3) 採血

接種 21 日後に採血を行い、血清分離・非働化を行った。

4) 中和試験

採取した血清の中和抗体価を HEp-2 細胞及びポリオウイルス（Sabin 株）を用いて測定した。

5) 解析

各血清の中和抗体価を用いて、プロビット法により DPT/IPV（lot CV02）の参照品候補に対する相対力価を算出した。

C．研究結果

調製した DPT/IPV（lot CV02）の成績を表2に示した。また、IPV の力価試験成績を表3に示した。

D．考察

表2に示した品質試験の結果から、DPT/IPV（lot CV02）の DPT 成分の品質は、いずれも DPT の生物学的製剤基準に適合していた。また、データは示していないが当所製市販 DPT ロットともほぼ同等の成績であった。よって、DPT 成分については、混合前後での品質の変化はないと考えられた。

表3に示した IPV 力価試験成績から、DPT/IPV（lot CV02）は、Ⅰ型、Ⅱ型、Ⅲ型のいずれも国内参照品候補と同等以上の力価があることが確認された。また、いずれの型も相対力価が2前後と混合前より上昇しており、これは DPT 中に含まれるアルミニウムゲルのアジュバント効果によるものと推察される。IPV 力価試験の解析法には今回プロビット法を採用した。各型毎の cut off 値は WHO で推奨されているように、参照品の段階希釈毎の各群における中和抗体価（10 匹の平均値）の最大値及び最小値の中点として設定した。参考として中和抗体価を用いた平行線定量法による解析も行ったが、その結果はプロビット法で解析した場合とほぼ同等の相対力価が算出され、95%信頼区間は平行線定量法で解析した場合の方が若干狭い範囲となった（表3参照）。この結果から、解析法として平行線定量法も採用可能であることが示唆された。

E．結論

今回の研究結果より、当所製 DPT バルクとポリオ研製 IPV バルクを混合しても、DPT 各成分に問題となるような品質の変化はないと考えられる。よって、DPT の生物学的製剤基準をベースに IPV の品質試験を上乗せすることで、DPT/IPV の生物学的製剤基準（案）を設定可能と考える。

IPV の力価試験法は混合ワクチンの力価試験としても適応可能と考えられるが、今後、試験条件や解析法の統一が必要である。

F．健康危惧情報

なし

G．研究発表

なし

H．知的財産権の出願・登録状況

なし

表1．各試料の抗原量

試料名	lot 番号など	抗原量（ /0.5mL）					
		D (Lf)	P (μgPN)	T (Lf)	IPV（Du）		
					Ⅰ型	Ⅱ型	Ⅲ型
DPT/IPV	CV02	12.5	6.3	1.5	2.8	90	113
IPV 力価試験 国内参照品候補	TIPV ＃ 04C (1:5)	-	-	-	3.6	103	113

表2．品質試験成績

試験項目	単位	DPT の 生物学的製剤基準	DPT/IPV (lot CV02)
pH 試験	－	5.4 ~ 7.4	6.93
ｱﾙﾐﾆｳﾑ含量試験	mg/mL	0.3 以下	0.21
ﾎﾙﾑｱﾙﾃﾞﾋﾄﾞ含量試験	W/V%	0.01 以下	0.0017
無菌試験	－	菌の発育を認めない	菌の発育を認めず
異常毒性否定試験（ﾓﾙﾓｯﾄ）	－	異常を認めない	異常を認めず
ｴﾝﾄﾞﾄｷｼﾝ試験	EU/mL	4.0 以下	0.021
ﾏｳｽ体重減少試験	BWDU/mL	10 以下	6.8
ﾏｳｽ白血球数増加試験	LPU/mL	0.5 以下	0.10
ﾏｳｽﾋｽﾀﾐﾝ増感試験（加温）	HSU/mL	0.4 以下	0.04
ﾏｳｽﾋｽﾀﾐﾝ増感試験（非加温）	HSU/mL	0.4 以下	0.04
ｼﾞﾌﾃﾘｱ毒素無毒化試験	－	毒素による異常を示さない	毒素による異常を示さず
破傷風毒素無毒化試験	－	毒素による異常を示さない	毒素による異常を示さず
力価試験（ｼﾞﾌﾃﾘｱ）	単位/mL	47 以上	157.7
力価試験（百日せき）	単位/mL	8 以上	18.1
力価試験（破傷風）	単位/mL	27 以上	224.3

表３．IPV 力価試験成績

解析方法	型	参照品候補に対する相対力価	95%信頼区間	
			下限	上限
プロビット法[a)]	Ⅰ型	2.112	1.294	3.629
	Ⅱ型	1.762	0.942	2.935
	Ⅲ型	2.146	1.169	4.004
平行線定量法	Ⅰ型	2.125	1.500	3.028
	Ⅱ型	1.614	0.966	2.431
	Ⅲ型	2.292	1.490	3.605

a）cut off 値：Ⅰ型（5.5）、Ⅱ型（7.0）、Ⅲ型（2.5）

厚生労働省科学研究費補助金(医薬品等医療技術リスク評価研究事業)
協力研究報告書

混合ワクチンの品質確保に関する研究

協力研究者 末原章宏、山下利明 武田薬品工業株式会社

研究要旨 混合ワクチン中の不活化ポリオワクチン免疫原性試験法の評価に先立ち、不活化ポリオワクチンを 2 倍、6 倍、18 倍に希釈したものをラット後肢大腿部筋肉内ならびにマウス腹腔内に免疫し、追加免疫の後に採血して得られた血清について中和抗体価測定を実施した結果、いずれの動物でも全ての型で 2^2 以上の中和抗体価は確認され、免疫原性試験へのマウスの使用も可能であることが示唆された。

次に、不活化ポリオワクチンまたはアルミ沈降型混合ワクチンを原液、2 倍、4 倍に希釈したものをラットに免疫し、3 週目から 6 週目まで 1 週間毎に採血して、得られた血清について中和抗体価測定を実施した結果、1 型において、3 週目以降、不活化ポリオワクチンの中和抗体価の上昇に比べ混合ワクチンの中和抗体価の上昇が大きく、中和抗体価の比に変化が認められたことから、相対力価を算出するにあたり免疫期間の検討の必要性が考えられた。

最後に、不活化ポリオワクチン参照品を原液、2 倍、4 倍、8 倍に希釈したものを 8 週齢・雌のラットに免疫し、3 週後に得られた血清について中和抗体価測定を実施した結果、いずれの型も一部の用量で平均中和抗体価に用量反応性が認められたが、カットオフ値を 2^2 にした場合、1 型および 2 型の抗体陽性率に用量反応性が認められず、プロビット解析を適用するにはカットオフ値または免疫希釈の見直しが必要と考えられた。

A. 研究目的

3 価混合不活化ポリオワクチン sIPV(以下、不活化ポリオワクチン)、精製百日せきワクチン、ジフテリアトキソイド、破傷風トキソイドを混合し、アルミニウム沈降型とした混合ワクチンの品質確保に関する研究として、混合ワクチン中の不活化ポリオワクチン免疫原性試験法の評価にあたり、免疫原性試験法に使用する動物(ラット、マウス)の評価、免疫期間の評価、更に、不活化ポリオワクチン参照品を用いて免疫原性試験法における相対力価算出法の評価を行った。

B. 研究方法

1. 免疫原性試験法(使用動物評価)

不活化ポリオワクチンに関する免疫原性を測定するため、不活化ポリオワクチンを 2 倍、6 倍、18 倍に希釈したものを、各群 5 匹ずつのラット(ウィスター系・雄)の後肢大腿部筋肉内またはマウス(dd 系・雌)の腹腔内に免疫したのち、その 3 週後に追加免疫し、ラットはその 2 週間後、マウスはその 1 週間後に採血し、得られた血清について、HEp2cN 細胞および各型セービンウイルスを攻撃ウイルスとして用い中和抗体価の測定を行った。

2. 免疫原性試験法(免疫期間評価)

同様に、不活化ポリオワクチンまたはアルミ沈降型混合ワクチンを原液、2 倍、4 倍に希釈したものを各群 5 匹ずつのラットに免疫し、その 3 週目から 6 週目まで 1 週間毎に採血し、得られた血清について中和抗体価の測定を行った。

3. 免疫原性試験法(相対力価算出法の評価)

不活化ポリオワクチン参照品を原液、2 倍、4 倍、8 倍に希釈したものを各群 5 匹ずつのラット

(雌、8 週齢)に免疫し、その 3 週目に採血し、得られた血清について中和抗体価の測定を行った。

C. 研究結果

1. 免疫原性試験法(使用動物評価)

表 1 に示したとおり、ラットおよびマウス共に、2 回免疫により 1 型、2 型、3 型のすべてで陽性下限値とされる 2^2 を十分上回っていた。

表 1　中和抗体価の測定結果

中和抗体価 (2^n)

	希釈	1 型	2 型	3 型
ラット	2	9.5	10.1	8.5
	6	7.9	8.7	6.6
	18	7.3	8.1	6.1
マウス	2	9.7	6.5	4.7
	6	5.5	6.3	4.7
	18	7.7	6.0	4.0

2. 免疫原性試験法(免疫期間評価)

1 型において、3 週目以降、不活化ポリオワクチンの中和抗体価上昇に比べ、混合ワクチンの中和抗体価上昇が大きく、表 2 に示したとおり、6 週目では中和抗体価相対値に有意な差が認められた。

表 2　中和抗体価相対値の測定結果

測定試料		4 週	5 週	6 週
1 型	混合ワクチン	1.4	1.4	1.8
	ポリオワクチン	1.2	1.2	1.1
2 型	混合ワクチン	1.1	1.2	1.2
	ポリオワクチン	1.2	1.1	1.0
3 型	混合ワクチン	1.4	1.5	1.5
	ポリオワクチン	2.3	0.7	1.5

(3 週目中和抗体価を 1 とした中和抗体価相対値)

3. 免疫原性試験法(相対力価算出法の評価)

1 型、2 型、3 型のいずれの型も、一部の希釈域で平均中和抗体価に用量反応性が認められたが、カットオフ値を 2^2 とした場合の抗体陽性率では 1 型および 2 型で用量反応性が認められなかった。

D. 考察

3 価混合不活化ポリオワクチン、精製百日せきワクチン、ジフテリアトキソイド、破傷風トキソイドを混合したアルミニウム沈降型製剤である混合ワクチンの品質確保に関する研究として、免疫原性試験法の検討を行った。

単味不活化ポリオワクチンの最終バルクの規格として想定されているラット免疫原性試験法は、追加免疫の実施により、マウスでも、中和抗体価が認められたことから、マウスの使用も可能であることが示唆された。

次に、免疫期間は、1 型に関しては 3 週目以降も混合ワクチンの中和抗体価が上昇したが、不活化ポリオワクチンでは上昇が認められなかったことより、相対力価を算出する場合、免疫期間の検討も必要であると考えられた。

最後に、不活化ポリオワクチン参照品を原液、2 倍、4 倍、8 倍に希釈し免疫することにより、平均中和抗体価に用量反応性が一部の希釈域で認められたが、カットオフ値を 2^2 とした抗体陽性率には、2 型および 3 型で用量反応性が認められず、プロビット解析を適用するには、カットオフ値または免疫希釈の見直しが必要と考えられた。

E. 結論

1. 免疫原性試験における不活化ポリオワクチンの 2 回免疫により、マウスで中和抗体価が測定され、マウスの使用も可能であることが示唆された。
2. 免疫原性試験において、混合ワクチンの 1 型は 3 週目以降も不活化ポリオワクチンに対して、有意に中和抗体価が上昇したことから、免疫期間の検討の必要性が考えられた。
3. 不活化ポリオワクチン参照品による免疫原性試験において、カットオフ値を 2^2 とした場合、1 型および 2 型に用量反応性が認められず、カットオフ値または免疫希釈の見直しが必要と考えられた。

F. 健康危険情報

なし。

G. 研究発表

1. 論文発表

なし。

2. 学会発表

なし。

H. 知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得

予定なし。

2. 実用新案登録

予定なし。

3. その他

特記なし。

厚生労働省科学研究費補助金（厚生労働科学特別研究事業）
研究協力報告書

ｓＩＰＶを用いたＤＰＴ－ＩＰＶ試作品の性状解析に関する研究

研究協力者　東　　雍　（財）阪大微生物病研究会

研究要旨　現行の沈降精製百日せきジフテリア破傷風混合ワクチン（ＤＰＴ）に財団法人日本ポリオ研究所が開発中の弱毒株由来不活化ポリオワクチン（ｓＩＰＶ）を混合したＤＰＴ－ＩＰＶ試作品の免疫原性を調査したところ、暫定国内参照品と比べてＩ型は高く、Ⅱ，Ⅲ型ではほぼ同等であることが確認された。

Ａ．研究目的

当会は、ＤＰＴ－ＩＰＶの製剤化に向けてＤＰＴ－ＩＰＶ試作品の性状解析を行ってきた。２００３年度の研究協力報告書では、①ＤＰＴに対するｓＩＰＶの干渉作用が見られない、②ＤＰＴと混合することでｓＩＰＶの免疫原性が増強されることを示し、本ワクチン開発の可能性について報告した。

本研究では、ｓＩＰＶ力価試験用参照品と同量のＤ抗原量を含むＤＰＴ－ＩＰＶ試作品を作製し、その免疫原性を調査する。

Ｂ．研究方法

（１）ＤＰＴ－ＩＰＶの試作

財団法人日本ポリオ研究所からｓＩＰＶを購入し、現行のＤＰＴと混合してＤＰＴ－ＩＰＶ試作品を作製した。

ＤＰＴの各抗原含量は現行ワクチンと同量とした。ｓＩＰＶについては、力価試験用参照品と同量のＩＰＶ抗原（Ｄ抗原）含量（Ｉ型：3Du/dose、Ⅱ，Ⅲ型：100Du/dose）に調整した。チメロサール及び２－PE（フェノキシエタノール）等の防腐剤は添加しなかった。

（２）ＩＰＶの免疫原性

「混合ワクチンの品質確保に関する研究」班で決定したラット免疫原性試験法に準じて実施した。

暫定国内参照品及びＤＰＴ－ＩＰＶ試作品を各々４段階希釈（暫定国内参照品；１：１，１：２，１：４，１：８、ＤＰＴ－ＩＰＶ試作品；１：１，１：４，１：１６，１：６４）した後、各希釈につき、Wistar 系ラット１０匹（８週齢♀：１０匹）の後肢大腿部に筋肉内接種した（0.5mL／匹）。１回接種から３週間後に全採血した後、得られた個体別血清についてマイクロタイター法により中和抗体価を測定した。解析はプロビット法及び平行線定量法により行い、暫定国内参照品に対する各型の相対力価を算出した。

（倫理面への配慮）

実験動物であるラットに対しては、採血時に麻酔処置を施し、負担を最大限軽減した。

Ｃ．研究結果

ＤＰＴ－ＩＰＶ試作品の免疫原性

① プロビット法

各型毎にカットオフ値を決め、

得られた中和抗体価から抗体陽性率を算出し、プロビット法により相対力価を算出した。その結果、暫定国内参照品に対する相対力価はⅠ型：6.0、Ⅱ型：1.8、Ⅲ型：2.2であった。（表1～3）。

② 平行線定量法

個体別の中和抗体価をもとに、平行線定量法により暫定国内参照品に対するＤＰＴ－ＩＰＶ試作品の相対力価を算出したところ、上記①と同様の結果が得られた（Ⅰ型：5.4、Ⅱ型：2.4、Ⅲ型：1.4）。（表1～3）。

D．考察

今年度に入り、「混合ワクチンの品質確保に関する研究」班において、懸案事項であったｓＩＰＶの力価試験用参照品のD抗原量が、Ⅰ型、Ⅱ型、Ⅲ型それぞれ 3：100：100（Du/dose）とすることが決まり、ラット免疫原性試験法の骨子も決定した。本研究ではｓＩＰＶ力価試験用参照品と同量のD抗原量を含むＤＰＴ－ＩＰＶ試作品について免疫原性を調査した。免疫原性を評価する際の解析方法は、プロビット法もしくは平行線定量法のいずれの方法でも同様の結果が得られたことから平行性及び直線性に問題がなければどちらの方法でも評価が可能であると思われる。しかし、プロビット法についてはカットオフ値によって相対力価が変わる恐れがあるため注意が必要であろう。

E．結論

力価試験用参照品と同量の D 抗原含量のｓＩＰＶを含むＤＰＴ－ＩＰＶ試作品を作製し、その免疫原性を調査した。プロビット法及び平行線定量法を用いて各型の免疫原性を解析したところ、暫定国内参照品と比べてⅠ型は高く、Ⅱ，Ⅲ型ではほぼ同等であることが確認された。

F．健康危機情報

特になし

G．研究発表

（1）論文発表

なし

（2）学会発表

なし

H．知的財産権の出願・登録状況（予定を含む。）

（1）特許取得

なし

（2）実用新案登録

なし

（3）その他

なし

表1．ＤＰＴ－ＩＰＶ試作品の免疫原性（Ｉ型）

参照品		1 : 1	1 : 2	1 : 4	1 : 8
暫定国内参照品候補	平均抗体価（2^n）*1	7.2	6.3	4.1	3.2
試験検体		1 : 1	1 : 4	1 : 16	1 : 64
ＤＰＴ－ＩＰＶ試作品	平均抗体価（2^n）*1	8.3	7.9	5.2	3.3
	プロビット法*2	６．０（カットオフ値；≧６）			
	平行線定量法*2	５．４			

表2．ＤＰＴ－ＩＰＶ試作品の免疫原性（Ⅱ型）

参照品		1 : 1	1 : 2	1 : 4	1 : 8
暫定国内参照品候補	平均抗体価（2^n）*1	7.4	6.8	5.3	3.5
試験検体		1 : 1	1 : 4	1 : 16	1 : 64
ＤＰＴ－ＩＰＶ試作品	平均抗体価（2^n）*1	7.7	6.6	5.5	3.7
	プロビット法*2	１．８（カットオフ値；≧７）			
	平行線定量法*2	２．４			

表3．ＤＰＴ－ＩＰＶ試作品の免疫原性（Ⅲ型）

参照品		1 : 1	1 : 2	1 : 4	1 : 8
暫定国内参照品候補	平均抗体価（2^n）*1	6.2	5.4	2.1	1.3
試験検体		1 : 1	1 : 4	1 : 16	1 : 64
ＤＰＴ－ＩＰＶ試作品	平均抗体価（2^n）*1	7.1	3.5	1.9	1.5
	プロビット法*2	２．２（カットオフ値；≧２）			
	平行線定量法*2	１．４			

＊１：各群（♀；１０匹）の幾何平均値

＊２：暫定国内参照品候補の免疫原性を１とした時の相対力価

厚生労働省科学研究費補助金（医薬品等医療技術リスク評価研究事業）
研究協力報告書

混合ワクチンの品質確保に関する研究

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研究要旨　沈降精製百日せきジフテリア破傷風混合（DPT）ワクチンに不活化ポリオワクチン（IPV）を混合し、DPT-IPV 混合試作ワクチンを調製した。混合ワクチンとすることで、DPT に関する力価は影響がなく、また IPV についても参照品と同等以上の力価が確認された。

A. 研究目的

DPT-IPV 混合ワクチンの品質確保を図るため、DPT と IPV を混合することで、DPT 及び IPV の品質に変化が生じないことを確認する。

B. 研究方法

(1) DPT-IPV 混合試作ワクチンの調製

濃縮 IPV 原液は財団法人日本ポリオ研究所のロット#04B を、沈降 DPT ワクチンは自社調製品を用いた。保存剤として 2-フェノキシエタノールを 0.5%含有する。

(2) DPT の力価試験

生物学的製剤基準の該当する力価試験を実施した。

(3) IPV の免疫原性試験

DPT-IPV 混合ワクチンまたは IPV について、2 倍階段希釈した検体を各群 10 匹ずつラット（Wister 系、メス、8 週令）の後肢大腿部筋肉内に接種した。参照品は日本ポリオ研究所のロット#04C を用いた。3 週後採血を行い、得られた血清について、Hep2CN 細胞及び各型の Sabin ウイルスを攻撃ウイルスとして、中和抗体価の測定を行った。

(4)IPV の D 抗原量測定

日本ポリオ研究所による D 抗原測定試薬を用い、ELISA 法により実施した。

C. 研究結果

(1) DPT の力価試験

DPT-IPV 混合ワクチンと DPT ワクチンで力価試験成績を比較したところ、DPT に関する力価試験の成績に影響は見られなかった。

(2) IPV の中和力価

DPT-IPV 混合ワクチンの中和力価の成績を平行線定量法で解析したところ、相対力価はⅠ型で 4.05、Ⅱ型で 1.87、Ⅲ型は 2.35 と参照品と同等以上であることが示された（表 1）。一方、プロビット解析は血清希釈 2^2 をカットオフ値とすると、陰性となる血清が少なく計算値が算出されなかった。

表 1．IPV の相対力価

検体	Ⅰ型	Ⅱ型	Ⅲ型
DPT-IPV	4.05	1.87	2.35
IPV*	1.15	1.29	2.31

*参考値

また DPT-IPV 混合ワクチンは、I 型の相対力価が IPV 単味に比べ高くなる傾向が伺えた。

(3) IPV の D 抗原量(表 2)

DPT-IPV 混合ワクチンを沈降品の状態で ELISA 系により測定したところ、いずれの抗原型も反応性を示した。またアルミゲルを除去した上清では、II 型、III 型抗原が最終バルク濃度の近似値を示したことから、本試作ワクチンにおいて II 型・III 型抗原はアルミゲルに吸着していないものと推察された。

表 2. D 抗原量測定 (Du/mL)

検体	I 型	II 型	III 型
DPT-IPV 沈降品	4.2	297.6	170.8
DPT-IPV 遠心後上清	2.0	278.4	171.6
IPV 最終バルク濃度	5.3	229.0	183.8

さらに生ポリオウイルスと不活化ポリオ原液は用量反応性に明らかな違いが認められなかったことから、標準品を不活化型としても支障がないものと考えられた。

D. 考察

DPT-IPV 混合ワクチンとした場合、IPV は I 型の相対力価が IPV 単独に比べ高くなる傾向が伺えたことから、DPT-IPV 混合ワクチンの調製濃度は、今後検討が必要と考えられた。

IPV の力価試験で 1 回免疫による中和抗体測定が可能であり、試験系として運用の見通しが示された。一方、D 抗原測定を定量系として適用するには、小分製品の測定について更なる検討が必要と考えられる。

E. 結論

DPT と IPV を混合し DPT-IPV 混合試作ワクチンを調製した。DPT と IPV を混合しても、DPT ならびに IPV の力価に影響しないものと考えられた。

F. 健康危機情報

なし。

G. 研究論文

(1) 論文発表

なし

(2) 学会発表

なし

H. 知的財産権の出願・登録状況

(予定を含む)

(1) 特許取得

なし

(2) 実用新案

なし

(3) その他

なし

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Recombinant cholera toxin B subunit (rCTB) as a mucosal adjuvant enhances induction of diphtheria and tetanus antitoxin antibodies in mice by intranasal administration with diphtheria–pertussis–tetanus (DPT) combination vaccine

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Abstract

Recombinant cholera toxin B subunit (rCTB) which is produced by *Bacillus brevis* carrying pNU212-CTB acts as a mucosal adjuvant capable of enhancing host immune responses specific to unrelated, mucosally co-administered vaccine antigens. When mice were administered intranasally with diphtheria–pertussis–tetanus (DPT) combination vaccine consisting of diphtheria toxoid (DTd), tetanus toxoid (TTd), pertussis toxoid (PTd), and formalin-treated filamentous hemagglutinin (fFHA), the presence of rCTB elevated constantly high values of DTd- and TTd-specific serum ELISA IgG antibody titres, and protective levels of diphtheria and tetanus toxin-neutralizing antibodies but the absence of rCTB did not. Moreover, the addition of rCTB protected all mice against tetanic symptoms and deaths. DPT combination vaccine raised high levels of serum anti-PT IgG antibody titres regardless of rCTB and protected mice from *Bordetella pertussis* challenge. These results suggest that co-administration of rCTB as an adjuvant is necessary for induction of diphtheria and tetanus antitoxin antibodies on the occasion of intranasal administration of DPT combination vaccine.
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Keywords: DPT combination vaccine; Recombinant cholera toxin B subunit (rCTB); Intranasal administration; Diphtheria and tetanus antitoxin antibodies

1. Introduction

Cholera toxin (CT), a major enterotoxin produced by *Vibrio cholerae*, is composed of two subunits—a toxigenic A subunit (CTA) which activates ADP-ribosylation activity and a pentameric B subunit (CTB) which is responsible for CT binding to the cell membrane GM1 gangliosides. The basic chemical structure of heat-labile toxin (LT) which is produced by enterotoxigenic *Escherichia coli* is almost the same as that of CT. Both CT and LT act as a mucosal immunogen and a mucosal adjuvant, and induce antigen-specific systemic IgG and secretory IgA antibody responses at various mucosal sites to unrelated vaccine antigens when administered along with them via the nasal or oral route [1–5]. Only an A subunit of LT (LTA) and various mutant LTs and CTs lacking ADP-ribosylation activity also retain the mucosal adjuvant properties of wild CT and LT [5–11]. Moreover, it has been shown that recombinant CTB (rCTB) and B subunit of LT (rLTB) also have potent adjuvant activity towards intranasally or orally administered antigens [12–14].

We demonstrated that rCTB, which is produced by *Bacillus brevis* HPD31 carrying pNU212-CTB [15] and purified with affinity chromatography using D-galactose immobilized agarose [16], effectively delivers tetanus toxoid (TTd) and diphtheria toxoid (DTd) to mucosal inductive sites and elicits antigen-specific serum IgG and mucosal IgA antibody responses when given intranasally along

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with the toxoids [17,18]. With intranasal co-administration of an acellular pertussis vaccine the adjuvant activity of rCTB was vague because of the mucosal adjuvanticity of pertussis toxoid (PTd) [19]. No distinct local histopathological reactions were observed in the muscle, the nasal cavity and the small intestinal loop given rCTB [20], and frequent nasal administrations of rCTB-containing same and different vaccine-induced antigen-specific immune responses without induction of systemic tolerance and suppression by pre-existing anti-rCTB immunity [21]. In this study we investigated the adjuvant effect of rCTB on intranasal administration of a 30 μl (15 μl per nare) of a diphtheria–pertussis–tetanus (DPT) combination vaccine consisting of DTd, TTd, PTd, and formalin-treated filamentous hemagglutinin (fFHA) and found that rCTB has an important role in the induction of diphtheria and tetanus antitoxin antibodies.

2. Materials and methods

2.1. Animals, immunogens, immunization, and sample collection

All procedures on animals were conducted according to the Guideline for the Care and Use of Laboratory Animals of the Nagoya City University Medical School under protocols approved by the Institutional Animal Care and Use Committee at the Nagoya City University Medical School. Female BALB/c mice (SLC, Shizuoka, Japan) aged 7 weeks were used in this study. Each group consisted of 5, 10, 15 or 16 mice. TTd containing 200 Lf units ml^{-1} (66 μg protein nitrogen (PN) ml^{-1} and purity: 3025 Lf mg^{-1} PN), DTd containing 200 Lf units ml^{-1} (67 μg PN ml^{-1} and purity: 2985 Lf mg^{-1} PN), PTd containing 16 μg PN ml^{-1}, pertussis toxin (PT) containing 30 μg PN ml^{-1}, formalin-non-treated filamentous hemagglutinin (FHA) containing 32 μg PN ml^{-1} and fFHA containing 75 μg PN ml^{-1} were provided by The Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). PT and FHA were used for an enzyme-linked immunosorbent assay (ELISA) and TTd, DTd, and PTd were not adsorbed to aluminum salt. Mice were administered and boosted intranasally with 30 μl of solution consisting of 5 Lf DTd, 5 Lf TTd, 0.5 μg PTd, and 5 μg fFHA (DPT combination vaccine) with and without 10 μg rCTB under light ether anesthesia. Intranasal administrations were performed on days 0, 14, 21, and 28. Mice were sacrificed on day 35 and blood was collected. Almost the same experiments were repeated four times and designated as Experiments 1–4. Experiment 4 was mainly carried out for a challenge test of tetanus toxin (TT). In Experiment 1 collection of lung and nasal cavity lavages, small and large intestinal washes, saliva and vaginal secretions, and feces was done for measurement of mucosal IgA titres as described before [12,17]. These samples were stored at −20 °C until use.

2.2. Measurement of antigen-specific IgG, IgA, and IgE antibody levels

Antigen-specific serum IgG and IgA, and mucosal IgA antibody titres were measured using ELISA (in-house ELISA) as described before [17,22] and antigen-specific serum IgE antibody was detected by fluorometric capture ELISA as described by Sakaguchi et al. [23] except the use of 5-[5-(*N*-succinimidyloxycarbonyl) pentylamido]hexyl D-biotinamide (Dojindo Laboratories, Kumamoto, Japan) in place of *N*-hydroxysuccinimidobiotin to conjugate antigen with biotin; serum and mucosal IgA, and serum IgE antibody titres were determined only in Experiment 1. Calibration of the mean and standard deviation (S.D.) of values at 450 and 405 nm with sera and each washing sample of five non-immunized mice, setting of antibody-positive cut off values and expression of antigen-specific antibody ELISA titres and the geometric mean (G.M.) ± S.D. were carried out according to the previous description [19].

2.3. Cell culture method (CCM) for measurement of diphtheria antitoxin titres

In Experiments 1–4, the titration of diphtheria antitoxin of mouse serum was performed by CCM using Vero cells as described by Miyamura et al. [24] and diphtheria antitoxin titres were expressed as international neutralizing antibody units (IU) ml^{-1}.

2.4. Determination of anti-tetanus toxin antibody

The TT neutralization (TTN) assay was carried out in mice as described in "Minimum Requirement for Biological Products [25]". Briefly, six-fold dilution (in Experiments 2 and 3) and two-fold dilution (in Experiment 4) of pooled sera were performed and assayed for TT level of L+/1000. Tetanus antitoxin titres were calculated relatively for the standard tetanus antitoxin [26,27] and expressed in IU ml^{-1}.

2.5. TT direct challenge test

Five days after final collection of blood on day 35, mice were challenged by subcutaneous injection of approximately 100 LD_{50} of TT and was observed through out their life and death after 7 days.

2.6. Kaketsuken particle agglutination (KPA) method

The particle agglutination test with poly (γ-methyl L-glutamate), which was developed and shortened to KPA by KAKETSUKEN (The Chemo-Sero-Therapeutic Research Institute) [28], was carried out beforehand for the purpose of reference to measure tetanus antitoxin titres in

Experiments 1–4 and diphtheria antitoxin titres in Experiments 2–4, using Tetanus and Diphtheria Antibody Assay kits, respectively. The procedure was done according to the manufacturer's instructions. The highest dilution showing positive agglutination was determined and the unitage of serum sample was calculated against the reference serum and expressed as IU ml^{-1}. There is a significant correlation [28] between the level of anti-tetanus KPA titres and that of tetanus antitoxin titres by hemagglutination [29] and between the level of anti-diphtheria KPA titres, and that of diphtheria antitoxin titres by CCM [24].

2.7. Measurement of anti-PT and anti-FHA antibody titres by calibration against a reference mouse serum

Serum PT- and FHA-specific IgG antibody titres were also expressed as ELISA units (EU) ml^{-1} on the basis of a comparison of the response curve of the test serum to that of the reference mouse serum provided by the National Institute of Infectious Diseases, Tokyo, Japan (anti-PT IgG antibody: 886 EU ml^{-1} and anti-FHA IgG antibody: 21 900 EU ml^{-1}). This was done according to a modified version of the parallel line assay procedure described by Sato and Sato [30].

2.8. Intranasal challenge of Bordetella pertussis

Intranasal infection of *B. pertussis* 18 323 phase I was performed using mice immunized intranasally with DPT combination vaccine with and without rCTB according to the method described before [19] and three mice per group were used.

2.9. Statistics

Analysis of antibody titres was performed on logarithmically transformed data and the G.M. and S.D. values were calculated. Mann–Whitney's *U*-test was used to compare mean values of different groups with serum and mucosal antibody titres. Statistical significance was designated as $P < 0.01$ or $P < 0.05$.

3. Results

3.1. DTd-specific serum IgG antibody and diphtheria antitoxin responses: comparison among in-house ELISA, CCM, and KPA titres

We assessed the immune responses to DTd induced by intranasally delivered DPT combination vaccine. Mice immunized with DPT plus rCTB showed much higher DTd-specific serum IgG antibody titres by in-house ELISA than those without rCTB except Experiment 1, indicating the statistically significant adjuvant effect of rCTB in Experiments 2 and 3 (Fig. 1A).

Mice immunized with DPT plus rCTB showed the mean $\log_{10}$ diphtheria antitoxin titres ± S.D. values of 0.47 ± 0.36, 0.80 ± 0.37, and -0.21 ± 0.65 IU ml^{-1} in Experiments 1–3, respectively, which were measured by CCM (Fig. 1B). These titres were sufficiently greater than 0.01 ($\log_{10} - 2$) IU ml^{-1} of serum and significantly higher than those obtained from mice treated with DPT alone, clearly showing that rCTB specifically induces diphtheria antitoxin antibody even if in-house ELISA antibody titres are almost the same irrespective of rCTB (Fig. 1A, Experiment 1). Diphtheria antitoxin titres measured by KPA in Experiments 2 and 3 also showed significant differences between mice immunized with DPT in the presence and absence of rCTB (Fig. 1C).

3.2. TTd-specific serum IgG antibody and tetanus antitoxin responses: comparison among in-house ELISA, KPA, and TTN titres

The addition of rCTB to DPT combination vaccine induced sufficiently high serum anti-TTd IgG in-house ELISA antibody titres, which were significantly high only in Experiment 3 when compared with no addition of rCTB (Fig. 2A).

The mean $\log_{10}$ tetanus antitoxin titres ± S.D. values measured by KPA were 0.65 (pool sera), -1.75 ± 0.69 and -1.47 ± 0.96 in Experiments 1–3, respectively, in the absence of rCTB and 2.53 (pool sera), 0.89 ± 0.05 and 0.75 ± 0.31 in Experiments 1–3, respectively, in the presence of

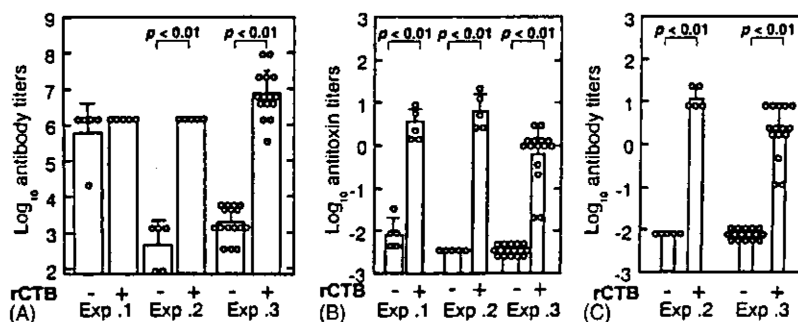

Fig. 1. Correlation among serum DTd-specific IgG in-house ELISA antibody titres (A), diphtheria antitoxin titres by CCM (B), and anti-diphtheria KPA titres (C) with and without rCTB. Mice were administered intranasally with 5 Lf DTd + 5 Lf TTd + 0.5 μg PTd + 5 μg fFHA ± 10 μg rCTB on days 0, 14, 21, and 28 and sacrificed on day 35. The same experiments were repeated three times but anti-diphtheria KPA titres were not measured in Experiment 1.

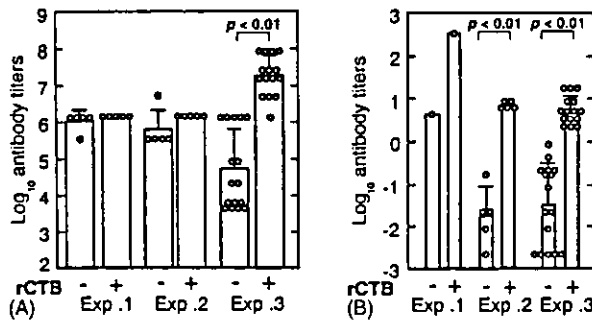

Fig. 2. Correlation between serum TTd-specific IgG in-house ELISA antibody titres (A) and anti-tetanus KPA titres (B) with and without rCTB. Mice were administered intranasally with 5 Lf DTd + 5 Lf TTd + 0.5 μg PTd + 5 μg fFHA ± 10 μg rCTB on days 0, 14, 21, and 28 and sacrificed on day 35. The same experiments were repeated three times. In Experiment 1 pooled sera from five mice were used to measure anti-tetanus KPA titres.

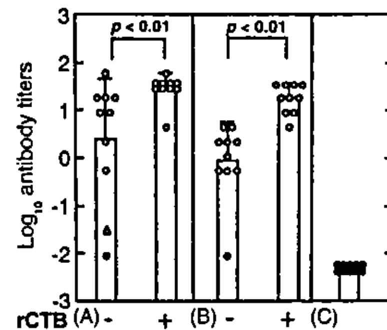

Fig. 3. Correlation between serum anti-tetanus KPA titres and life and death of mice in Experiment 4. Mice were administered intranasally with 5 Lf DTd + 5 Lf TTd + 0.5 μg PTd + 5 μg fFHA ± 10 μg rCTB (A) and with 5 Lf DTd + 5 Lf TTd + 0.5 μg PTd ± 10 μg rCTB (B) or administered intranasally with PBS (C) on days 0, 14, 21, and 28. Serum was collected from each mouse on day 35 and used for the measurement of anti-tetanus KPA titres. Pooled sera were used for TTN test. Tetanus toxin challenge test was performed on day 40 and observed through out the life and death of mice after 7 days. (○) Mice showing no symptoms, (△) paralyzed mouse, and (●) dead mice. Anti-diphtheria KPA titres were also examined in Experiment 4 but not shown because almost similar results to those of Fig. 1C were obtained.

rCTB (Fig. 2B). These tetanus antitoxin titres were significantly different between the presence and absence of rCTB unlike serum anti-TTd IgG in-house ELISA antibody titres. Subsequently, the toxin neutralization tests were carried out by six-fold dilution using the pool sera of mice obtained from Experiments 2 and 3. In Experiment 2 the TTN titres of pool sera from each five mice were less than 0.01 IU ml^{-1} without rCTB and 0.36–2.16 IU ml^{-1} with rCTB (Table 1). In Experiment 3, 15 or 16 mice were divided into two groups by KPA titres, i.e. into a higher and lower one. In mice immunized with DPT alone, TTN titres obtained from pool sera of 5 mice showing KPA titres less than 0.0025 unit ml^{-1}, and 11 mice showing KPA titres from 0.01 to 1.0 units ml^{-1} were less than 0.01 IU ml^{-1} (Table 1). On the other hand, in mice immunized with DPT in the presence of rCTB both higher group (5 mice) showing KPA titres between 8 and 16 unit ml^{-1} and lower one (10 mice) showing KPA titres between 2 and 4 unit ml^{-1} demonstrated TTN titres from 1.80 to 10.8 IU ml^{-1} (Table 1). Judging from these results, the addition of rCTB is essential to elevate tetanus antitoxin titres unlike anti-TTd antibody in-house ELISA titres.

Table 1
Correlation of serum anti-tetanus KPA titres with tetanus toxin neutralization (TTN) titres of pooled sera

Experiment number	rCTB	Number of mice	KPA titres (units ml^{-1})[a]	TTN titres (IU ml^{-1})[b]
Experiment 2	–	5	0.0025–0.16	<0.01
	+	5	6.4–8.0	0.36–2.16
Experiment 3	–	5	<0.0025	<0.01
	–	11	0.01–1.0	<0.01
	+	10	2.0–4.0	1.8–10.8
	+	5	8.0–16.0	1.8–10.8

[a] See Fig. 3B.
[b] Pooled sera were used and TTN tests were carried out by six-fold dilution.

3.3. Correlation between TTd-specific KPA and TTN titres

In Experiment 4, the correlation between TTd-specific KPA and TTN titres was investigated in detail in combination with DTd + TTd + PTd ± fFHA ± rCTB. As shown in Fig. 3, tetanus antibody titres measured by the KPA method with rCTB were significantly higher in comparison with those in the absence of rCTB irrespective of fFHA. Two of the 10 mice immunized with the mixture of DTd, TTd, PTd, and fFHA without rCTB showed very low anti-tetanus KPA titres, and one of them died on day 1 and the other one was paralyzed (Fig. 3A). Only one of the 10 mice immunized with DTd, TTd, and PTd in the absence of both fFHA and rCTB showed very low anti-tetanus KPA titre and died on day 1 (Fig. 3B). All mice inoculated with phosphate buffer saline died on day 1 (Fig. 3C). However, the addition of rCTB to DPT combination vaccine surely protected all mice against tetanic symptoms and deaths regardless of fFHA (Fig. 3A and B). TTN titres obtained from pooled sera of each ten mice immunized without rCTB were 2.77 IU ml^{-1} in the presence of fFHA and 0.36 IU ml^{-1} in the absence of fFHA. The addition of rCTB showed high TTN titres of 11.60 and 10.30 in the presence and absence of fFHA, respectively. Diphtheria antitoxin titres measured by CCM and KPA in the same samples were significantly higher in the presence of rCTB like Experiments 1–3 (data not shown).

Together with the results obtained from Experiments 1–3, these results made it clear that the addition of rCTB to DPT combination vaccine is necessary to protect against tetanus and diphtheria.

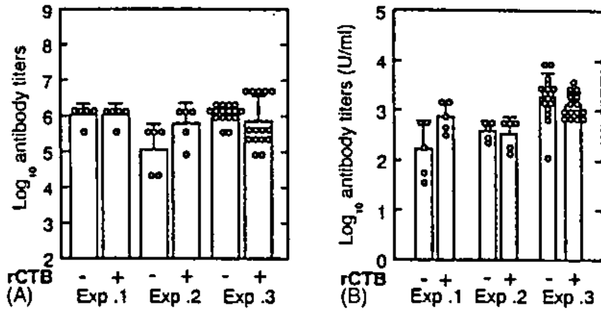

Fig. 4. Correlation between serum PT-specific antibody titres by in-house ELISA (A) and ELISA units by calibration against a reference mouse serum (B) with and without rCTB. Mice were administered intranasally with 5 Lf DTd + 5 Lf TTd + 0.5 μg PTd + 5 μg fFHA ± 10 μg rCTB on days 0, 14, 21, and 28 and sacrificed on day 35. The same experiments were repeated three times. Both titres were not measured in Experiment 4.

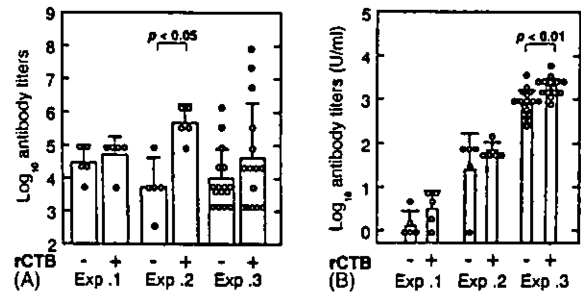

Fig. 5. Correlation between serum FHA-specific antibody titres by in-house ELISA (A) and ELISA units by calibration against a reference mouse serum (B) with and without rCTB. Mice were administered intranasally with 5 Lf DTd + 5 Lf TTd + 0.5 μg PTd + 5 μg fFHA ± 10 μg rCTB on days 0, 14, 21, and 28 and sacrificed on day 35. The same experiments were repeated three times. Both titres were not measured in Experiment 4.

3.4. Serum PT-specific IgG antibody titres by in-house ELISA and serum anti-PT ELISA units by calibration against a reference mouse serum

High levels of anti-PT serum IgG antibody titres by in-house ELISA were induced irrespective of rCTB through Experiments 1–3 and there were no significant differences in the presence and absence of rCTB (Fig. 4A). Serum anti-PT ELISA units from calibration against a reference mouse serum also increased and the presence and absence of rCTB showed no significant differences (Fig. 4B).

3.5. FHA-specific serum IgG antibody titres by in-house ELISA and serum anti-FHA ELISA units by calibration against a reference mouse serum

Serum FHA-specific IgG antibody titres by in-house ELISA increased substantially irrespective of rCTB and there was a significant difference in the presence and absence of rCTB only in Experiment 2 (Fig. 5A). Anti-FHA ELISA units obtained from calibration against a reference mouse serum were also elevated regardless of the presence or absence of rCTB and a significant increase with rCTB was observed only in Experiment 3 (Fig. 5B).

3.6. Protection from intranasal challenge of B. pertussis in mice intranasally administered with DPT combination vaccine with and without rCTB

The bacteria were not removed from the lungs of non-vaccinated control mice even on day 14 of infection. However, regardless of rCTB, intranasal administration with DPT combination vaccine showed complete clearance of the bacteria from the lungs by 6 days postinfection (data not shown).

3.7. DTd-, TTd-, PT-, and FHA-specific serum IgA and IgE antibody responses

Serum IgA antibodies specific to all components of DPT vaccine were produced independent of rCTB and anti-DTd, TTd, and FHA IgA antibody titres were significantly higher in the presence of rCTB (Fig. 6A). Serum IgE antibody responses to DTd, TTd, and PT were seen in the presence of rCTB and TTd- and PT-specific IgE antibody titres were significantly higher (Fig. 6B).

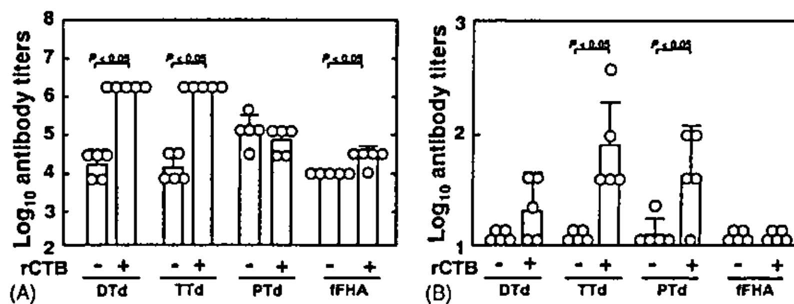

Fig. 6. Serum DTd-, TTd-, PT- and FHA-specific IgA (A) and IgE (B) antibody titres with and without rCTB. Mice were administered intranasally with 5 Lf DTd + 5 Lf TTd + 0.5 μg PTd + 5 μg fFHA ± 10 μg rCTB on days 0, 14, 21, and 28 and sacrificed on day 35. Both titres were measured only in Experiment 1.

3.8. Mucosal DTd-, TTd-, PT-, and FHA-specific IgA antibody responses

Mucosal antigen-specific IgA antibody titres were measured only in Experiment 1. Significantly higher levels of mucosal DTd-specific IgA antibody titres were observed in the lungs, small intestines, feces and the saliva and vaginal secretions in the presence of rCTB (data not shown), almost coinciding with the results obtained from intranasal immunization with DTd ± rCTB [18]. Mucosal TTd-specific IgA antibody titres rose significantly in the nasal cavities, lungs, small intestines, feces, and the saliva and vaginal secretions in the presence of rCTB (data not shown), being different from the previous results from intranasal immunization with TTd ± rCTB [17]. The difference between two data may be due to the presence of PTd. High or moderate levels of PT-specific mucosal IgA antibody responses were induced in the lungs and the vaginal secretions regardless of rCTB and mucosal anti-FHA IgA antibody titres in the presence of rCTB increased significantly in the lungs and small intestines but not in the other mucosal sites (data not shown). These results were roughly in agreement with the those from intranasal immunization with PTd + fFHA ± rCTB [19].

4. Discussion

In this study the mucosal adjuvant effect of rCTB on intranasal administration of DPT combination vaccine consisting of DTd, TTd, PTd, and fFHA was investigated and it was clearly shown that co-administration of rCTB is essential for induction of diphtheria and tetanus antitoxin antibodies. Moreover, all mice intranasallly administered with a mixture of DPT combination vaccine and rCTB were surely protected from challenge of TT and diphtheria antitoxin titres above a protective level of 0.1 $\mathrm{IU\,ml^{-1}}$, which is the smallest level necessary to protect humans from diphtheria, were induced. These results coincided with those from intranasal co-administration of rCTB and DTd or TTd except a little high serum IgE antibody titres [17,18]. Our previous studies showed that subcutaneous injection of aluminium-adjuvanted DTd elicited high levels of DTd-specific serum IgG, no or slight levels of DTd-specific serum and mucosal IgA and significantly high levels of DTd-specific serum IgE antibody responses [31], and moreover, caused severe histopathological reactions [20]. Accordingly, rCTB seems to be an excellent adjuvant. The mucosal adjuvant effect of rCTB for mixed PTd and fFHA is inconspicuous because of the mucosal adjuvanticity of PTd and induction of moderately high levels of serum PT-specific IgE antibody titres [19].

As far as we know about intranasal administration of DPT combination vaccine and a mucosal adjuvant in animal experiments, there is only one paper reported by Nagai et al. [32]. They used some onjisaponins, from the root of *Polygala tenuifolia* Willdenow, as a mucosal adjuvant and showed significant increases in serum anti-DTd, -TTd, -PTd IgG, and nasal IgA antibody titres. However, there were no data on diphtheria and tetanus antitoxin antibody and anti-DTd, -TTd, and -PTd serum IgE antibody titres.

It is generally known that mucosal IgA plays an important role in protection against infections by enteropathogens and viruses both in human and animal models [33–37]. The inhibition of bacterial adherence by mucosal IgA is considered to be one of the most important defense mechanisms against mucosal bacterial invasion and in vitro, has been shown to limit the attachment of bacteria to epitherial cells isolated from various mucosal sites [38–43]. Moreover, mucosal IgA can also neutralize toxins by blocking their binding to cell receptors [44]. On the other hand, a functional role for serum IgA is still unclear but the IgA, not the IgG, serum fraction, from patients with *Clostridium difficile* has been reported to neutralize the cytotoxic and enterotoxic properties of the major virulence factor, toxin A, of the bacterium [45]. In this study, rCTB significantly enhanced production of serum IgA antibodies and mucosal ones to DTd in the respiratory tract and the vagina. In our preliminary experiment, IgAs purified from three mouse sera with 13.0, 1.63, and 3.26 CCM $\mathrm{IU\,ml^{-1}}$, which were intranasally administered with DTd + rCTB, showed 0.102, 0.005, and 0.026 CCM $\mathrm{IU\,ml^{-1}}$, respectively, and mucosal IgAs purified from the pulmonary lavages of 4 mice intranasally immunized with DTd + rCTB, showed 0.006, 0.102, 0.026, and 0.026 CCM $\mathrm{IU\,ml^{-1}}$, suggesting the possibility of neutralizing diphtheria toxin of IgA. However, it has been suggested that IgA is not essential for preventing viral infection, reduction in the severity of disease, or both because of no differences between wild-type $\mathrm{IgA^{+/+}}$ and transgenic $\mathrm{IgA^{-/-}}$ knockout mice [46]. The role of systemic and mucosal IgA merits further research.

There have been several studies that have tracked the tissue distribution of a marker following intranasal delivery to mice but the results obtained from these studies have been varied [47–51]. Visweswaraiiah et al. [52] reported in detail that all parameters such as level of anesthesia, position of the animal during and post dye administration, dosing schedule and total volume administered are important for a solution delivered intranasally to be retained in the nasal cavity of a mouse. In case of a total volume of 30 μl, it is important to hold a mouse under heavy anesthesia and in a supine position both during and post dye administration and to administer according to the optimal schedule of administration, i.e. 2 μl per nare at 5 min intervals [52]. However, in our experiment a 15 μl bolus of vaccine per nare (a total volume of 30 μl) was delivered intranasally to moderately anesthetized mice held upright during administration and placed on its stomach in the cage post administration. Accordingly, a part of intranasally administered vaccine may have been swallowed or aspirated into the lungs.

Phase 1 safety studies conducted with intranasally administered rCTB and LTR192G revealed only minor side effects including self-limiting increased nasal secretions, itching,

runny nose and sneezing within a certain amount of them but not visible effects on the nasal mucosa, systemic adverse events and long-term adverse events [53]. Clinical trials with an inactivated, virosome-formulated, LT-adjuvanted, intranasal subunit influenza vaccine showed solicited and unsolicited symptoms such as nasal discomfort, sneezing, nasal pain, stuffy nose, runny nose, shivering, malaise, and so on. Moreover, Bell's Palsy (facial paresis) was observed in a tiny minority [53]. However, a detailed analysis of the observed cases has not revealed a distinct pattern for an influenza vaccine-induced facial paresis. Pre-clinical studies in animal models are needed to evaluate the safety of intranasally administered CT and LT adjuvants, and derivatives prior to the initiation of clinical trials. Intranasal administration of rCTB to BALB/c mice caused no distinct local histopathological reactions in the nasal cavity [20]. If rCTB is confirmed to be completely non-toxic for humans, an intranasal DPT combination vaccine with rCTB could be easily licensed for human use. The needle-free intranasal administration is an attractive alternative procedure, especially in developing countries.

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