2) virological, immunological, and pharmacokinetic studies were not performed; (3) long-term adverse events, including disorders of central nervous system function, were not evaluated; (4) the vaccination history of the mothers of infants was not collected; and (5) the prevalent subtype was pandemic influenza A/H1N1 2009 virus. Further prospective, randomized, controlled studies and long-term follow-up with a larger group of infants aged < 3 months with influenza are required to confirm the safety and outcome of oseltamivir treatment.

In conclusion, this is the first report evaluating the safety and outcome of treatment with oseltamivir in Japanese infants aged < 3 months with influenza. We found that infants aged < 3 months without underlying diseases were susceptible to pandemic influenza A/H1N1 2009 virus infection. Treatment of influenza with oseltamivir 1.5–2 mg/kg twice daily may be safe in infants aged < 3 months.

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Experience Publishing

マルチプレックスPCRを用いた呼吸器感染症ウイルスの検討

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抄　　録

夏季に発熱を主訴で受診し呼吸器感染が疑われた小児の鼻腔吸引液からマルチプレックスPCRを用いてウイルス遺伝子の検出を試みた。47人中37人でウイルス遺伝子を検出でき，その内12人では複数のウイルス遺伝子が検出された。ウイルスの重複感染による臨床症状の違いは明らかでなかった。今後，ウイルス遺伝子が鼻腔内で検出される臨床的意義を検討する必要がある。

キーワード：呼吸器感染症，ウイルス，マルチプレックスPCR

はじめに

呼吸器感染症を引き起こすウイルスは110種類以上の血清型を有するライノウイルス（RV）が多く，その他にコロナウイルス（CoV），パラインフルエンザウイルス（PIV），インフルエンザウイルス，Respiratory Syncytial Virus（RSV）などがある。さらに近年では，ヒトメタニューモウイルス（MPV），CoV-NL63，CoV-HKU1，ヒトボカウイルス（BoV）など新たに発見されたウイルスもあり，多くのウイルスが呼吸器感染症に関与していることが明らかになってきた[1]。

呼吸器感染ウイルスの検出は従来から組織培養を用いたウイルス分離と抗原検出が行われてきたが，ウイルス分離は時間がかかること，抗原検出は感度が低いことが問題であった。また，BoVのようにウイルス分離法が確立されていないものもある。しかし，近年ではreverse transcription-polymerase chain reaction（RT-PCR）法やreal-time RT-PCR法などの遺伝子学的検査手法が発達し病因解明に寄与している[2]。さらに最近はマルチプレックスPCRにより網羅的に多数のウイルスを同時検出することが試みられている[3)4)]。

ウイルスの検出は季節によって特長があり，インフルエンザウイルス，RSVは冬期，エコー，コクサッキーなどエンテロウイルス（EV）は夏季に検出頻度が高い。様々なウイルスが呼吸器感染を起こすことが知られているが検出ウイルスと臨床所見との関係については十分検討されていない。

今回，15種類の呼吸器系ウイルスを検出できるマルチプレックスPCRを用い，夏季の小児呼吸器感染症でのウイルス検出を試みたので報告する。

対象と方法

1．対象

対象は2010年７月から８月までの間に，すずかこどもクリニックを受診した７歳未満の発熱を主訴とする患者である。咽頭結膜熱，ヘルパンギーナ，手足口病など臨床診断が容易なものは除いた。これらの患者から鼻腔吸引液を採取し生理食塩水２mLで希釈し検体とした。

2．核酸（RNA/DNA）の抽出

核酸の抽出は検体200μLからQIAamp MinElute Virus Spin Kit（QIAGEN）を用い，最終的に60μLのelusion buffer中に核酸を抽出した。

3．マルチプレックスPCR

RevertAid™ First Strand cDNA Synthesis kits（Fermentas）を用いてcDNAを合成し，マルチプレックスPCRはSeeplex® RV15 ACE Detection（Seegene）を使用した。これは３種類のプライマーセットを用い15種類のウイルスを増幅できる（**表１**）。

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4．検出

増幅されたPCR産物は，マイクロチップ電気泳動装置　MCE®-202 MultiNa（Shimadzu）を用いて検出した。

結　　果

対象は47人（男25人，女22人）で平均年齢3.7±1.4歳，87.7%が保育園などの集団生活をしていた。臨床症状では発熱のみが44.7%で，受診前の最高体温は中央値で39.0℃であった。受診時の咳，鼻水，嘔吐，下痢の頻度はそれぞれ，27.7%，38.3%，10.6%，4.3%であった。37.5℃以上の発熱を認めてから検体採取までの時間は中央値で12.5時間（範囲0.1－94.1時間）であった。

47人中37人（78.7%）に1種類以上のウイルスが検出され，ウイルスの検出の有無で年齢および性比に有意差はなく，臨床症状ではウイルスが検出されなかった群では発熱のみである傾向があり，咳の頻度は有意に低かった（**表2**）。37人から検出されたウイルスはサブタイプ別も含めて9種類，51件であった。最も検出率が高かったのはEV，ついでRVであった。またウイルスが検出された37人中12人（32.4%）から複数ウイルスが検出され10人は2種類，2人は3種類のウイルスが同時に検出された（**表3**）。

ウイルス別の重複感染率ではエンテロウイルス（EV）が検出された15人中9人（60.0%）で他のウイルスが検出され，同様にRVでは12人中8人（66.7%），PIVでは10人中1人（10.0%），アデノウイルス（AdV）では8人中2人（25.5%），BoVでは5人中5人（100%）だった（**表3**）。重複感染を認めた12人においてEVとRVの重複が最も多く，次いでEVとBoVの重複であった（**表4**）。また，重複感染の有無で臨床症状の違いは認められなかった。

47名中43人（ウイルス検出34例，非検出9例）について経過を確認でき，3人に抗菌薬投与，3人に輸液を行い，37人は経過観察で軽快した。抗菌薬投与例は伝染性膿痂疹合併が2人，急性中耳炎合併の1人であり，伝染性膿痂疹合併2例からPIV-1が，急性中耳炎合併1例からRVが検出された。輸液を行った3人の内1例は嘔吐下痢と喘息発作を伴った症例でPIV-1が検出され，2例目は多型紅斑を伴い6日間発熱が続き水分摂取不良であった症例でAdVが検出された。残りの1例は発熱が8日間持続し初診時認めなかった咳が増悪したものの胸部XP（第5病日）に異常を認めなかった症例でウイルスは検出されなかった。

表1　Seeplex® RV15 ACE Detection

プライマーセットA	増幅サイズ（bp）
Internal control	850
Human adenovirus	534
Human coronavirus 229E/NL63	375
Human parainfluenza virus 2	268
Human parainfluenza virus 3	188
Human parainfluenza virus 1	139
プライマーセットB	**増幅サイズ（bp）**
Internal control	850
Human coronavirus OC43	578
Human rhinovirus A/B/C	394
Human respiratory syncytial virus A	273
Influenza A virus	206
Human respiratory syncytial virus B	143
プライマーセットC	**増幅サイズ（bp）**
Internal control	850
Human bocavirus 1/2/3/4	579
Influenza B virus	456
Human metapneumovirus	351
Human parainfluenza virus 4	249
Human enterovirus	194

表2　ウイルス検出有無での比較

ウイルス検出	あり	なし
人数	37人	10人
性比　男／女	1.1	1.5
年齢 mean±SD	3.5±1.4歳	4.4±1.2歳
体温 median	39.0℃	38.9℃
咳	35.1%	0.0%*
鼻水	43.2%	20.0%
下痢	2.7%	10.0%
嘔吐	10.6%	10.0%
発熱のみ	37.8%	70.0%

*$p<0.05$

考　　察

夏季に見られる小児の熱性疾患には，咽頭結膜熱，ヘルパンギーナ，手足口病などがあり，これらの疾患は臨床所見から診断が容易である。これら以外の発熱を主とする疾患については，いわゆる“夏風邪”と診断されていることが多い。今回，この“夏風邪”について15種類の呼吸器系ウイルスを検索できるマルチプレックスPCRを用いて検討した。その結果47人中37人（78.7%）に1種類以上のウイルスを検出できた。検

表3　検出されたウイルスと重複感染率

検出ウイルス	検出数（%）[a]	重複感染数（%）	重複感染ウイルス（例数）
EV	15（31.9）	9（60.0）	RV（6），BoV（4）
RV	12（25.5）	8（66.7）	EV（6），AdV（2）
PIV-1	6（12.8）	0	
PIV-2	1（2.1）	0	
PIV-3	2（4.3）	1（10.0）[b]	RV（1）
PIV-4	1（2.1）	0	
AdV	8（17.0）	2（25.5）	RV（2），EV（1）
BoV	5（10.6）	5（100）	EV（4），RV（1），CoV-OC43（1）
CoV-OC43	1（2.1）	1（100）	BoV（1）
Negative	10（21.3）		

a）全検体47に対する割合
b）サブタイプをまとめた割合

出されたウイルスは，夏季に流行するEV[5]が最も多く，次いでRV，PIV，AdVが検出された。一方，夏季には流行が小さいCoV，RSVおよびMPV[6)~8)]は1検体でCoV-OC43が検出されたのみであった。

15種類のウイルスを検索できるマルチプレックスPCRを用いても10人ではウイルスを検出できず，これらは検査感度の問題か，未知のウイルスもしくはプライマーセットに入っていないC型インフルエンザ等の可能性もある。C型インフルエンザは通年性に呼吸器感染症において検出されているが疫学や症状についてはまだ不明なことが多い[9]。10例中経過不明の1例を除き抗菌薬の投与なく改善したのでおそらくウイルス感染と考えられるが，初診時に咳がなかったことから溶連菌などの細菌感染も否定できない。経過中，咳の増悪があり8日間発熱が続きマイコプラズマ，クラミジアが疑われた症例もあったがレントゲン所見では否定的であった。

ウイルスが検出された37人中12人（32%）で複数のウイルスが同時に検出され，最も多い組合せはRVとEVで，12人中6人で認めた。ウイルス別に重複感染率を見るとBoVを検出した場合100%，RVを検出した場合66.7%と高率に他のウイルスを検出した。他の報告でもBoVの重複感染率は40~83%で[10)~12)]，またBoVは健康小児でも5%に検出され，呼吸器感染症では14日以上検出される場合もあり持続感染も示唆されている[13]。同様にRVも重複感染率は40~57%で[14),15)]，健康小児でも22%に検出され，呼吸器感染症においては前後約100日間検出されることがあると報告されている[16]。このように呼吸器感染から複数ウイルスが同時に検出されることはめずらしくないことが報告されており，複数ウイルスが検出された場合臨床症状と最

表4　重複感染症例

No.	性	年齢	検出ウイルス		
1	女	2.0	EV	RV	BoV
2	女	4.2	EV	RV	AdV
3	女	1.7	EV	RV	
4	男	3.4	EV	RV	
5	男	3.7	EV	RV	
6	男	5.4	EV	RV	
7	男	1.7	EV	BoV	
8	男	2.0	EV	BoV	
9	女	4.7	EV	BoV	
10	男	2.0	RV	AdV	
11	女	3.2	RV	PIV-3	
12	男	3.1	BoV	CoV-OC43	

も関連するウイルスを決定することは困難である。上気道には無症状でも呼吸器系ウイルスが存在することがあり，従来から言われたウイルスは上気道に常在しない，または干渉作用のため複数のウイルスは同時感染しないという常識は変わってきたと考えられる。

上気道炎の90%以上はウイルス性で，今回の検討でも約8割でウイルスを証明でき原則抗菌薬投与なしで経過観察可能であった。また上気道炎に対する抗菌薬投与は予後も合併症の有無も改善しないとされている[17]ことから，耐性菌の増加予防のためにも抗菌薬は投与すべきではない。

ウイルスの遺伝子学的検査手法の発達で従来の方法に比べ熟練した技術も必要せず簡便にウイルスを同定できるようになり，外来で見られる軽症疾患においてもウイルス学的知見が増えてきた。その一方でウイルス遺伝子の存在や複数検出の臨床的意義について不明

な点も多く出てきており，今後も詳細な臨床所見とウイルス学的検査を両立させ更に検討する必要がある。

終わりに

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

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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 WIV, 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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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%)

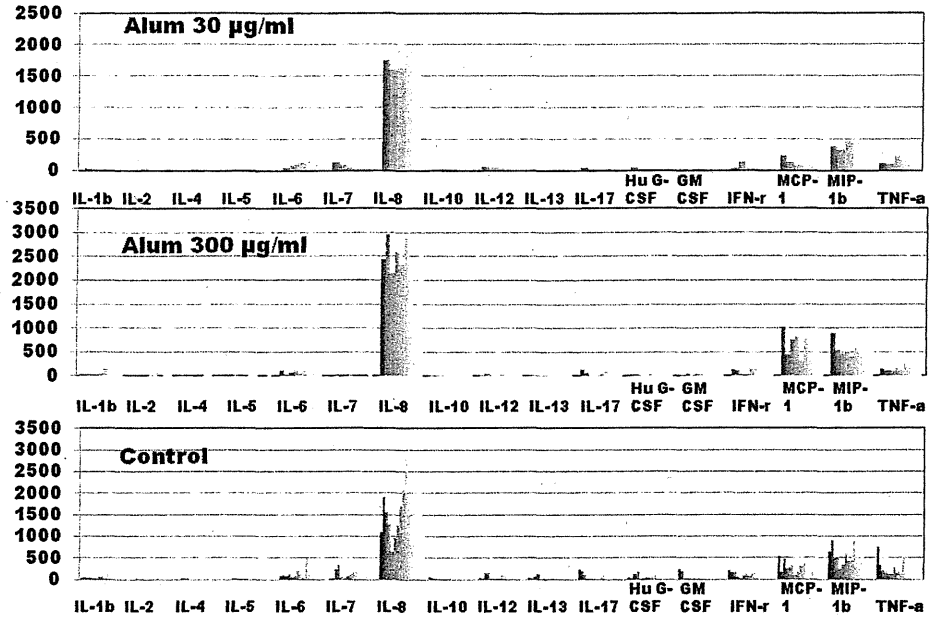

Fig. 1. Cytokine profile in PBMC cultures stimulated with aluminum solution. PBMC were stimulated with 0.1 ml of Alum adjuvants of 300 μg/ml (similar concentration as alum-adjuvanted H5 vaccine) and 30 μg/ml (1:10 dilution).

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.

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.

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.

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

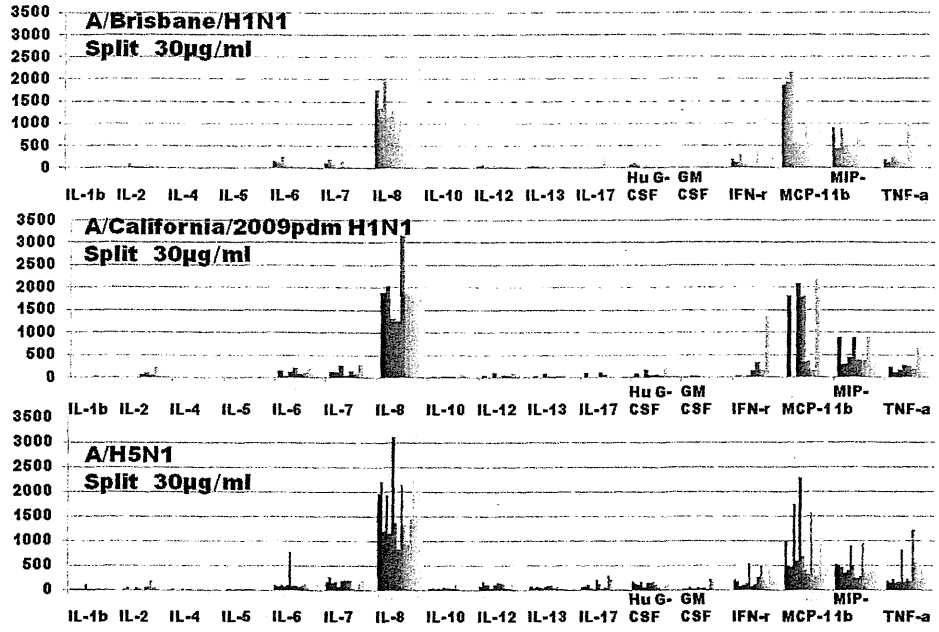

Fig. 2. Cytokine profile of PBMC cultures stimulated with split influenza vaccines. Split vaccine materials were used: H5N1 pandemic NIBRG-14 strain, originated from H5/A/Vietnam/1194/2004, A/H1N1/Brisbane/2007, and 2009 pandemic A/California/07/2009. Each antigen was prepared at the concentration of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

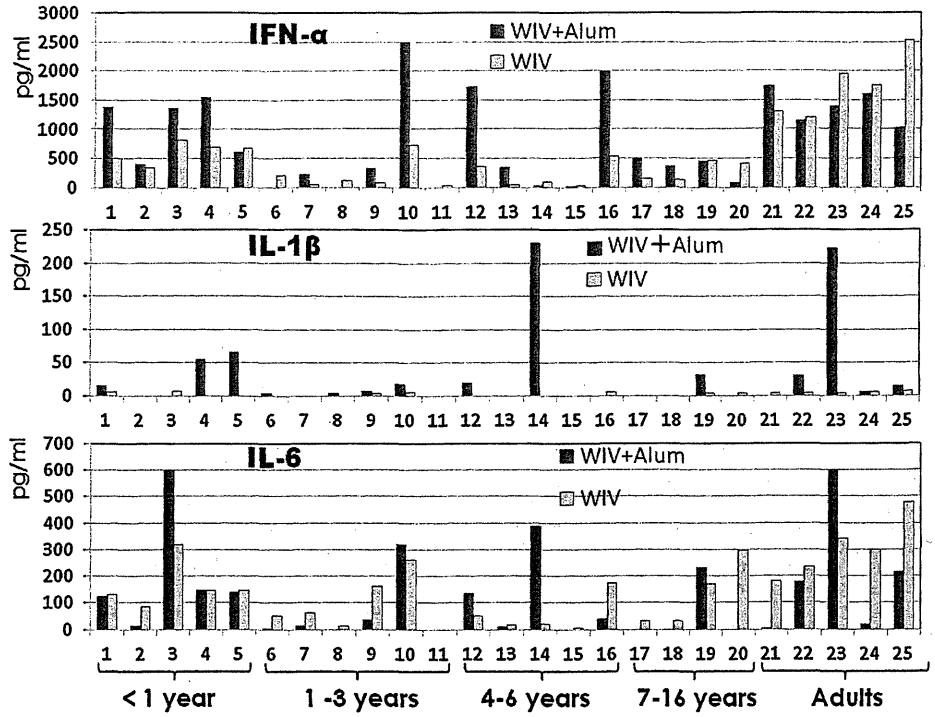

Fig. 3. IFN-α, IL-1β, and IL-6 production. IFN-α, IL-1β, and IL-6 were measured by EIA in PBMC cultures. PBMC were stimulated with H5 WIV and alum-adjuvanted WIV vaccine materials. Samples 1–5 were obtained from healthy individuals less than one year, those 6–11 from 1 to 3 years of age, those 12–16 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.

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