クチン開発の特徴でもある非常に高い安全性の担保という側面においても同様といえる．また，より効果的なワクチンを開発し医療現場へと還元していくためには，個々の研究室だけではなく，多様な研究領域がコンソーシアムを築き連携しあうことが必須であり，かつ産学官による合理的な開発・認可体制の構築が望まれる．

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＊＊次号の特集予告(234巻6号)＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊

【8月第1土曜特集】

◆ここまで進んだ　不整脈研究の最新動向

(企画：井上　博／富山大学大学院医学薬学研究部内科学第二)

＊＊

わが国で誕生したiPS細胞を利用して遺伝性不整脈をもつ患者の体細胞から心筋細胞を分化誘導し，電気生理的検討をはじめとするさまざまな研究がすでに開始されている．工学技術との共同研究として，コンピュータ・シミュレーションにより不整脈の発生機序の解明が試みられている．光学マッピングの応用も同様で，このような解析を通して不整脈の発生機序，薬剤効果の解明などが可能となってきた．臨床では，あらたに心房に特異的に作用する抗不整脈薬が開発され，また一方で新規の作用機序をもつ経口抗凝固薬の開発が進行中である．Brugada症候群の疫学調査の成績もまとまり，海外とは異なる臨床像が明らかになり，また，IT技術の応用である遠隔モニタリングも臨床現場に導入されはじめ，重症不整脈例の管理もきめ細かく行えるようになりつつある．本特集では，不整脈の基礎と臨床の分野で現在，注目されているトピックを解説する．

新しいワクチン開発

アジュバントに関する最新の話題

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要旨 1926年にアルミニウム化合物がアジュバント効果を有していることが見出されてから80年以上が経った．近年，免疫学の急速な発展により，様々な分子や化合物が新規アジュバントとなり得ることが見出されてきた．本稿では，アジュバント候補の作用機序や用途など最新の話題をふまえ，各項目に分けて説明する．

はじめに

現在，新興・再興感染症の予防に効果的なワクチンが必要とされているが，生ワクチン作製は限られた病原体にしか対応できず，不活化ワクチン，もしくはコンポーネント（サブユニット）ワクチンなどは，その免疫原性の低さから，感染症の制圧に資する手法は，いまだ確立されていない．そのため，効果的なワクチンの開発とともに，ワクチンの効果を増強させるためのアジュバントの開発も重要な課題であると考えられる．ワクチンアジュバントは，ワクチン効果の向上のため必須であるが，我が国ではアルミニウム化合物が臨床応用されて以来それ以外に汎用されているものはない．最近になり免疫学，特にアジュバントのメカニズムを担う自然免疫周辺の研究の進歩により，多くのアジュバントが臨床試験で検討され，また多くの候補物質が実験レベルで検討されてきた．本稿では最新のアジュバントの話題について筆者が行った最新の研究成果とともに解説したい．

これまでアルミニウム化合物は様々な種類のワクチンにアジュバントとして応用されてきた．アルミニウム化合物のアジュバント効果は，ワクチン抗原の生物学的，免疫学的半減期を延長させる（徐放効果）ことにあり，体液性免疫（Th2型免疫応答）を誘導することができる．また強力に抗原特異的IgE抗体産生を誘導することができる．最近の研究により，アルミニウム化合物は細胞質内に存在するNLPR3インフラマソームを活性化することでCaspase-1依存的にIL-1βやIL-18などの炎症性サイトカイン産生を誘導することが報告された[1]．この報告によりアルミニウム化合物におけるアジュバント効果はNLRP3インフラマソームに依存的である可能性が示唆されたが，後の研究でこの可能性を否定する報告がなされた[2]．現在ではアジュバント効果とNLRP3インフラマソームとの関連の詳細は明らかとなっていないが，アルミニウム化合物によるアジュバント効果の引き金となる分子を明らかにすることは，安全性の面からも重要な課題である．

ある種の感染症においてはTh2型の免疫応答だけでは予防効果が十分でなく，細胞性免疫（Th1型免疫応答）を誘導することが必要であることも明らかとなっており，作用の異なる新たなアジュバントが必要とされている．ワクチンアジ

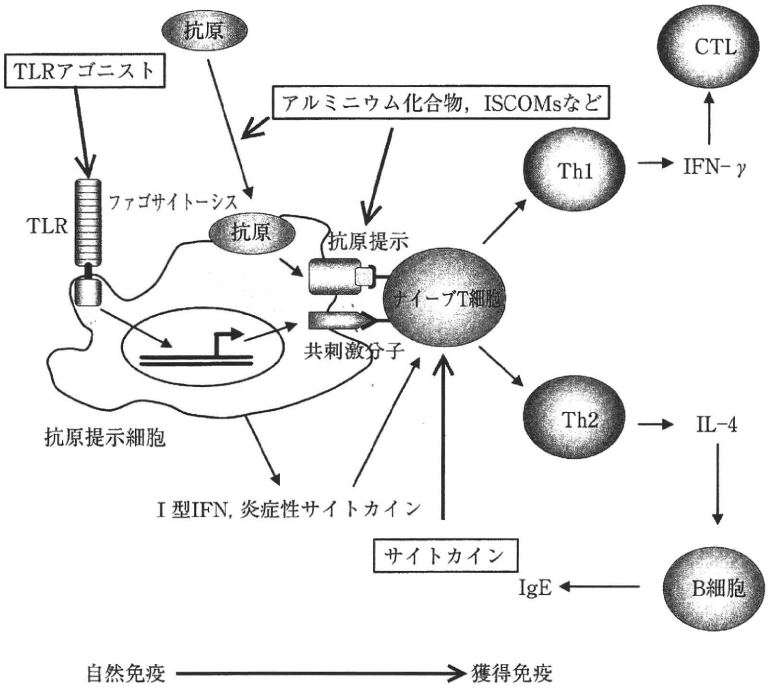

図1　アジュバントの作用機序

ュバントの効果および機構は，3つに大別することができる．①アルミニウム化合物のような徐放効果，②抗原提示細胞による抗原提示の増強，③炎症性サイトカイン産生の誘導である（**図1**）．また，アジュバントの種類により，ワクチン抗原に対する免疫応答をTh2型だけでなくTh1型または両方の免疫応答を著しく活性化することができ，この作用には産生される炎症性サイトカインの種類が鍵となっている．これまでに様々な種類のアジュバントが開発され，動物試験や臨床試験が行われている（**表1**）．次に，様々な種類のアジュバントについて解説する．

■毒　　素

これまでにコレラトキシンのBサブユニット（CTB）や大腸菌易熱性毒素がインフルエンザウイルス粘膜ワクチンのアジュバントとして検討されてきた．実際に，ワクチンとCTBを経鼻免疫することにより，粘膜表面への抗原特異的分泌型IgA産生を誘導することが明らかとなった．それゆえ，コレラトキシンの毒性をなくし，アジュバント活性のみを有している変異体を作製する試みが行われてきた．しかしながら，スイスのワクチンメーカーが行った大腸菌易熱性毒素をアジュバントとして用いた経鼻ワクチンの臨床試験において，一部のヒトに顔面神経麻痺（ベル麻痺）の発症が見られた．発症とワクチン＋アジュバントとの関連が否定できないことから，細菌毒素のアジュバントは，まだ臨床応用されていない．

■エマルジョン

現在O/Wエマルジョンタイプのアジュバントとして MF59，AS03，Provax などが知られている．ノバルティス社によって開発された MF59 はスクアレンポリソルベート（Tween80）とソルビタントリオレアート（Span85）を基剤としており，強力な Th2 型免疫応答を誘導することができる．また，毒性も低く有用性が高いと考えられているため，欧州ではインフルエンザワクチンのアジュバントとして認可されている[3]．AS03 は Tween80 にビタミン E が添加されており，2008年に H5N1 インフルエンザウイルスのアジ

表1　アジュバントの種類と開発状況

分類	アジュバント	特徴
鉱酸塩	水酸化アルミニウム，リン酸アルミニウムなど	IgE 産生誘導が強い．蛋白抗原と沈降物を形成し，徐放性に抗原を放出する．1920年代に見出された[1,2]．
毒素	CTB，大腸菌易熱性毒素	ワクチンと経鼻投与することにより IgA 産生を誘導．臨床試験で顔面神経麻痺が起き，臨床応用はされていない．
O/W エマルジョン	MF59	粒子が小さく細胞に取り込まれやすく，体液性免疫を誘導．インフルエンザワクチンのアジュバントとして使用されている[3]．
	AS03	2008年に欧州で認可された H5N1 ウイルスワクチンのアジュバント[4]．
	Provax	CTL 誘導活性が強い．現在開発中[5]．
W/O エマルジョン	Montanide ISA 51/ミネラルオイルと植物由来界面活性剤	阪大，久留米大が開発中の癌ペプチドワクチンのアジュバント．樹状細胞を活性化．
Bio polymer	Advax/biopolymer	HBV ワクチン，インフルエンザワクチンのアジュバントとして開発中．
植物成分（サポニン）	QS2	成分は QuiA 由来サポニン．CTL を誘導することができる．現在開発中[6]．
	ISCOM/脂質＋サポニンのミセル	直径 40nm ほどの粒子．CTL を誘導することができる．現在開発中[7]．
Lipid A	AS04/MPL＋アルミニウム塩	細胞性免疫を誘導．MPL とアルミニウム塩の混合剤．HBV，インフルエンザワクチンのアジュバントとして欧州で認可[4]．
	RC-529/ MPL アナログ	細胞性免疫を誘導．HBV ワクチンのアジュバントとしてアルゼンチンで認可．
	AS02/スクアレン＋QS21＋MPL（W/O）	MPL と QS21 との混合剤．マラリアワクチンのアジュバントとして開発中[4]．
	AS01/リポソーム＋QS2＋MPL	マラリアワクチンのアジュバントとして開発中[4]．
鞭毛成分	フラジェリン	TLR5 のリガンド．細胞性免疫を誘導．現在開発中．
核酸	dsRNA	TLR3 のリガンド．インターフェロン誘導薬としては認可されている．アジュバントとして細胞性免疫を誘導．現在開発中[9]．
	CpG ODN	細菌に特有な非メチル化 CpG オリゴデオキシヌクレオチド．細胞性免疫を誘導．CpG 2006 はヒト用として認可．抗癌薬としても特許がとられている．CpG7909 は HBV，インフルエンザワクチンのアジュバントとして開発中[10,11]．
結晶	ヘモゾイン	ヘムの2量体のポリマー．炎症性サイトカイン産生を誘導し，アジュバントとして用いることで体液性免疫を誘導．50～200nm の結晶が高いアジュバント活性を有している[13]．
	尿酸結晶	痛風の原因物質．IL-1β や IL-18 産生を誘導．アジュバントとして用いることで体液性免疫を誘導[14～16]．
β-グルカン	SPG	シゾフィランとして抗悪性腫瘍剤として応用されている多糖類．樹状細胞から炎症性サイトカイン産生を誘導．CpG と複合体形成することにより，新規 CpG アジュバントとして応用可能．また，DDS としても注目されている[17]．
サイトカイン	IL-12，GM-CSF	IL-12 は細胞性免疫を誘導し，IgG2 や IgG3 産生を誘導する[18]．
カチオン	DOTAP，DDA	DNA ワクチンの安定性や抗原の発現量を増大させる．細胞性免疫を誘導．現在開発中[19,20]．
ポリペプチド	N'-CARD-PTD	PTD が付加していることにより，細胞内に取り込まれやすく，細胞性免疫を誘導[21]．

ュバントとして欧州で認可された[4]．Provax はスクアレンと Pluronic L121 で構成されており，強力に Th1 および Th2 型免疫応答を誘導し，また強力な CTL（細胞傷害性 T リンパ球）を誘導することが，マウスを用いた実験により明らかとなっている[5]．エマルジョンタイプのアジュバントはこれまでに欧州で認可されている．また，日本が輸入した新型インフルエンザワクチンにはアジュバントとして MF59 が添加されている．毒性が低いとの報告があるとはいえ，日本での使用例はないことから副作用の発症が懸念される．

■ISCOMs

植物成分であるサポニンの類縁体である QS21 は *Quillaja saponaria* の樹皮成分であり現在アジュバントとしての開発が進んでいる．QS21 は抗原提示細胞による抗原提示を促進し，CTL を誘導することが動物実験により明らかとなってい

る[6]．一般的にコレステロールや脂質，サポニンは直径40nmほどの球形粒子を形成し，その中に抗原を取り入れることができる．このような球形粒子をimmunostimulatory complexes(ISCOMs)と呼ぶ．これまでに，ISCOMsは有効なアジュバントであることが実験から明らかとなっており，抗原をMHC class Iの経路に組み込むことができる．結果として細胞性免疫，特にCTLを誘導することが明らかとなっている[7]．また，サポニンなどに類似した成分であるトマト含有アルカロイド，トマチンが強力なアジュバント活性を有していることが明らかとなっている．実際に可溶性のリコンビナント蛋白質に対して，強力に抗原特異的抗体産生を誘導するだけでなく，抗原特異的CTLを誘導することが明らかとなった[8]．このようにISCOMsやトマチンのようなISCOMsに似たアジュバントは細胞性免疫を惹起することができるが，その詳細な作用機序はわかっていない．

■TLRアゴニスト

過去10年でTLRが自然免疫，そして獲得免疫に密接に関与していることが明らかとなってきた．TLRはpathogen-associated microbial patterns（PAMPs）と呼ばれる病原微生物にのみ発現する高度に保存された構造を認識する．具体的には，糖脂質成分や核酸，蛋白質などの多彩なPAMPsをそれぞれ異なるTLRが認識する．TLRがPAMPsを認識するとアダプター分子を介して，自然免疫応答を惹起しI型インターフェロンや炎症性サイトカイン産生を誘導する．これまでに様々なTLRアゴニストが同定され，ワクチンアジュバントとして試験されてきた．その中で，グラム陰性菌である*Salmonella minnesota* R595株のリポポリサッカライド（LPS）から得られた，3-O-desacyl-4'-monophosphoryl lipid A（MPL）はアジュバント効果を保持しながら，LPSに比べ劇的に毒性が減少している．LPSやMPLはTLR4のアゴニストであり，強力に体液性，細胞性免疫を誘導することができるが，主としてTh1型免疫応答を誘導する．実際にグラクソスミスクライン（GSK）社によりMPLとアルミニウム化合物の混合物であるAS04がB型肝炎ウイルスワクチンやヒトパピローマウイルスワクチンのアジュバントとして認可されている．またMPLと他のアジュバントの組み合わせにより，マラリアワクチンのアジュバントとしても開発が進んでいる[4]．

また，近年核酸医薬への注目が高まっており，TLR3アゴニストである合成二本鎖RNA，Poly（I：C）はアジュバントとしての応用も試みられている．すでにAmpligen（Poly I：PolyC12U）は米国でインターフェロン誘導薬として第Ⅲ相臨床試験が終了しており，それ自身の安全性は確立されている．実際にインフルエンザウイルスワクチンとの経鼻免疫により，Th1型免疫応答を誘導し，また粘膜における抗原特異的分泌型IgA抗体の産生を誘導することが明らかとなっている[9]．これらの結果をふまえ，現在前臨床試験が行われている．

TLR9アゴニストであるCpG oligodeoxynucleotide（ODN）はエンドサイトーシスによって細胞に取り込まれ，エンドソーム内でTLR9と結合すると考えられており，この結合が免疫応答を惹起し様々な免疫細胞の成熟，分化，増殖を誘導し，様々な炎症性サイトカイン産生を誘導する．CpG ODNは自身の有する自然免疫活性化能により，癌などを含めた様々な疾患への治療が期待されている．また，CpG ODNはワクチンのアジュバントとしても開発が進んでおり，CpG ODNをワクチン抗原とともに免疫することにより強力にTh1型免疫応答を誘導することがすでにマウスなどの実験で明らかとなっている．CpG ODNは大きく3つに分けることができ，それぞれの特性が異なる．その中でClass-B CpG ODN 2006はすでにヒト用として認可されている．このCpG ODNはColey Pharmaceutical Groupによって開発され，癌の治療に対してはファイザー社が，アジュバントとしてはノバルティス社が特許を取

得しており，幅広い種において免疫応答を増強する結果が得られている．また，B型肝炎ワクチンやインフルエンザウイルスワクチンのアジュバントとしての開発が進んでいる．このCpG ODNは生体内でDNaseによる分解を抑えるためにすべての塩基がホスホロチオエート化されており，実際に生体内での半減期の上昇が確認されている．しかしながら，ホスホロチオエート化による副作用の発症も懸念されている[10,11]．

これまでに様々なTLRリガンドもしくはその誘導体が臨床治験で検討されてきた．しかしながら，TLRを標的にした分子は，マウスでの結果から予想された効果はヒトの臨床治験で得られていない[12]．このことは，ヒトとマウスでのTLR発現様式や機能の違いが重大な問題の一つであると考えられている．また，ヒトとマウスなどのCpG motifは必ずしも一致しないことも明らかとなっており，安全かつ効果的に使用できるアジュバントを作製するにあたって，動物間での効果の違いを検討することも重要である．

■ヘモゾイン

最近の研究により我々は，マラリア毒素とも呼ばれる，マラリア原虫が赤血球のヘモグロビンを消費した後にヘムの代謝産物として生成するヘモゾインが，アジュバント効果を有することを見出した．ヘモゾインはヘムの2量体のポリマーでnm-mmサイズの結晶体であり，TLR9に結合することが明らかとなった．また，ヘモゾインは自身でサイトカイン産生能を有しており，抗原とともに免疫することにより，強力にTh2型免疫応答を誘導する．また，結晶のサイズによりアジュバント活性が異なり，50～200nmの結晶が著しいアジュバント活性を有していることが明らかとなった[13]．

■尿酸結晶

尿酸結晶は痛風の原因物質であることはすでに知られており，強い炎症性反応を誘導する．最近の報告により，尿酸結晶はCaspase-1依存的に炎症性サイトカインであるIL-1βやIL-18の産生を誘導することが明らかとなった[14]．また，この炎症反応はNLRP3インフラマソームを活性化することにより引き起こされることが明らかとなっている[15]．尿酸結晶はインフラマソームだけでなく，TLR4やTLR2を活性化し，共通のアダプター分子であるMyD88を介して，TNF-α，IL-6，IL-8などのサイトカイン産生を誘導することも報告されている[16]．実際に抗原とともに免疫することによりTh2型免疫応答を誘導することが明らかとなっている．しかしながら，アルミニウム化合物同様，尿酸結晶によるアジュバント効果とNLRP3インフラマソームとの関与も明らかとなっていない．また，痛風患者で生成される関節腔内の尿酸結晶が内因性のアジュバントとして働くかは明らかとなっていないため，痛風の治療のためにも詳細な作用機序の解明は重要な課題である．

■β-グルカン

シゾフィラン（SPG）は現在，抗悪性腫瘍剤として応用されているスエヒロタケ由来の多糖類である．D-グルコースが主にβ1→3結合で連結した構造をしており，強固な三重螺旋構造を形成している．SPGをマウス樹状細胞に添加することにより，炎症性サイトカイン産生を誘導することが報告されている．また，我々の研究によりSPGのみでなく，TLR9のアゴニストであるCpG ODNとSPGの複合体が新規アジュバントになり得ることを見出した．SPGは三量体を形成しているが，その1本をCpG ODNに変えたCpG-SPGは複合化前に比べ，有意に樹状細胞からのサイトカイン産生が増大した．またTLR9欠損細胞ではその活性が減少したことから，SPGではなくCpGによる活性であることが考えられる[17]．近年，SPGはオリゴと三量体を形成できることから，ドラッグデリバリーシステム（DDS）の分野でも注目されている．SPGを用い

ることで球形粒子を形成することができ，内部に抗原などを封入することができる．この技術を応用することにより，抗原を抗原提示細胞に効率よく取り込ませることが可能になり，新たなアジュバントとしても期待される．

■アジュバントとしてのサイトカイン

サイトカインはTh0細胞のTh1やTh2への成熟に関与しており，ワクチンアジュバントとして直接応用することができると考えられている．すでに様々なサイトカインが評価を得ており，その中で，IL-12をアジュバントとして用いることにより，Th1型の免疫応答を誘導することができ，IgG2やIgG3などの産生を誘導することがマウスの実験により明らかとなっている[18]．このように，直接免疫系に作用できる，サイトカインはシンプルなアジュバントにはなり得るが，実際の臨床応用には至っていない．

■DNAワクチンのためのアジュバント

いくつかのカチオン性アジュバントはDNAワクチンの免疫原性を高めることが明らかとなっている．その中で1,2-dioleoyl-3-trimethyl-ammonium-propane（DOTAP）やdimethyl, dioctadecyl ammonium bromide（DDA）はカチオン性のエマルジョンを形成し，DNAワクチンを封入することでDNAワクチンの安定性を高め，DNAワクチンの取り込みや組み込まれた抗原の発現量を高めることができ，細胞性免疫を誘導することができる[19]．また，モノオレインとオレイラミンの1対1の混合物であるN3脂質エマルジョンはヒト免疫不全ウイルス（HIV）のgp120とともに経鼻免疫することにより，CTLの誘導と粘膜における抗原特異的IgA抗体の産生を誘導することが明らかとなっている[20]．DNAワクチンは自身で細胞性免疫を誘導することができ，また安価で安全性も高いと考えられるが，DNAワクチンの作用機序もまだ不明な点が多く残っており，効果的なワクチン，およびアジュバントの開発には至っていない．

■新規シグナルペプチドアジュバント

近年，TLR非依存的な自然免疫応答の研究が数多く報告されてきた．その中でアダプター分子であるIFN-β promoter stimulator-1（IPS-1）は，このシグナル伝達経路において中心的な役割を果たしており，IPS-1の持つcaspase recruitment domain（CARD）がシグナル伝達に重要であることが明らかとなっている．そこで我々はTLR非依存的自然免疫シグナル伝達を調節するための新たな試みとして，IPS-1のシグナル活性化に必須なCARD領域に様々なオルガネラ移行シグナルを付加した融合分子を作製し，そのシグナル活性化を解析した．その結果，核移行シグナルを付加した融合分子であるN'-CARDが強力にI型インターフェロン産生シグナルを活性化することを明らかにした．また，相互作用分子の網羅的解析により，このN'-CARDはnuclear DNA helicase Ⅱ（NDH）を介してIFN-β産生シグナルの活性化を誘導していることを確認した．またNDHを介するシグナル経路は，TLRなどの既知の自然免疫応答シグナルには関与せず，N'-CARD独自のシグナル経路であることを明らかにした．そこで，このN'-CARDを蛋白アジュバントとして応用するために，protein transduction domain（PTD）を付加させたN'-CARD-PTDポリペプチドを作製した．実際にN'-CARD-PTDを細胞培養液中に添加することにより，マクロファージや樹状細胞を活性化して，I型インターフェロン産生を誘導し，抗原提示細胞としての成熟を促進した．また，マウスにインフルエンザワクチン（Flu vax）とN'-CARD-PTDを同時投与することにより，ワクチン単独投与群に比べて有意に高いTh1型抗インフルエンザウイルス抗体の産生が認められ，ウイルスに対する強力な感染防御的免疫応答を誘導した（図2）．また，腫瘍モデル抗原であるE7ペプチドとともに投与することにより，特異的細胞性免疫応答を強力に

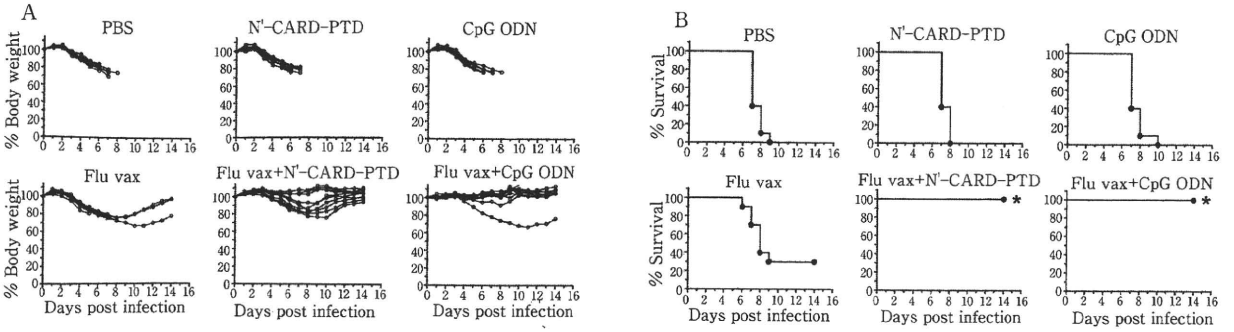

図2 N'-CARD-PTD はアジュバント活性を有している（Kobiyama K *et al.*, 2010[21]より引用）
AとB：マウスに PBS, N'-CARD-PTD（5 μg), CpG ODN(5 μg), Flu vax（0.7 μg), Flu vax（0.7 μg）+N'-CARD-PTD（5 μg), または Flu vax（0.7 μg）+CpG ODN（5 μg）を 10 日おきに 2 回免疫し，最終免疫後 10 日目にインフルエンザウイルス（1×10⁴pfu）を経鼻感染させた．その後 14 日間の体重変化（A）と，生存率（B）を測定または観察した．

誘導し，腫瘍抗原を発現する移植腫瘍の有意な退縮効果が確認された．これらの結果から，N'-CARD は既知の自然免疫受容体を介することなく，NDH を介するシグナルを直接活性化し，ワクチンアジュバントとして応用できる可能性があることが示唆された[21]．

■アジュバントの安全性

アジュバントは病原体の抗原とともに投与されるため，ワクチン抗原の免疫原性の増強効果が必須であるが，それと同時に安全性の確立が必須である．ゆえに，アジュバントを添加することに免疫活性化と，アジュバントの成分による副作用のリスクとのバランスをどのようにとるかが重要である．局所の副作用としては，投与部位における炎症が知られているが，まれに肉芽腫や膿腫が誘発させることが報告されている．また，動物実験において倦怠感や発熱，関節炎，前部ブドウ膜炎が全身性の副作用として報告されている．これらの反応はアジュバント自身による反応やアジュバントと抗原との反応，アジュバントによって産生された複数のサイトカインが起因していると考えられている．たとえワクチンやアジュバントがそれぞれ前臨床における毒性試験や安全性試験がすでに行われていたとしても，臨床試験の前にワクチン製剤（ワクチンとアジュバントの合剤）としての安全性評価が必要である．

おわりに

このように様々な候補物質がアジュバントとして実験動物を使用しての検討，開発が進められてきた．新規アジュバントのいくつかはすでに海外で認可されているが，我が国ではようやく GSK の AS04（MPL，HPV ワクチン）が認可され，同じく GSK の AS03，ノバルティスの MF59 が新型インフルワクチンとして特例承認を受けている．これまでに，HIV や B 型肝炎ウイルス，C 型肝炎ウイルスなどの慢性感染疾患に対するワクチン開発は数多く行われているが，効果的なワクチンはまだ開発されていない．また，インフルエンザウイルスなどのような，ワクチンを接種しても感染，発症してしまうこともある．それゆえワクチンのみではなく効果的なアジュバントの開発がこれらの疾患で苦しむ人々を救う手助けになる．また，投与経路などもアジュバントとワクチンとの併用により変えることも可能になり，より効果的にワクチンを投与することができる．

しかしながら，効果的なアジュバントの開発にはまだ様々な課題が残っている．完全に作用機序が明らかになっていないもの，また機序は明らかとなっているが，ワクチンとの併用による副作用の出現などである．これらの問題をクリアすることが我が国におけるアジュバントの認可には必要である．

新規ワクチンアジュバントが応用されれば，投

与するワクチン量を低下させてアレルギー反応を軽減し，安全性を高められ，感染防御応答を高めることにより感染症の拡大および罹患率を低下させ，国民の健康向上および医療費削減につながる．また，これまで抗原性が低く臨床応用できなかったワクチンについても，効果的な感染防御応答の誘導が可能となり，未開発のワクチンが応用可能となり得ることができる．そのため，ワクチン開発とともにアジュバントの開発も重要な課題である．

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Protease and helicase domains are related to the temperature sensitivity of wild-type rubella viruses

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ABSTRACT

Wild-type rubella viruses grow well at 39 °C (non-temperature sensitivity: non-*ts*), while vaccine strains do not (temperature sensitivity: *ts*). Histidine at position 1042 of the p150 region of the KRT vaccine strain was found to be responsible for *ts*, while wild-type viruses had tyrosine at position 1042 (Vaccine 27; 234–42, 2009). The point-mutated virus (Y1042H) based on the wild-type unexpectedly showed little reduction in growth at 39 °C. In this report, several recombinant viruses were characterized, and point-mutated Y1042H together with the p90 region of KRT significantly reduced virus growth, compared to the parental wild-type virus. There was one amino acid difference at position 1497 of the helicase domain in the p90 region. Double mutation involving both positions 1042 and 1497 markedly reduced virus growth at 39 °C, but single substitution at 1497 did not. The other vaccine strain (TO-336vac) was investigated, and serine at position 1159 of the protease domain in p150 was a crucial amino acid for *ts* and non-*ts* characteristics among four amino acid substitutions between TO-336vac and the wild-type. Our results suggest that protease and helicase domains in non-structural protein were consistent with *ts* phenotype, possibly related to the attenuation process of wild-type viruses.

1. Introduction

Rubella virus (RV) is the sole member of the genus *Rubivirus* in the family *Togaviridae*. The RV genome is single-stranded, positive-sense RNA of approximately 10 kb. The genome encodes two open reading frames (ORFs). One ORF is located at the 5′ end, encoding two non-structural proteins (NSPs), p150 and p90, required for replicating genomic RNAs. The motifs of methyltransferase and protease are located in p150, and the domains of helicase and RNA-dependent RNA polymerase are in p90. Another ORF is located at the 3′ end, encoding three structural proteins (SPs), capsid, E1, and E2, for the virion components. There are three untranslated regions (UTRs) at the 5′ and 3′ ends, and junction UTR (J-UTR) between the two ORFs [1,2].

RV infection occurs mostly in infants and children. Patients with RV infection develop low-grade fever, malaise, maculopapular rash, arthralgia, and post-auricular lymphadenopahty. Although most patients recover within several days without sequela, infection in unimmunized women during the first trimester of pregnancy causes severe fetal defects known as congenital rubella syndrome (CRS) or fetal death. The common defects of CRS are deafness, cataracts, cardiac disease, and neurological abnormalities [3–6]. For the control of rubella outbreaks and prevention of CRS, live attenuated vaccines were developed and have been used in many countries [6,7].

Four live attenuated rubella vaccine strains have been used in Japan. Although the attenuation process was found to differ for each strain with serial passages of the wild-type rubella viruses in different primary cells at 35 °C or lower [8], all Japanese rubella vaccine strains exhibited unique but common characteristics of temperature sensitivity (*ts*) [9]. While wild-type viruses showed approximately 1/10 infective titers at 39 °C in comparison with that observed at a permissive temperature of 35 or 37 °C, vaccine strains with the *ts* phenotype demonstrated lower virus growth at 39 °C with less than 1/1000 at 35 or 37 °C.

The complete genomic sequences were determined for both the KRT live attenuated rubella vaccine and the wild-type RVi/Matsue.JPN/68 strain isolated at the same time and in the same district as the progenitor wild-type of KRT [10]. In order to determine the region responsible for the *ts* of KRT, a series of recombinant and point-mutated viruses were generated by reverse genetics (RG) [11–16], and infection experiments with cultured cells were carried out. The p150 gene, especially the histidine at position 1042 (His^{1042}), was determined to be responsible for the *ts* phenotype of

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the KRT strain [10]. Conversely, it was not confirmed whether the introduction of His^{1042} into the wild-type viruses influenced the growth of these viruses at 39 °C.

The objective of the present study was to identify whether the substitution of His^{1042} was necessary and sufficient for the phenotype change from non-*ts* to *ts*, possibly causing attenuation of the wild-type viruses. Mutated recombinant viruses were generated based on the wild-type, RVi/Matsue.JPN/68, with subsequent investigation of virus growth at 39 °C, compared to that at 35 °C. The substitutions of His at position 1042 together with Ile at position 1497 were required for the acquisition of the *ts* phenotype. Moreover, Ser at position 1159 of the other vaccine strain (TO-336vac) was identified to be a crucial amino acid for the *ts* phenotype. These two positions (1042 and 1159) were located in the protease domain of p150, and this region was believed to play a key role in the non-*ts* and *ts* phenotypes, possibly related to the attenuation process.

2. Materials and methods

2.1. Cells and viruses

Vero and RK13 cells were maintained in Eagle's minimum essential medium (MEM) (Sigma–Aldrich, MO, USA) supplemented with 5% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 U/ml). Recombinant and point-mutated viruses were generated by RG, as reported previously [10]. Constructed RVs were propagated and stocked after one or two passages in Vero cells. The KRT vaccine seed strain was supplied by the Kitasato Institute, Research Center for Biologicals, and wild-type RVi/Matsue.JPN/68 strain was from the National Institute of Infectious Diseases Japan.

2.2. Construction of infectious cDNA clones of recombinant and point-mutated viruses

The infectious cDNA clones of RVi/Matsue.JPN/68 and KRT vaccine strains were constructed in a previous study and designated as pRViM and pKRT [10]. pH1042Y having a substitution at position 1042 of RVi/Matsue.JPN/68 was generated based on the pKRT, and the one with reversion, pY1042H, was constructed based on the pRViM, as shown Fig. 1.

Two recombinant clones, pY1042H-KRT p90 and pY1042H-KRT SP, were constructed, using the restriction enzyme sites *Bsm* I (gp 3243) and *Not* I (gp 6623) for recombination of the p90 region, and *Xmn* I (gp 6514) and *Eco*R I (3'end) for recombination of the SP region, as shown in Fig. 2.

The point-mutated clone in the p90 region, pT1497I, based on pRViM was constructed by PCR amplification with the GeneTailor™ Site-Directed Mutagenesis System (Invitrogen, CA, USA), using a set of forward, 5′-CGAGCGCACCGGCAtCTTCGCCTGCAACC-3′ (gp 4516–4543) and reverse, 5′-TGCCGGTGCGCTCGCCCTCGATGTCATAA-3′ (gp 4501–4529), primers, with the mutation site indicated as a lowercase letter. The double-mutated cDNA clone, pY1042H-T1497I, was developed with replacement of the fragments after digestion with restriction enzyme sites *Bsm* I (gp 3243) and *Bgl* II (gp 5355). The substitution sites of these constructions are shown in Fig. 3.

Four point-mutated clones (pC501R, pH573Y, pN1159S, and pN1351D) were generated by introducing the substitutions of TO-336 vaccine strain into pRViM, using the GeneTailor™ Site-Directed Mutagenesis System (Invitogen). Four substitutions at each of these sites are shown in Fig. 4.

Along with the genome structure of RV, we generated eight recombinant infectious cDNA clones, designated as pRViM rec1-8, replacing each region from 5′ UTR, p150, p90, J-UTR, C, E2, E1, to 3′ UTR of the RVi/Matsue.JPN/68 with the respective KRT region

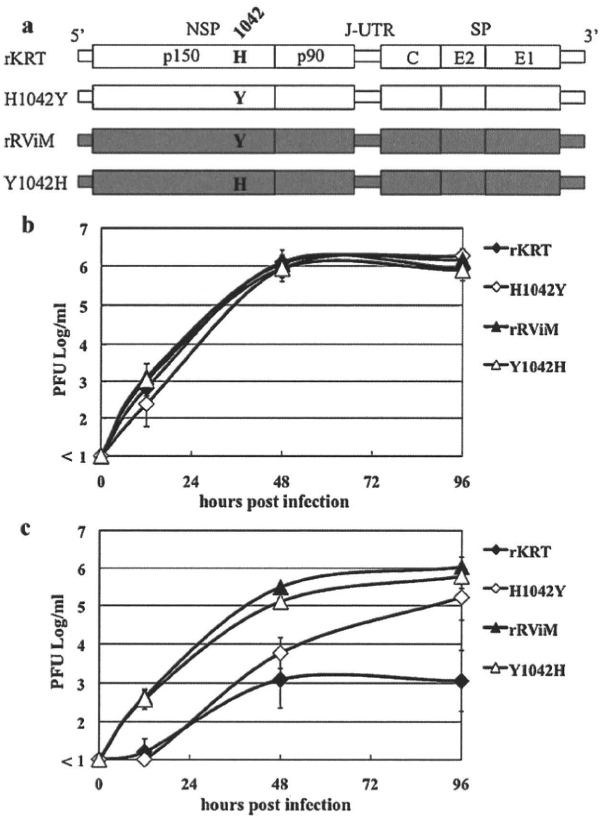

Fig. 1. The influence of the critical residue responsible for the *ts* of KRT on the growth of RVi/Matsue.JPN/68. (a) Construction of point-mutated viruses, H1042Y and Y1042H, based on rKRT and rRViM. The genomic structure of RV is indicated in the panel. The broad boxes demonstrate ORFs including NSP (p150 and p90) at the 5′ end and SP (capsid, E2, and E1) at the 3′ end. Narrow boxes outer than and between two ORFs show untranslated regions (5′ UTR, J-UTR, and 3′ UTR). H1042Y was constructed based on rKRT by introducing a substitution at position 1042 of rRViM. Y1042H was inversely constructed based on rRViM. The backbone based on rRViM is indicated as a gray bar, and open bars indicate the regions based on rKRT. (b) Growth kinetics of the point-mutated and parental viruses at 35 °C. RK13 cells were infected at a MOI of 0.01. The culture medium was harvested at 12, 48, and 96 h post-infection (hpi), and the infective titer was measured by the plaque assay. The results show the average of three independent experiments and the error bar indicates ± standard deviation (S.D.) (c) Growth kinetics of the point-mutated and parental viruses at 39 °C.

(Supplementary Fig. 1). A series of recombinant clones were constructed employing similar procedures to a previous report [10].

For the construction of the chimerical p150 region between pRViM and pKRT, seven recombinant viruses were constructed, introducing the complimentary region of pKRT into those of pRViM or pY1042H, using the restriction enzyme sites *Mfe* I (gp 126), *Nde* I (gp 1872), *Nhe* I (gp 2803), *Bsm* I (gp 3243), and *Eco*R V (gp 4213). These cDNA clones were named pRViM-KRT p150 rec 1, 2, 3, and 4, and pY1042H-KRT p150 rec 1, 2, and 4. pD1007G-Y1042H having the two substitutions of KRT was generated based on pY1042H instead of recombinant virus between *Nhe* I and *Bsm* I. The construction schemes are shown in Supplementary Figs. 2 and 3.

2.3. Recovery of clone viruses from infectious cDNA clones

Full-length viral genomic RNA was synthesized from the infectious cDNA clones with the mMESSAGE mMACHINE T7 kit (Applied Biosystems), following the instruction manual. Vero cells were cultured at 8.0×10^5 cells/well in 6-well plates 24 h before RNA transfection. After the cells were washed with 2.0 ml of OPTI-MEM,

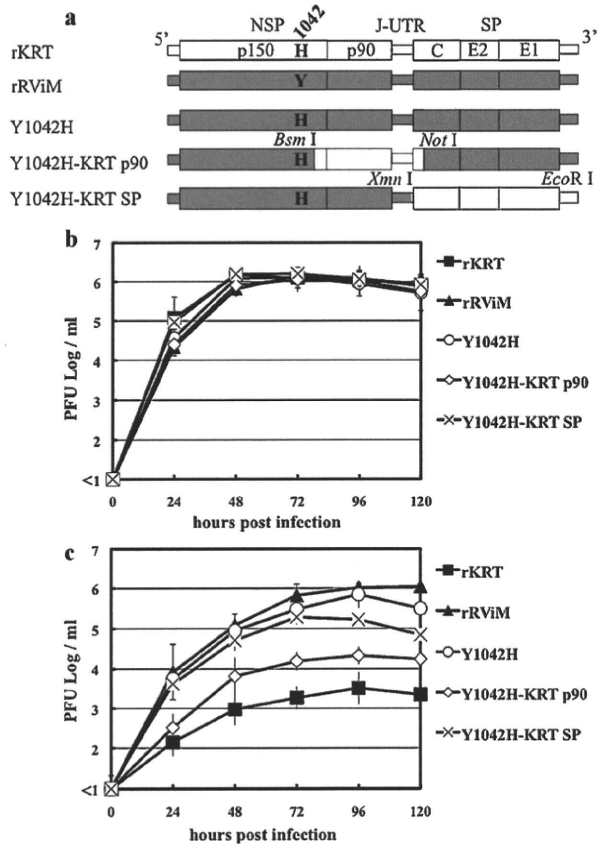

Fig. 2. Contributions of KRT genomic regions together with Y1042H on growth at 39 °C. (a) Construction of recombinant viruses with Y1042H by replacing the p90 and SP of rRViM with those of rKRT. rKRT and rRViM were parental viruses, and Y1042H was the backbone virus for generating the recombinant viruses. Two recombinant viruses, Y1042H-KRT p90 and Y1042H-KRT SP, each having p90 and SP of KRT, were generated using the appropriate restriction enzyme sites (*Bsm* I, *Not* I, *Xmn* I, and *EcoR* I). The number in bold is the site of crucial for the *ts* phenotype of KRT. The gray bars represent those from rRViM and open bars are those from rKRT. (b) Growth kinetics of the recombinant viruses with Y1042H at 35 °C. The culture medium was harvested every 24 h until 120 hpi. The titration of the medium was carried out using a plaque assay. The infective titer is shown as the average for three independent experiments and the error bar indicates ±S.D. (c) Growth kinetics of recombinant viruses at 39 °C.

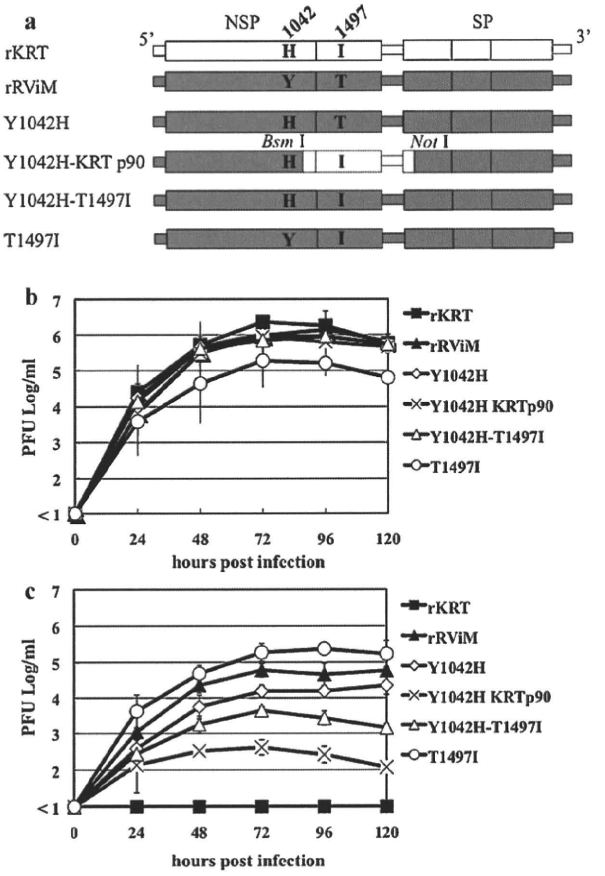

Fig. 3. Requirement of double mutations in p150 and p90 for the suppression of RVi/Matsue.JPN/68 growth at a high temperature. (a) Two point-mutated viruses, Y1042H-T1497I and T1497I, were constructed. The numbers in bold indicate sites where residues of wild-type viruses were replaced with those of KRT. Y1042H-T1497I was constructed based on Y1042H by replacing the threonine at position 1497 of RVi/Matsue.JPN/68 with the isoleucine of KRT. T1497I was generated by introducing the threonine at 1497 of KRT into rRViM. (b) Growth kinetics of the recombinant and mutated viruses at 35 °C. The culture medium was harvested at 24, 48, 72, 96, and 120 hpi. The average infective titers in three independent experiments are shown and the error bar indicates ±S.D. (c) Growth kinetics of the recombinant and point-mutated viruses at 39 °C.

RNA transfection was carried out with a mixture of 12.5 μg of synthesized RNA and 15.0 μl of DMRIE-C (Invitrogen) in 1.0 ml of OPTI-MEM. After incubation at 35 °C for 4 h, the mixture was removed and replaced with 2.0 ml of MEM containing 5% FBS. Culture media were harvested four days after transfection and stocked as master seed viruses.

2.4. Analysis of temperature sensitivity

Monolayers of RK13 cells in 6-well plates were infected at a multiplicity of infection (MOI) of 0.01. After adsorption, each well was washed twice with 2.0 ml of PBS and replaced with 2.0 ml of MEM containing 5% FBS and antibiotics. The plates were incubated at 35 or 39 °C in a 5% CO_2 incubator, and the culture medium was collected. The infective titer of the medium was determined based on the plaque assay.

2.5. Viral titration by plaque assay

Monolayers of RK13 cells in 6-well plates were infected with 100 μl of 10-fold serial dilutions of samples. The inoculum was removed after contact for 1 h at room temperature and replaced with 3.0 ml of MEM containing 2% FBS, 40 μg/ml of DEAE dextran, 0.07% sodium bicarbonate, 0.7% agarose, penicillin at 100 U/ml, and streptomycin at 100 U/ml. The plates were incubated at 35 °C in a 5% CO_2 incubator. On day 7 post-infection, plaques were visualized by staining with PBS containing 0.1% crystal violet and 4% formalin [12,17,18].

2.6. Nucleotide sequence accession numbers

The accession number of the sequence and genotype used in this study for comparison are summarized in Table 1 [10,19–22].

3. Results

3.1. Influence of histidine at position 1042 on growth of wild-type virus at 39 °C

Our previous results showed that only the p150 region was responsible for the *ts* phenotype of a KRT vaccine strain among all genomic regions. Detailed analysis of four recombinant viruses in the p150 region was revealed that the region between *Nhe* I and

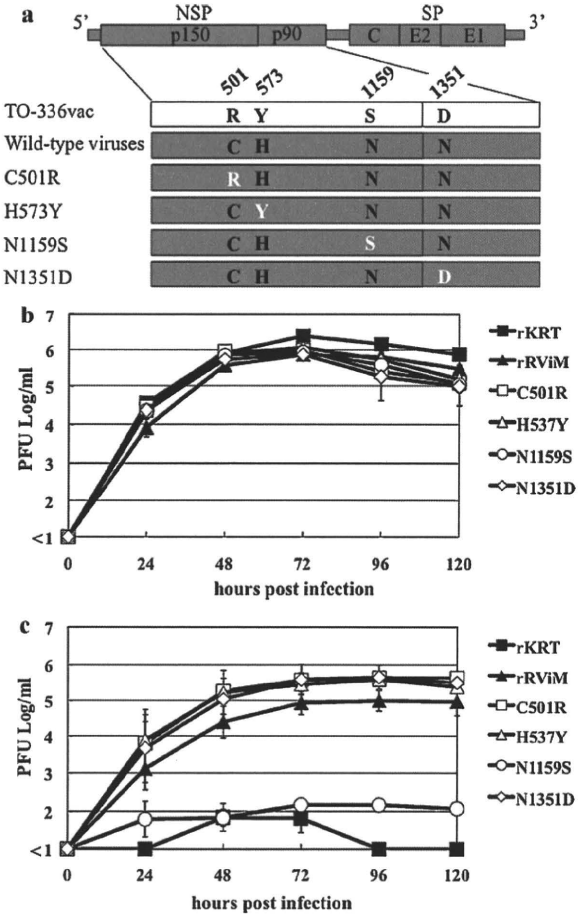

Fig. 4. Identification of crucial amino acid residues for the growth of wild-type viruses at 39 °C based on TO-336vac. (a) Amino acid residues shown in the white bar represent unique residues in the NSP region of TO-336vac, and the gray bar indicates residues conserved among wild-type viruses. The numbers in bold show amino acid positions. White letters in gray bars represent the replacement of residues of wild-type viruses with those of TO-336vac. Four point-mutated viruses were generated based on rRViM. (b) Growth kinetics of the point-mutated viruses at 35 °C. The infective titer is shown as the average of three independent experiments, and the error bar indicates ±S.D. (c) Growth kinetics of point-mutated viruses at 39 °C.

Bsm I (genome position 2803–3243) was important for the *ts* of KRT and there were two amino acid substitutions at positions 1007 and 1042 (D and Y for RVi/Matsue.JPN/68, and G and H for KRT, respectively). Two residues of RVi/Matsue.JPN/68 were introduced into KRT solely or in combination to confirm the effect on the *ts* of KRT. The influence of aspartic acid residue at position 1007 on the *ts* was not observed, while tyrosine at position 1042 exerted a strong effect on the *ts*. Thus, we concluded that histidine at position 1042 (His^{1042}) was critical for the *ts* of KRT. However, it remained to be confirmed whether His^{1042} is conversely crucial for growth of the wild-type virus at a restrictive temperature of 39 °C. For this reason, the present study was focused on the influence of residue at position1042 on the growth of wild-type virus at 39 °C. To assess the influence of His^{1042} on the growth of wild-type viruses at 39 °C, we generated point-mutated viruses (Y1042H and H1042Y) based on pRViM and pKRT, respectively. RK13 cells were infected with the point-mutated viruses Y1042H and H1042Y, as well as parental viruses (rRViM and rKRT) at a moi of 0.01 and cultured at 35 and 39 °C. Culture media were harvested at 12, 48, and 96 hpi, and the results of virus growth are shown in Fig. 1. The growth kinetics of all viruses were the same at 35 °C, whereas the growth properties varied at 39 °C. rKRT with its *ts* phenotype exhibited a significant reduction in growth at 39 °C, with the peak titer of the virus infectivity at 39 °C being approximately 1/1000 of that at 35 °C. However, rRViM with its non-*ts* phenotype demonstrated virus growth at 39 °C similar to that at 35 °C, and the highest titer of the virus at 39 °C was about 1/5 of that at 35 °C. H1042Y also showed efficient growth at 39 °C, and the peak titer of the virus was 1/10 in comparison with that observed at 35 °C. These results were identical to our previous ones, but, unexpectedly, the peak titer of Y1042H at 39 °C did not decrease compared to that at 35 °C, and the growth kinetics of Y1042H at 39 °C were slightly lower than those of rRViM, with no significant differences.

3.2. Contribution of other genomic regions of KRT in combination with Y1042H to growth at 39 °C

The essential determinant of the *ts* phenotype of KRT was appeared to be histidine at position 1042, since the growth of H1042Y at 39 °C was markedly higher than that of KRT (Fig. 1). Moreover, it was observed that no genomic regions, except p150, contributed to the *ts* phenotype of KRT in our previous report [10]. Hence, we investigated whether the other region(s) participated in the growth of RVi/Matsue.JPN/68 at 39 °C in combination with His^{1042}. Two recombinant viruses, Y1042H-KRT p90 and Y1042H-KRT SP, were generated employing a point-mutated virus based on rRViM with Y1042H. Y1042H-KRT p90 was constructed by exchanging the p90 region of RVi/Matsue.JPN/68 with that of KRT. Y1042H-KRT SP was generated by replacing the structural proteins (capsid, E2, and E1) of RVi/Matsue.JPN/68 with those of KRT. The growth kinetics of these viruses were examined at 35 and 39 °C (Fig. 2). There were no significant differences in the growth kinetics among the recombinant viruses (Y1042H, Y1042H-KRT p90, and Y1042H-KRT SP) and parental viruses (rKRT and rRViM) at 35 C. At 39 °C, the growth kinetics of rRViM and Y1042H showed a very similar pattern, and there were no significant reductions in comparison with those at 35 °C. The replacement of the SP region of rRViM with that of KRT showed a small influence on the growth of

Table 1
Summary of genomic information on rubella viruses in this study.

Strain	Wild-type/vaccine	Clade	Genotype	GenBank accession no.
KRT	Vaccine	I	a	AB222608
TO-336vac	Vaccine	I	a	AB047329
RVi/Matsue.JPN/68	Wild-type	I	a	AB222609
RVi/TO-336wt.JPN/67	Wild-type	I	a	AB047330
RVi/SUR.SVK/74	Wild-type	I	a	AF435866
RVi/GUZ.GER/92	Wild-type	I	B	DQ388280
RVi/Anim.MEEX/97	Wild-type	I	C	DQ085341
RVi/JC2.NZL/91	Wild-type	I	D	DQ388281
RVi/6423.ITA/97	Wild-type	I	E	DQ085343
RVi/BRI.CN/79	Wild-type	II	A	AY258322
RVi/AN5.KOR/96	Wild-type	II	B	DQ085342
RVi/C4.RUS/67	Wild-type	II	c	DQ388279

rRViM at 39 °C, while that of the p90 region brought about a marked reduction in virus growth similar to that of rKRT at 39 °C. These results indicated that the non-*ts* phenotype of RVi/Matsue.JPN/68 altered the *ts* phenotype by the amino acid substitution of histidine at position 1042 together with the p90 region of KRT.

3.3. Determination based on KRT vaccine strain for wild-type viruses representing ts phenotype

There was a single amino acid difference at position 1497 in the p90 region between the two strains. The amino acid at position 1497 of p90 was changed from tyrosine (T) of RVi/Matsue.JPN/68 to isoleucine (I) of KRT. In order to decide on the amino acid residue responsible for altering the non-*ts* to *ts* phenotype, two mutated viruses, T1497I and Y1042H-T1497I, containing a single amino acid substitution at position 1497 or both at positions 1042 and 1497 of KRT were constructed, and the growth kinetics at 35 and 39 °C were investigated (Fig. 3). The growth kinetics of T1497I were slightly lower than those of the other viruses at 35 °C. They showed different patterns of growth at 39 °C. The maximum titer of rRViM and Y1042H at 39 °C was approximately 1/20 and 1/50 of those observed at 35 °C, respectively, and there was no significant change in the peak infective titer between the two. As for the growth kinetics of T1497I, there were no differences in the peak titer between 35 and 39 °C. The maximum titers of Y1042H-KRT p90 and Y1042H-T1497I at 39 °C were markedly lower than those at 35 °C. The peak titer of Y1042H-KRT p90 at 39 °C was less than 1/2000 of that at 35 °C. As for the amino acid difference in p90, the growth of Y1042H-T1497I clearly decreased, and the peak titer at 39 °C was approximately 1/200 of that at 35 °C. However, virus growth of Y1042H-T1497I at 39 °C was not identical to that of Y1042H-KRT p90.

To rule out any potential regions of KRT influencing virus growth of RVi/Matsue.JPN/68 at 39 °C, the effects of each genomic region of KRT were investigated on the growth of RVi/Matsue.JPN/68 at 39 °C, constructing a series of recombinant viruses (Supplementary Fig. 1). As in our previous study, there were no genomic regions except for the p150 region that reduced the growth of RVi/Matsue.JPN/68 at 39 °C. Replacement of the p90 region of pRViM with that of KRT did not alter the growth of RVi/Matsue.JPN/68 at 39 °C by itself. The effects of other fragments in the p150 region of KRT were investigated by generating a series of recombinant viruses (Supplementary Figs. 2 and 3). In the series of recombinant viruses based on pRViM and pY1042H, the kinetics of growth at 35 °C were very similar, and the peak titer at that temperature was approximately 10^6 PFU/ml. At 39 °C, the maximum titers of all recombinant viruses roughly exhibited a range of $10^{4.5-5}$ PFU/ml. Among those viruses, Y1042H and rRViM-KRT p150-rec 3, with His^{1042}, tended to show slightly weaker growth, and there were no regions that decreased the growth at 39 °C together with His^{1042}. Thus, the results strongly suggested that two mutations at positions 1042 and 1497 were essential for RVi/Matsue.JPN/68 representing the *ts* phenotype. The amino acid sequences of the NSP region were compared among KRT and wild-type viruses (Clade I: 1a, 1B, 1C, 1D, and 1E; Clade II: 2A, 2B, and 2c). Genomic information on those viruses is summarized in Table 1, and the characteristic amino acid residues of KRT that were otherwise conserved among wild-type viruses (Table 2). There were six amino acid differences in the NSP region, five residues in the p150 region, and one in the p90 region. Positions 295, 483, and 674 were located in a non-identified region, while those of 961, 1042, and 1497 were identified in X, protease, and helicase domains, respectively, which were highly conserved among wild-type viruses of all genotypes. These data conclusively demonstrated that two mutations at positions 1042 and 1497 were necessary for any wild-type viruses altering their non-*ts* phenotype to the *ts* of a KRT vaccine strain.

Table 2
Distinct amino acid residues in the NSP region of KRT that differed from wild-type viruses.

Position	Region	Domain	Amino acid differences	
			KRT	Wild-type viruses
295	p150	None	A	T
483	p150	None	A	T
674	p150	None	V	I
961	p150	X	V	A
1042	p150	Protease	H	Y
1497	p90	Helicase	I	T

Table 3
Unique amino acid residues in the NSP region of TO-336vac that differed from wild-type viruses.

Position	Region	Domain	Amino acid differences	
			TO-336vac[a]	Wild-type viruses
501	p150	None	R	C
573	p150	None	Y	H
1159	p150	Protease	S	N
1351	p90	Helicase	D	N

[a] The genomic sequence of TO-336 vaccine strain was cited from reference [19].

3.4. Other mutations in attenuation process of TO-336

It was hypothesized that the characteristic residue(s) for the *ts* phenotype of other vaccine strains would be located in NSP, especially protease and helicase domains, responsible for the growth of wild-type viruses at a high temperature. The TO-336 vaccine strain (TO-336vac) was investigated because all Japanese vaccine strains had the *ts* phenotype and the complete genomic sequences of TO-336vac and its parental wild-type virus (RVi/TO-336wt.JPN/67) were identified [19]. A comparison of the amino acid residues in NSP between TO-336vac and wild-type viruses is shown in Table 3. There were four substitutions at positions 501, 573, 1159, and 1351, and two residues at positions 1159 and 1351 were located in the protease and helicase domains, respectively. Each residue was introduced into pRViM, and the influence on growth at 39 °C is shown in Fig. 4. At 35 °C, the growth patterns of all viruses were similar and their peak titers were approximately 10^6 PFU/ml at 72 hpi. As for the growth at 39 °C, C501R, H537Y, and N1351D grew well and the peak titers of these viruses were approximately 4-hold higher than that of rRViM. On the other hand, the growth of N1159S was extremely poor at 39 °C, as well as that of rKRT. These results clearly demonstrated that serine at position 1159 was the critical determinant of a significant reduction in the growth of wild-type viruses at 39 °C, and it was responsible for the *ts* phenotype of the TO-336 vaccine strain.

4. Discussion

Vaccine strains have several biological differences such as cell tropism, plaque morphology, the *ts* phenotype, and immunogenic markers of rabbits and guinea pigs in comparison with wild-type viruses [8–11,13,23,24]. All rubella vaccine strains, RA27/3, KRT, TO-336vac, Matsuura, and Matsuba, which are available on the market, have the common feature of temperature sensitivity (*ts*). The *ts* phenotype is invaluable for analysis of the attenuation mechanisms of rubella viruses at the molecular level, because rubella vaccines were established through cold adaptation whereby isolated rubella wild-type viruses were serially passaged with human diploid or primary animal cells at 35 °C or less [8,25]. The acquisition of the *ts* phenotype during cold adaption was strongly correlated with the attenuation of their progenitor wild-type viruses, although the molecular mechanisms or

essential residues of the *ts* phenotype differ in each vaccine strain [10,19,26].

Through our previous experiments using the KRT vaccine strain, the genomic region responsible for the *ts* phenotype of the vaccine was identified using RG. The introduction of tyrosine at position 1042 of the RVi/Matsue.JPN/68 into rKRT resulted in growth recovery at 39 °C, and the rate of restoration was 100-fold higher than that of KRT. From these observations, a marked reduction was expected in the growth of RVi/Matsue.JPN/68 at 39 °C by introducing histidine at position 1042 (His^{1042}) into rRViM. However, this did not influence virus growth at 39 °C, and the growth kinetics of Y1042H at 39 °C barely reduced. Nevertheless, this result did not lessen the importance of our previous findings of the necessity of histidine at position 1042 for the *ts* of KRT, because H1042Y based on rKRT showed efficient growth at 39 °C in the present study (Fig. 1). These results suggested the possibility of additional modification at position 1042. The propensities of Y1042H-KRT p90 and Y1042H-T1497I at 39 °C were comparable, showing a significant reduction in virus growth compared to parental rRViM and Y1042H. Nonetheless, their growth patterns did not entirely coincide with that of rKRT (Figs. 2 and 3). This may be due to the differences in the genetic background between KRT and RVi/Matsue.JPN/68, because the growth properties at 39 °C showed diverse patterns in wild-type viruses and vaccine strains [24]. In fact, there was only one difference at the amino acid level in the p90 region between the two, but there were 65 differences at the nucleotide level. These nucleotide differences occurred through cold adaptation; thus, the accumulation of these mutations may be beneficial to replicate genomic RNAs at a low temperature, with the loss of stability of the genomic structure and efficiency of genomic replication at a high temperature. Alternatively, it remains a possibility that there is compatibility of conformational interaction between the viral genome and NSP. Consequently, the individual impacts of the p90 region and isoleucine at position 1497 of KRT on the growth of rRViM at 39 °C were not specifically identified (rRViM-rec3 in Supplementary Fig. 3 and T1497I in Fig. 3), and the influence of the mutation at 1497 on the growth at the temperature was exerted together with histidine at position 1042 with certainty. It is especially noteworthy that the combination of the two substitutions, at positions 1042 and 1497, with brought about more than a 100-fold reduction in growth at 39 °C compared with the parental rRViM.

Histidine at position 1042 and isoleucine at position 1497 were essential for altering the non-*ts* phenotype of any wild-type viruses with different genotypes to the *ts* phenotype, since those positions of wild-type viruses were highly conserved. These two amino acids were unique for the KRT strain. Next, the region(s) was investigated that was responsible for the *ts* of other vaccines. The TO-336 Japanese vaccine strain (TO-336vac) had four amino acid substitutions compared to wild-type viruses and each mutation was introduced into rRViM. A point-mutated virus (N1159S) was generated by introducing the serine at position 1159 of TO-336vac into rRViM, which exhibited a very similar pattern of growth at 39 °C to that of rKRT (Fig. 4). Interestingly, the effect of the single residue on virus growth of TO-336vac was more efficacious than the combination of two residues of KRT, and position 1159 was also located in the protease domain.

In our advanced study, the molecular function of the residue at position 1042 (H1042Y and Y1042H) was investigated regarding the steps of viral life cycles. Through the results of analysis concerning the efficiency of genomic replication, expression level of viral proteins in infected cells, and production of infective viruses in culture medium, the poor virus growth of the *ts* phenotype of KRT was due to the reduction of viral RNA synthesis, and RNA replication of the H1042Y at 39 °C was restored at a significant level (data not shown). On the other hand, Y1042H showed a declined genomic replication at 39 C in comparison to that of rRViM; however, the infective titer in the medium barely decreased. The efficacy of genomic replication was evidently affected by the substitution at position 1042. In the genomic replication of rubella virus, the non-cleaved NSP (p200) is essential for synthesizing the complementary genomic RNA (cRNA) as the template of viral genomic RNA (vRNA), while the cleavage complex of p150 and p90 is required for the efficient production of new vRNA as the template of synthesized cRNA. The replication stage of genomic RNA for both vRNA and cRNA is temporally controlled by the regulation of NSP processing [27]. Thus, the substitutions at position 1042 or 1159 would be correlated with the reduction in protease activity to cleave p200 into p150 and p90 and/or in the conformational stability of non-processed and processed NSP to limit viral RNA replication in each phase at a high temperature.

In other members of the family *Togaviridae*, Sindbis and Semliki Forest viruses, many *ts* mutants have a single mutation responsible for the *ts* phenotype in the protease or helicase domains, not combinations [28–33]. The *ts* phenotype represented by the combination of two mutations in protease and helicase domains may be characteristic of rubella virus. Interestingly, the protease domain of hepatitis C viral NS3 accelerated NS3 helicase activity, just as the helicase activity enhanced protease activity [34–36]. Protease and helicase domains of hepatitis C virus were located on one molecule of NS3, while the two domains of rubella virus locate on p150 and p90. These observations provide insights into structural and biochemical interactions. To account for the differences between H1042Y and Y1042H in *ts* or non-*ts* characteristics, the analysis of T1497I, Y1042H-T1497I, their inversion constructions in the viral life cycle, and biochemical characterization of the protease and helicase domains are currently under investigation.

In conclusion, using two vaccine strains, KRT and TO-336, we found that the mutations in the protease and helicase domains are necessary for the conversion of non-*ts* of wild-type viruses to the *ts* phenotype of vaccine strains. This may be applied to the other vaccine strains (RA27/3, Matsuura, and Matsuba) through further investigations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2010.11.074.

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Safe and effective booster immunization using DTaP in teenagers

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ABSTRACT

The incidence of reported cases with pertussis has increased in young adults in Japan and the lack of additional booster immunizations containing pertussis components is suspected to be one of the causal reasons. Instead of DT immunization at 11–12 years of age, safety and immunogenicity were investigated using 0.2 ml and 0.5 ml of DTaP. 176 subjects in DTaP 0.5 ml, 178 in DTaP 0.2 ml, and 197 in DT 0.1 ml groups were enrolled in clinical trial. The relative risk of local reactions in the DTaP 0.2 ml group compared to the DT 0.1 ml group was 1.13 (95% CI: 0.97–1.30), and that of the DTaP 0.5 ml to the DT 0.1 ml group was 1.34 (95% CI: 1.18–1.53). The relative risks of local pain and heat were 1.62 (95% CI: 1.33–1.98) and 1.59 (95% CI: 1.19–2.13), respectively, in the DTaP 0.5 ml group compared to the DT 0.1 ml group. Seropositive rates against PT and FHA were 54% and 82% before immunization and increased to >95% for both after vaccination with no significant difference in GMT. Instead of the scheduled DT program, 0.2 ml of DTaP was acceptable and demonstrated efficient immunogenicity.

1. Introduction

Pertussis is still a serious illness in young infants, causing whooping cough, apnea, cyanosis, chocking, and encephalopathy [1]. In Japan, whole cell pertussis vaccine was developed in 1950s and combined with diphtheria and tetanus toxoids (DTwP). DTwP became accepted, resulting in a reduction of reported cases of pertussis [2,3]. Approximately 10% of recipients experienced a febrile illness, with 50–60% showing redness and 20% induration [2]. In 1974–75, two accidental deaths after DTwP administration were reported and, thereafter, DTwP was discontinued for a while. It was re-introduced for children at 2 years of age, but the number of pertussis patients increased because of low vaccine coverage [2,3]. In 1981, a new type of acellular pertussis was developed, and combined vaccine (DTaP) was introduced into recommended immunization practice. Principally, two types of DTaP vaccines were developed: the B-type consisted of two major antigens, pertussis toxin (PT) and filamentous hemagglutinin (FHA) and the T-type contained pertactin and fimbrie besides PT and FHA [4–6]. Nationwide monitoring of clinical adverse events demonstrated low reactogenicity and sufficient antibody responses similar to natural infection. Since 1981, the number of pertussis patients decreased after the acceptance of DTaP. The incidence of pertussis in adults has been increasing gradually from 2002 in Japan, and several outbreaks on college campuses, and in high schools and offices have been reported [7,8]. In addition, the incidence in young infants less than 1 year of age increased as well as adult cases in 2009.

Pertussis is principally an infectious children's illness causing whooping and prolonged cough and the Advisory Committee on Immunization Practices (ACIP) recommended a 5-dose DTaP schedule, at ages 2, 4, 6, and 15–18 months and 4–6 years, instead of the previous DTwP in the US in 1997 [9]. In 1990s, the incidence of pertussis in older age increased in many countries because of waning immunity after primary childhood immunization and antigenic change of pertussis, and adolescent pertussis was identified as the source of transmission of pertussis to young infants through enhanced surveillance studies [10–16]. In 2005, tetanus toxoid, and a reduced concentration of diphtheria toxoid combined with reduced acellular pertussis (Tdap) vaccine was licensed, and the ACIP recommended that adolescents aged 11–18 years should receive a single dose of Tdap for booster immunization [17]. It was now recommended for all generations from 19 to 64 years [18].

It takes several years to obtain a license to introduce a new vaccine from foreign countries into Japan, even though Tdap is

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used worldwide. The immunization schedule in Japan incorporates no booster dose of pertussis components after the completion of the initial primary immunization (three times over 3 months of age and additional dose after approximately 12 months after the third dose), and vaccine containing pertussis components should be scheduled to cope with an outbreak of pertussis. In this study, safety and immunogenicity were investigated in clinical trials using 0.2 and 0.5 ml of DTaP at the age of 11–12 years, in comparison with 0.1 ml of DT.

2. Subjects and methods

2.1. Subjects

The study was conducted from September 2008 to August 2009, involving 29 pediatric outpatient clinics and departments of pediatrics of regional public and university hospitals. Subjects of this study included 555 children, 11–18 years of age, mostly 11–12 years of age, who had completed primary immunization of more than three doses of DTaP and had not undergone DT immunization. The study protocol was checked by the ethical committee of National Mie Hospital as a central organization and also checked by ethical committee of each hospital. Written informed consent was obtained from their parents or guardians. A total of 555 children were enrolled, but four children were excluded: three did not complete the primary immunization (one or two doses of DTaP), and one had already been immunized with DT. They were divided into two study groups: group 1 consisted of 266 subjects undergoing serological examination: 29 receiving 0.1 ml of DT, 119 for 0.2 ml of DTaP, and 118 for 0.5 ml of DTaP. Group 2 comprised 285 immunized without serological examinations, and totaling 551 subjects, with 197 receiving 0.1 ml DT, 178 for 0.2 ml of DTaP, and 176 for 0.5 ml of DTaP, were examined for safety. They are summarized in Fig. 1.

2.2. Vaccines

Five brands of DTaP were on the market in Japan, and the components of each antigen were different for each brand, as shown in Table 1. Subjects were allocated equally to each brand. The B-type (Biken and Kaketsu) vaccine consisted of PT and FHA and the T-type (Takeda, Denka, and Kitasato) contained other components, and the composition of pertussis antigens differed from the brands of DTaP available abroad [5,6,19]. The PT antigen contents varied from 3 to 23.5 μg/dose, and FHA from 23.5 to 51.5 μg/dose, but the amount of diphtheria and tetanus toxoids was 15 and 2.5 Lf/dose, respectively, without a difference among DTaP brands. 0.2 ml of DTaP contained 1.2–9.4 μg of PT, 9.4–20.6 μg of FHA, 6–6.6 Lf of diphtheria toxoid, and 1.0 Lf of tetanus toxoid. Antigen contents of FHA and diphtheria toxoid were slightly higher in 0.2 ml of DTaP than Tdap, Boostrix and Adacel (2.5–8 μg of PT, 5–8 μg of FHA, 2–2.5 Lf of diphtheria toxoid, and 5 Lf of tetanus toxoid) [17]. A 0.2-ml volume of DTaP contained similar amounts to Tdap. The antigen content of tetanus toxoid was lower in 0.2 ml of DTaP than Tdap available abroad, similar to 0.1 ml of DT.

2.3. Study design

The study was designed as a randomized open trial. Subjects were allocated randomly to DT 0.1 ml, DTaP 0.2 ml, and DTaP 0.5 ml groups. They were observed for 30 min for the appearance of anaphylaxis. To assess the safety afterwards, they were asked to check their body temperature and for adverse clinical events based on the healthcare diary every day for 7 days after immunization. In study group I, paired sera were obtained immediately before immunization and principally 4–6 weeks after immunization and kept at −20 °C. The paired sera were divided into two aliquots and transferred to the National Institute of Infectious Diseases, Department of Bacteriology II to examine antibodies against diphtheria and tetanus toxoids and to Kitasato-Otsuka Bio-Medical Assay Laboratories for the examination of pertussis antibodies (PT and FHA).

2.4. Serology

Antibodies against tetanus toxoid were determined with a KPA kit (Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) [20]. The kit comprised polypeptide artificial carrier particles stained with Reaction Blue solution, sensitized with highly purified tetanus toxoid (3000 Lf/mg PN), and provided in lyophilized form. The test was performed as instructed by the

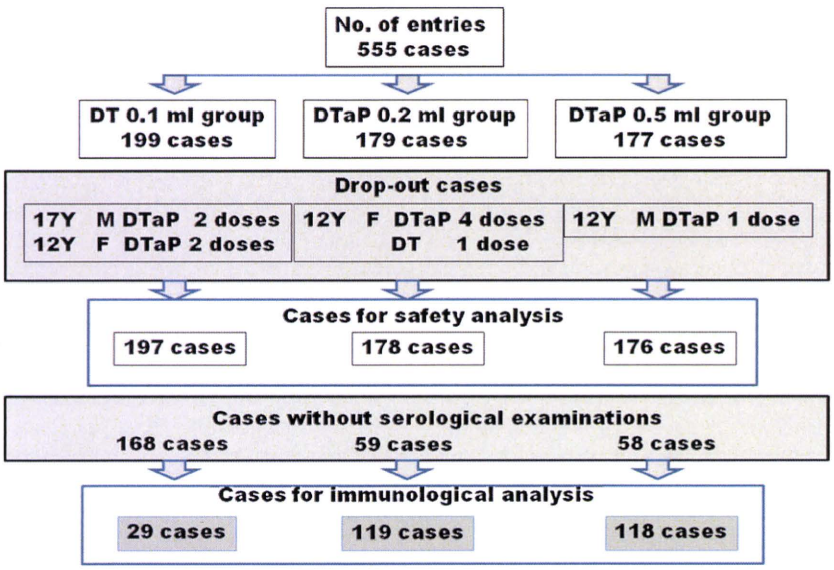

Fig. 1. Number of subjects in the study. A total of 555 subjects were enrolled, of whom four were excluded. Therefore, 551 subjects were evaluated regarding safety. Among the 551, 197 were immunized with 0.1 ml of DT, 178 with 0.2 ml of DTaP, and 176 with 0.5 ml of DTaP. Study group 1 consisted of 266 subjects for serological examination: 29 with 0.1 ml of DT, 119 with 0.2 ml of DTaP, and 118 with 0.5 ml of DTaP.

Table 1
Contents of PT, FHA, and diphtheria and tetanus toxoids.

DTaP/DT (manufacturers)	PT (μg)	FHA (μg)	Pertactin	Fimbrie	D (Lf)	T (Lf)
DTaP 0.5 ml (Kaketsu)	8	32			≦16.7	≒2.5
DTaP 0.5 ml (Biken)	23.5	23.5			≦15	≦2.5
DTaP 0.5 ml (Takeda)	3	34.5	7.5	1	≒15	≒2.5
DTaP 0.5 ml (Denka)	9	32	15	1	≦15	≦2.5
DTaP 0.5 ml (Kitasato)	6	51.5	5	1	≒15	≒2.5
Adacel (Aventis)	2.5	5	3		2	5
Boostrix (GSK)	8	8	2.5		2.5	5
DTaP 0.2 ml	1.2–9.4	9.4–20.6			6–6.6	1.0
DT 0.1 ml					3.2	0.7

Table 2
Background of the subjects.

	DTaP 0.2 ml (*N* = 178)	DTaP 0.5 ml (*N* = 176)	DT 0.1 ml (*N* = 197)	Total (*N* = 551)
Gender				
Male	93 (52.2%)	95 (54.0%)	113 (57.4%)	301 (54.6%)
Female	85 (47.8%)	81 (46.0%)	84 (42.6%)	250 (45.4%)
Age				
11 years	97 (54.5%)	95 (54.0%)	73 (37.1%)	265 (48.1%)
12 years	68 (38.2%)	68 (38.6%)	111 (56.3%)	247 (44.8%)
Others	13 (7.3%)	13 (7.4%)	13 (6.6%)	39 (7.1%)
Mean age ± SD	11.6 ± 0.8	11.6 ± 0.8	11.8 ± 0.8	11.6 ± 0.8
Median age	11.0	11.0	12.0	12.0
Range (min–max)	(11–15)	(11–15)	(11–17)	(11–17)
DPT history				
I-1	178 (100.0%)	176 (100.0%)	197 (100.0%)	551 (100.0%)
I-2	178 (100.0%)	176 (100.0%)	197 (100.0%)	551 (100.0%)
I-3	172 (96.6%)	172 (97.7%)	193 (98.0%)	537 (97.5%)
I-boost	172 (96.6%)	168 (95.5%)	191 (97.0%)	531 (96.4%)

manufacturers. Antibodies against diphtheria toxoid were examined using the micro cell-culture method with Vero cells, and diphtheria antitoxin titers were expressed as international units (IU)/ml [21]. Antibodies against PT and FHA were examined using enzyme-linked immunosorbent assay (EIA) kits (Wako Chemicals, Japan) as instructed by the manufacturers. Positive levels were defined as ≥0.1 IU/ml for antibodies against diphtheria toxoid, ≥0.01 IU/ml for those against tetanus toxoid, and ≥10 EU/ml for those against PT and FHA [22,23].

2.5. Statistical analysis

The sero-positivity rate and the incidence of solicited adverse events (fever as systemic reaction, and redness, swelling, pain, heat, and itching as local reactions) were compared by using Fisher's Extraction test. Geometric mean titers (GMTs) of antibodies before and after immunization were compared by converting to a logarithmic scale using Wilcoxon rank test. The *t* student Welch method was employed to evaluate significance and the significant level was set at $p < 0.05$.

3. Results

3.1. Background of the subjects

The subjects included 555 children aged 11–18 years of age, as shown in Fig. 1. A total of 555 subjects were enrolled, but four were excluded. Therefore, 551 subjects were evaluated for safety. Among the 551, 197 were immunized with 0.1 ml of DT, 178 with 0.2 ml of DTaP, and 176 with 0.5 ml of DTaP. The backgrounds of the subjects are shown in Table 2. A total of 301 (54.6%) were male, and the gender ratio was similar among the three groups with no significant differences in ages, which ranged from 11 to 17 years. They had all completed their primary immunizations (three or four doses of DTaP), confirmed by checking their immunization records.

3.2. Incidence of adverse events

The incidences of adverse events are summarized in Table 3. Febrile reactions were noted in 8 (4.1%) of 197 in the DT 0.1 ml group, 7 (3.9%) of 178 in the DTaP 0.2 ml group, and 7 (4.0%) of 176 in the DTaP 0.5 ml group, and the relative risks in DTaP

Table 3
Incidence of clinical adverse events.

Adverse events	DTaP 0.2 ml (1)	DTaP 0.5 ml (2)	DT 0.1 ml (3)	Risk ratio (95% CI)		
	(*N* = 178)	(*N* = 176)	(*N* = 197)	(2) vs. (1)	(1) vs. (3)	(2) vs. (3)
Fever	7 (3.9%)	7 (4.0%)	8 (4.1%)	1.01 (0.36,2.82)	0.97 (0.36,2.62)	0.98 (0.36,2.65)
Local reactions	123 (69.1%)	145 (82.4%)	121 (61.4%)	1.19 (1.06,1.34)	1.13 (0.97,1.30)	1.34 (1.18,1.53)
Redness	95 (53.4%)	109 (61.9%)	92 (46.7%)	1.16 (0.97,1.39)	1.14 (0.93,1.40)	1.33 (1.10,1.60)
Swelling	90 (50.6%)	95 (54.0%)	76 (38.6%)	1.07 (0.87,1.30)	1.31 (1.04,1.65)	1.40 (1.12,1.75)
Pain	83 (46.6%)	116 (65.9%)	80 (40.6%)	1.41 (1.17,1.71)	1.15 (0.91,1.45)	1.62 (1.33,1.98)
Heat	50 (28.1%)	74 (42.0%)	52 (26.4%)	1.50 (1.12,2.00)	1.06 (0.76,1.48)	1.59 (1.19,2.13)
Itching	81 (45.5%)	83 (47.2%)	75 (38.1%)	1.02 (0.82,1.28)	1.21 (0.95,1.54)	1.24 (0.98,1.57)

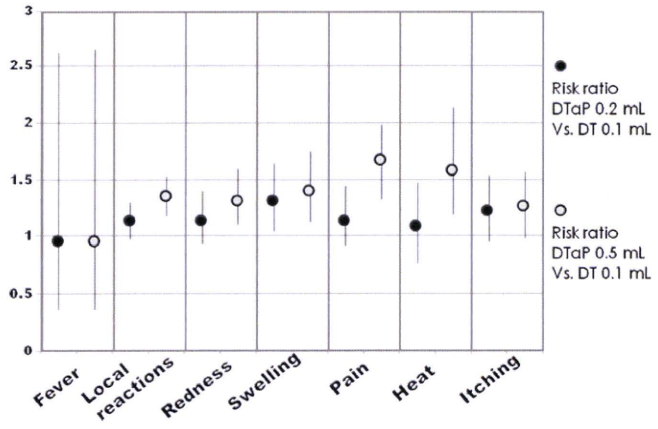

Fig. 2. Summary of the risk ratio regarding the incidence of adverse reactions. The relative risks of the incidence of adverse reactions after immunization with 0.2 ml (●) and 0.5 ml (○) of DTaP in comparison with those observed after immunization with 0.1 ml of DT are summarized. Vertical lines represent 95% CI.

0.2 ml and DTaP 0.5 ml groups were 0.97 and 0.98, respectively, in comparison with that observed in the DT 0.1 ml group. The relative risk of local reactions after immunization with DTaP at 0.2 ml was 1.13 (95% CI: 0.97–1.30) in comparison with the incidence after immunization with DT at 0.1 ml, and that of the DTaP 0.5 ml compared to the DT 0.1 ml group was 1.34 (95% CI: 1.18–1.53). Relative risks of redness, swelling, local pain, heat, and itching in the DTaP 0.2 ml group compared to the DT 0.1 ml group were 1.14 (95% CI: 0.93–1.40), 1.31 (95% CI: 1.04–1.65), 1.15 (95% CI: 0.91–1.45), 1.06 (95% CI: 0.76–1.48), and 1.21 (95% CI: 0.95–1.54), respectively. However, the relative risks of redness, swelling, local pain, heat, and itching in the DTaP 0.5 ml group compared to the DT 0.1 ml group were 1.33 (95% CI: 1.10–1.60), 1.40 (95% CI: 1.12–1.75), 1.62 (95% CI: 1.33–1.98), 1.59 (95% CI: 1.19–2.13), and 1.24 (95% CI: 0.98–1.57), respectively. The relative risks of the adverse reactions after immunization in the DTaP 0.2 ml and 0.5 ml groups in comparison with those observed after immunization in the DT 0.1 ml group are summarized in Fig. 2. Thus, the incidence of local reactions after immunization with 0.2 ml of DTaP was similar to that observed after immunization with 0.1 ml of DT, but those observed after immunization with 0.5 ml of DTaP were higher than after immunization with 0.1 ml of DT, notably regarding the incidences of local pain and heat, demonstrating the relative risks: 1.62 (95% CI: 1.33–1.98) and 1.59 (95% CI: 1.19–2.13), respectively.

3.3. Onset of adverse reactions

The immunization day was defined as day 0. The onset of adverse reactions was examined, and the results are shown in Fig. 3. Febrile reactions were noted from days 0 to 7 without any case accumulation, but the incidence of local reactions peaked on days 1 and 2. Systemic adverse events were reported sporadically: headache in 25 (9 in DT 0.1 ml group, 9 in DTaP 0.2 ml group, and 7 in DTaP 0.5 ml group), fatigue in 11 (3 in DT 0.1 ml group, 4 in DTaP 0.2 ml group, and 4 in DTaP 0.5 ml group), rhinorrhea in 10 (1 in DT 0.1 ml, 2 in DTaP 0.2 ml, and 7 in DTaP 0.5 ml group), sore throat in 8, cough in 7, and nasal obstruction in 7. Three subjects with urticaria eruption were reported: two on day 0 (one for each DT 0.1 ml and DTaP 0.5 ml group) and one on day 1 in DTaP 0.5 ml group. Generalized eruption was reported on day 1 in DTaP 0.5 ml group. The relative risk of local reactions on day 0 after immunization with 0.2 ml of DTaP compared to that observed after 0.1 ml of DT was 1.08 (95% CI: 0.74–1.58), 1.18 (95% CI: 0.96–1.44) on day 1, 1.09 (95% CI: 0.91–1.30) on day 2, 1.19 (95% CI: 0.97–1.47) on day 3, 1.3 (95% CI: 0.99–1.71) on day 4, 1.56 (95% CI: 1.09-2.23) on day 5, 1.42 (95% CI: 0.87–2.29) on day 6, and 1.54 (95% CI: 0.87–2.72) on day 7. The incidence of local reaction for each day after immunization with 0.2 ml of DTaP was similar to that observed after 0.1 ml of DT. The incidence of local reactions after immunization with 0.5 ml of DTaP was higher than that observed in the DT 0.1 ml group, especially on days 1 and 2, with a relative risk of 1.61 (95% CI: 1.35–1.92) on day 1, and 1.33 (95% CI: 1.13–1.92) on day 2. Most local adverse reactions appeared on day 1 and continued for 3–4 days, but those observed in the DTaP 0.5 ml group became prolonged, showing a relative risk of 2.15 (95% CI: 1.39–3.33) on day 6.

In this study, the extents of redness and swelling were monitored when they appeared and the degree of adverse reactions was evaluated (Fig. 4). There was no significant difference in the incidence of redness and swelling of <2.0 cm and 2–5 cm among the

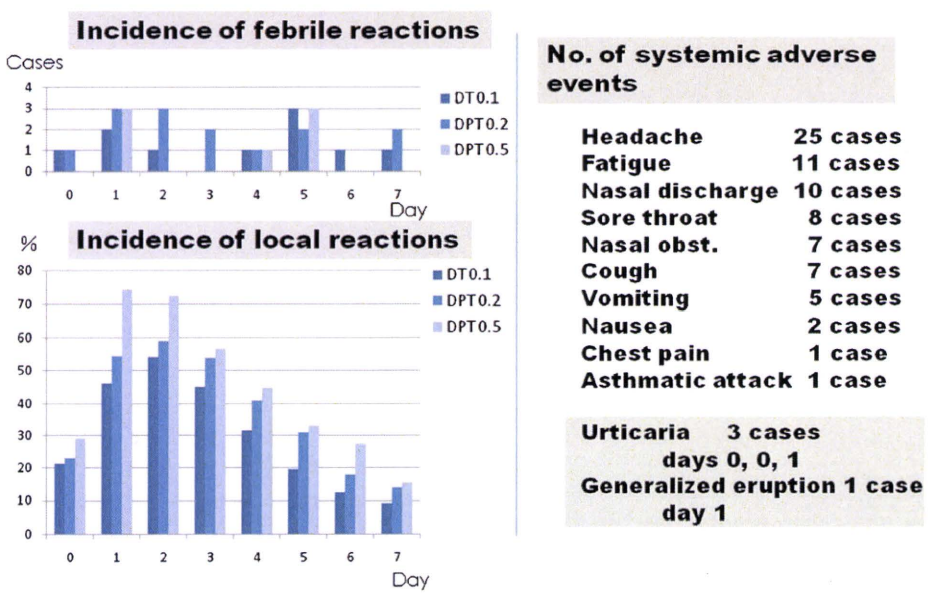

Fig. 3. Onset of febrile and local reactions within 7 days after immunization and the no. of cases with systemic adverse events.