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Multi-center study on the simultaneous administration of DPT-IPV and Hib PRP-T vaccines

Part 1. Immunogenicity

J. Labadie, L.C. Sundermann, H.C. Rümke
and the DPT-IPV ~ Hib vaccine study group*

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* **DPT-IPV~Hib vaccine study group:**
G.A.M.Berbers, P.E.Vermeer-de Bondt, A.D. Plantinga, M.H.J. de Bruijne, J.F.P.Schellekens, N.Elzinga-Gholizadea, C.C.A.M. Peeters, J.Westdijk, D.J.A.Bolscher, E.de Natris, M.Wala, R.E.Juttmann, R.de Groot.

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RIVM (National Institute of Public Health and the Environment)
PO Box 1, 3720 BA Bilthoven, The Netherlands
tel +31 30 274 2202, fax +31 30 274 4430

Mailing list

1	Hoofdinspecteur Preventieve en Curatieve Gezondheidszorg
2	Directeur-Generaal Volksgezondheid
3	Inspectie Gezondheidszorg, Inspecteur Infectieziekten
4	Gezondheidsraad, Den Haag
5	Medisch Ethische Commissie AZR/EUR, Rotterdam
6-9	Dr R.de Groot
10-13	D.J.A.Bolscher
14-17	Stichting Thuiszorg Rotterdam
18-21	GGD Rotterdam en omstreken
22-25	Capelse Kruisvereniging
26-29	Districts Kruisvereniging Oost-Veluwe
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47-48	Hoofd KRZ
49	Hoofd CIE
50	Hoofd LIS
51	Hoofd LIO
52	Dr J.F.P.Schellekens
53	Dr N. Elzinga-Gholizadea
54	Dr C.C.A.M. Peeters
55	M.H.J. de Bruijne
56	J. Westdijk
57	Dr M.A.E Conyn-van Spaendonck
58	H.E. de Melker
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Participating organisations and investigators

1. ROTTERDAM / CAPELLE AAN DE IJSSEL

- Sophia Kinderziekenhuis
R.de Groot MD PhD, pediatrician, principal investigator

- Stichting Thuiszorg Rotterdam
R.E.Juttmann MD, M.Wala MD, child health consultants

- GGD Rotterdam
mr.M.E.van den Akker-Tuijl, head section infectious disease
G.Oskamp, head immunisation administration
C.F.Engelhard MD

- Capelse Kruisvereniging
E.de Natris MD, child health consultant

- Provinciale Entadministratie Zuid-Holland
W.Hessing, head immunisation administration
R.S.Boeken Kruger-Mangunkusumo MD, pediatrician,
medical advisor immunisation administration

2. APELDOORN

- Districts Kruisvereniging Oost-Veluwe,
D.J.A.Bolscher MD, child health consultant, principal investigator

- Stichting Provinciale Entadministratie Gelderland
C.Verhaaff, head immunisation administration

3. BILTHOVEN

- LVO: Laboratory for Clinical Vaccine Research
- LPO: Laboratory for Product and Process Development
- LBA: Laboratory for Bacteriology and Antimicrobials
- LCB: Laboratory for Control of Biological products
- LVM: Laboratory of Vaccine Development and Immune Mechanisms

ABBREVIATIONS *AFKORTINGEN*

AEQ	adverse event questionnaire
°C	degrees centrigrade
CB	baby clinic *(consultatiebureau)*
CKV	Capelse Kruisvereniging
CRF	Case Report Form
D-ag	poliovirus D-antigen units
DKV	Districts Kruisvereniging Oost-Veluwe
DPT-IPV	Diphtheria, Pertussis(-whole cell), Tetanus and Inactivated Poliovirus Vaccine *(Difterie, Kinkhoest, Tetanus, Polio (DKTP) vaccin)*
GGD Rotterdam	Municipal Public Health Service Rotterdam
Hib	Haemophilus influenzae type b
IU	International Units
IOU	International Opacity Units
KRZ	Bureau for Quality and Regulatory Affairs *(Bureau Kwaliteits en Registratiezaken RIVM)*
LBA	Laboratory for Bacteriology and Antimicrobials *(Laboratorium voor Bacteriologie en Antimicrobiële Middelen)*
LCB	Laboratory for Control of Biological products *(Laboratorium voor Controle Biologische Produkten)*
Lf	Flocculation units *(Limes flocculationes)*
LPO	Laboratory for Product and Process Development *(Laboratorium voor Produkt- en Procesontwikkeling)*
LVM	Laboratory of Vaccine Development and Immune Mechanisms *(Laboratorium voor Vaccinontwikkeling en Immuniteits Mechanismen)*
LVO	Laboratory for Clinical Vaccine Research *(Laboratorium voor Veldonderzoek vaccins)*
LVO-BI	LVO Bio- and Immunochemistry section *(LVO afdeling bio- en immunochemie)*
MIT	minimum immune titre
PEA	Immunisation Administration *(Provinciale Entadministratie)*
PRP	Polyribosyl-ribitol-phosphate
PRP-T	PRP conjugated to Tetanustoxoïd
RIVM	National Institute of Public Health and Environment *(Rijksinstituut voor Volksgezondheid en Milieu)*
RVP	National Childhood Immunisation Programme *(Rijksvaccinatieprogamma)*
SKZ	Sophia Kinderziekenhuis/Academisch Ziekenhuis Rotterdam
STR	Stichting Thuiszorg Rotterdam
UTN	Unique Trial Number

ABSTRACT

The present report gives results of a randomised multi-center clinical study into the antibody formation following two vaccination regimens with simultaneous administration of DPT-IPV and Hib PRP-T vaccines. The study was done in 543 infants, recruited from 42 baby clinics in Apeldoorn, Capelle and Rotterdam.
The antibody responses to all vaccine antigens were considered sufficient for protection on both study groups. A moderate but statistically significant interference was found with regard to tetanus antibodies, being lower in children who received combined injections than in children who received these separate injections.
This negative interference should be interpreted in the light of the advantages of the combined administration of these important vaccines reducing the number of injections.

SUMMARY

Background

In the Netherlands routine Hib vaccination was introduced in 1993.
Hib PRP-T vaccine was given at age 3-4-5-11 months together with quadruple DPT-IPV vaccine, by separate injections. We compared antibody formation to vaccine antigens after these vaccinations with separate and combined injections.

Methods

A controlled, randomised multi-centre study was done in Rotterdam, Capelle and Apeldoorn. In total, 543 infants were recruited from preventive health care clinics. The trial closed with 474 evaluable children. DPT-IPV and Hib were given to 181 infants at different injection sites (group B), 180 infants received these vaccines in one syringe mixed just before injection (group C), and as a control 182 children (group A) were given routine DPT-IPV alone, followed by later Hib vaccinations (6-7-13 months). Blood was taken at 3-6-11 and 12/14 months.

Results

The anti-Hib-PRP response was high and at similar levels in groups B and C. After 3 vaccinations, 160/163 vs 159/163 had >0.15 μg/ml anti-PRP antibodies, and 140 vs 141 >1.0 μg/ml, with GMT's of 2.60 vs 2.12 μg/ml. Group A had low anti-PRP levels at that age (before their Hib vaccination).
The responses to diphtheria, pertussis and polio antigens were similar in the three groups. The anti-tetanus response in group C was significantly lower than in group B. After 3 vaccinations 99% of 158 of the group B children had antibodies >0.1 IU/ml with a GMT of 1.30 IU/ml, vs 97% of 157, with GMT of 0.88 IU/ml in group C. Similar differences were found before the 4th dose: 96 vs 89% with titre >0.1 IU/ml, with GMT 0.66 and 0.37 IU/ml respectively, but at both occasions all children had antibodies above the protective level of 0.01 IU/ml.

Discussion

The vaccination regimens studied with DPT-IPV and Hib vaccine induced comparable antibody responses to all vaccine antigens except tetanus toxoid. The clinical relevance of this moderate interference will be discussed, in r lation to the advantages of the reduced number of injections.

SAMENVATTING

Achtergrond

Routinevaccinatie tegen Hib infecties zijn in Nederland ingevoerd in 1993. Hierbij wordt Hib PRP-T vaccin gegeven aan zuigelingen op de leeftijden van 3-4-5-11 maanden, tegelijk met DKTP vaccin. Beide vaccins worden via aparte injecties toegediend. In de huidige studie werd de antistofvorming tegen vaccin antigenen onderzocht, in toedieningsschema's met aparte injecties ten opzichte van gecombineerde injecties.

Methode

In Rotterdam, Capelle en Apeldoorn werd een gecontroleerde, gerandomiseerde studie uitgevoerd. Een totaal van 543 evalueerbare kinderen werden gerecruteerd uit de populatie die voor de reguliere preventieve zorg de Consultatiebureaus bezochten. De studie sloot af met 474 evalueerbare kinderen. DKTP en Hib vaccins werden op verschillende injectieplaatsen gegeven aan 181 kinderen (groep B), aan 180 kinderen via één injectie waarbij de vaccins kort tevoren gemengd werden (groep C), en aan 182 kinderen die eerst DKTP vaccin kregen, en later (op 6-7-13 maanden) Hib vaccin (groep A). Bloedmonsters werden onderzocht op antistoffen op 3-6-11 en 12/14 maanden.

Resultaten

In groep B en C was de Hib antiPRP respons hoog en op vergelijkbaar niveau. Na 3 vaccinaties hadden 160/163 vs 159/163 van de kinderen een anti-PRP antistofniveau >0.15 μg/ml, en 140 vs 141 een niveau >1.0 μg/ml, met GMT waarden van 2.60 vs 2.12 μg/ml. In groep A werden toen lage anti-PRP waarden gevonden (voor vaccinatie in deze groep). De antistof respons tegen difterie, kinkhoest en poliovirus antigenen waren vergelijkbaar in de drie studiegroepen. De anti-tetanus respons in groep C was significant lager dan in groep B. Na 3 vaccinaties had 99% van 158 kinderen in groep B antistoffen >0.1 IU/ml met een GMT van 1.30 IU/ml, vs 97% van 157 kinderen, met GMT van 0.88 IU/ml in groep C. Vergelijkbare verschillen werden gevonden voor de 4e vaccinatie: 96 vs 89% met een titer >0.1 IU/ml, met GMT waarden van 0.66 en 0.37 IU/ml resp, maar in beide bloedafnames hadden alle kinderen antistofniveaus boven het beschermende niveau van 0.01 IU/ml.

Discussie

De vaccinatie schema's met verschillende toedieningsvormen van DKTP en Hib PRP-T vaccin induceren vergelijkbare antistofniveaus tegen de difterie, kinkhoest, poliovirus en Hib antigenen. De respons op tetanus was lager in de groep kinderen die beide vaccinaties gecombineerd in één injectie kreeg toegediend, ten opzichte van aparte injecties. De klinische betekenis van deze lagere anti-tetanus respons moet worden afgewogen tegen de voordelen van het geven van minder injecties.

1 INTRODUCTION

In the Netherlands, before vaccination against Haemophilus influenzae type B (Hib) invasive Hib disease (meningitis, septicaemia, epiglottitis, pneumonia, otitis, arthritis, cellulitis) predominantly occurred in children in their first 4 years of life (1). In this age group Hib caused approximately 45% of bacterial meningitis cases, resulting in death in about 2% (5-10 children each year) and serious persistent neurologic sequelae (hearing loss, seizures, paralysis or mental retardation) in about 9% (20-30 children each year). In the Netherlands the peak incidence of Hib-meningitis was at the age of 7-9 months (2,3).

Protection against Hib disease is mediated by antibodies against polyribosyl-ribitol phosphate (PRP), the Hib capsular polysaccharide. Induction of protective antibodies by immunisation in young infants only became possible with the development of conjugated vaccines in which PRP was coupled to a protein carrier. Several conjugated Hib-vaccines with different protein carriers are licensed in many countries (4,5).

In 1991 the National Health Council advised to include Hib-vaccination in the national childhood immunisation program (RVP)(6,7). The PRP-T vaccine (in which the carrier protein is tetanus toxoid) produced by Pasteur Mérieux Serums et Vaccins (France) was chosen. This vaccine is registered and used in national childhood immunisation programs in several European countries. Simultaneous administration with the RIVM DPT-IPV vaccine at the ages of 3, 4, 5 and 11 months seemed feasible. However in a clinical trial in Chile interference of PRP-T with simultaneously administered DPT-vaccine resulted in a reduced pertussis antibody response (8,9). This led the Medicines Evaluation Board in the Netherlands to advise against simultaneous administration of the RIVM DPT-IPV vaccine with the PRP-T vaccine, and to observe an interval of 2 weeks between administration. Compliance with this recommendation on the PRP-T package insert would seriously impede effective introduction of the Hib-vaccine in the RVP and reduce the acceptance of the immunisation program. Therefore the 'Beraadsgroep Infectie en Immuniteit' of the Netherlands Health Council advised to administer both vaccines (DPT-IPV and PRP-T) simultaneously but at separate injection sites when used in the RVP. All children born after April 1st 1993 were offered simultaneous but separate administration of the PRP-T and DPT-IPV vaccine in the RVP. Acceptance of the RVP has not been reduced since and preliminary evaluation shows a marked reduction in invasive Hib disease (10,11).
The RIVM was asked to evaluate the serological effects of this strategy, the main reason for this study. A second question was to evaluate the effects of mixed administration (DPT-IPV used to reconstitute the lyophilised PRP-T) which could increase acceptance of the immunisation program by reducing the number of injections in the RVP.

2 MATERIALS AND METHODS

The study protocol "Multi center studie naar immunogeniteit en bijwerkingen van gecombineerde toediening van DKTP en Hib PRP-T vaccin" was approved by the institutional Ethics Review Board of the Sophia Children's hospital and the University hospital in Rotterdam.

2.1 Vaccines

The vaccines under study are
1. DPT-IPV (RIVM) lotnrs 165 and 167 of which one dose (1 ml) contains: diphtheria toxoid 15 Lf, whole cell pertussis 16 IOU, tetanus toxoid 5 Lf, inactivated polioviruses (type 1: 40; type 2: 4; type 3: 7,5 D-Ag units, respectively), aluminumphosphate 1,5 mg, 2-fenoxyethanol 5 mg, formaldehyde 0,025 mg, water for injection.
2. Hib PRP-T (Pasteur Mérieux Serums et vaccines) lotnr H0961 of which one dose (0,5 ml) contains: lyophilised PRP polysaccharide 10 microgram conjugated to 25 Lf tetanus protein for reconstitution in 0.4% saline.
These vaccine lots were not reserved for exclusive use in this study, but also distributed across the country according to regular RVP-channels. However the PEA's responsible for the vaccine supply for the participating 'Thuiszorg' (Homecare)-organisations provided for sufficient doses to accommodate all enrolled participants in each organisation. The vaccines were stored at the study sites at 4-8 °C throughout the study. Storage temperatures were recorded on temperature logs.

2.2 Study sites

The 'Thuiszorg' organisations in Apeldoorn, Capelle ad IJssel and Rotterdam selected baby clinics (CB) (and their staff) to participate in this study:

Districts Kruisvereniging Oost-Veluwe, Apeldoorn	5 CBs
Capelse Kruisvereniging, Capelle aan de IJssel	5 CBs
Stichting Thuiszorg Rotterdam, Rotterdam	32 CBs

The latter two organisations will be referred to as study site Rotterdam, the former organisation as study site Apeldoorn.
Children visited their CB for their regularly scheduled RVP immunisation visits and were attended to by the regular CB-staff. Blood was sampled by venipuncture by physicians or trained research nurses at the child's home or at the CB.
The CRF's were stored in the regular CB patient file and were accessible to the CB staff who regularly have access to the patient files.

2.3 Participants

Children born in February and March of 1993, living in the area where preventive infant care was provided by the participating organisations, were invited to participate in the

study by direct mailing from the PEA's. During the subsequent visit to the CB additional information was given to the parents, both in writing (in Dutch and in 5 additional languages: Arabic, Chinese, English, Portuguese, Turkish) as well as orally and parents questions were addressed. After evaluation of inclusion and exclusion criteria and signing of an informed consent form by the parents, a child was enrolled by assigning an Unique Trial Number (UTN) and randomisation to be vaccinated according to one of the 3 immunisation groups. Participants were randomised by a computer generated list, assigning them by UTN to one of the three study groups. Unique Trial Numbers were assigned in order of enrolment. The numbers 74-98 and 200-400 were reserved for participants in Apeldoorn.

The study forms: CRF (Case Report Form) and AEQ (Adverse Event Questionnaire) appropriate for the assigned immunisation group were forwarded to the CB-physician to be kept in the patient files of the child at the study site.

2.4 Study design and procedures

It had been reported prior to this study that concurrent or mixed administration of DPT and Hib PRP-T could influence the response to pertussis antigens (7,8). This interference was shown only after the primary series ("the first 3") of childhood immunisations. In the present study it was decided to address interference only after the primary series of vaccinations at 3, 4 and 5 months of ages. Three study groups were formed, two of which were given both DPT-IPV and Hib PRP-T vaccine at the same time, either by separate injections at different injection sites (group B), of mixed together in one syringe just before injection (group C). The control group of children (group A) was given DPT-IPV vaccine only, while Hib vaccine was given at the earliest occasion following the primary series according to a regimen appropriate for age (at 6, 7 and 13 months). Because of this vaccination regimen protection against Hib-infections could be effective in group A at a later age, leaving a longer window period of susceptibility for them, than for participants in groups B and C. However the participants in group A did not fulfil the requirements to receive the Hib PRP-T vaccine free of charge in the course of the regular RVP, whereas they did by participating in this study.
In each study group the antibody responses to relevant vaccine components and the occurrence and severity of adverse events were assessed.

2.4.1 Study size calculation

A statistical power calculation was made using table 26.1 nr 4 in "Essentials of Medical Statistics" Betty R. Kirkwood (Blackwood Scientific Publications) and based on results of prior studies. With 100 participating infants in each immunisation group the following differences can be detected with a power of 80% and 95% confidence interval (two-sided): pertussis agglutinin antibodies: 85%, anti-diphtheria toxoid antibodies: 75%, anti-tetanus toxoid antibodies: 65%, and poliovirus neutralising antibodies: 20% difference.
Based on the annual birth rates in the areas where preventive infant care was provided by the participating organisations it was calculated that during the enrolment period of two

months a maximum number of 1500 infants could be enrolled. Previous experience with field trials showed that an initial acceptance of less than 40% could be expected, resulting in 600 participants at most. This would allow for a drop-out of less than 50% to retain a number of 100 participants in each immunisation group (in accordance with the power calculation).

2.4.2 Study design by immunisation group

age	3	4	5	6	7	8	9	10	11	12	13	14 months
group A	**D**	**D**	**D**						**D**			M
				H	**H**						**H**	
	b			b					b			b
	q	q	q	q	q				q		q	q
group B	**D**	**D**	**D**						**D**			M
	H	**H**	**H**						**H**			
	b			b					b	b		
	q	q	q						q			
group C	**DH**	**DH**	**DH**						**DH**			M
	b			b					b	b		
	q	q	q						q			

D,H,M : immunisation with DPT-IPV, Hib PRP-T, MMR vaccine respectively
b : blood sampling by venipuncture
q : adverse event questionnaire

Group A: control group.
DPT-IPV was given following the 3-4-5-11 months schedule, Hib PRP-T vaccine was given at the age of 6, 7 and 13 months.

Group B: separate injections group, according to RVP, designated as **'separate'** group
DPT-IPV and Hib PRP-T were given simultaneously, but at separate injection sites (opposite extremity).

Group C: combined injection group, designated as **'combined'** group
DPT-IPV and Hib PRP-T vaccines were given mixed in the same syringe by using the DPT-IPV vaccine in stead of the regular reconstitution fluid to reconstitute the lyophilised Hib PRP-T vaccine.

Children in group A received 7 vaccine injections, children in group B received 8 vaccine injections and children in group C received 4 vaccine injections.
The MMR vaccination at 14 months was not part of the study. However, final blood sampling and filling of AEQ was done at the same occasion.

2.4.3 Blood sampling and storage

Blood was sampled by venipuncture for serological investigation.
Blood samples from Apeldoorn were sent to the RIVM in Bilthoven by regular mail. Upon arrival serum was separated and frozen at -20°C until antibody testing.
In Rotterdam and Capelle, blood samples were taken at home, and kept at ambient temperature. At the SKZ, plasma was separated from heparinized blood and stored frozen at -70°C until transport to the RIVM in Bilthoven for antibody testing. At the RIVM-LVO immunobiochemistry section (LVO-BI) the samples were kept frozen and stored at -20°C until distribution to the laboratories which were to perform specific antibody measurements.

2.4.4 Injections

Both the DPT-IPV and the Hib PRP-T vaccine were administered by intramuscular injection in the thigh (vastus lateralis muscle) or in the upper arm (deltoid muscle) depending on CB-physician or district nurse preference, but according to standard CB practice. The vaccine lot number, date and time of injection were registered in the CRF. Also the site of vaccine administration was registered in the CRF to be able to link local reactions at the injection site to one specific vaccine in case of simultaneous but separate administration of the two vaccines.

2.5 Antibody tests

Blood specimens were stored at -20°C at LVO-BI until distribution of aliquots to several RIVM laboratories for blinded specific antibody measurements. Consequently, tubes with blood specimens were marked with a code which did not reveal the immunisation group. Due to the limited yield of the venipunctures in the small infants participating in this study in some cases, the following order of priority for antibody testing was decided upon:

order	antibody test	RIVM laboratory
1	Hib PRP-antibodies	LVM
2	Pertussis agglutinin antibodies	LPO
3	IgG to pertussis toxin	LBA
3	IgA to ultrasonicated B.pertussis	LBA
4	Poliovirus neutralising antibodies	LCB
5	Diphtheria toxoid specific antibodies	LPO
5	Tetanus toxoid specific antibodies	LPO

Immunogenicity of the Hib PRP-T in the Dutch infant study population needed assessment at the moment of introduction in the RVP. The above order of precedence was decided upon the reported experience on interference, notably with pertussis specific antibody-responses to a primary series of immunisations (Chile). At that time, there were no reports on interference from studies in which an IPV-containing combined vaccine was used.

2.5.1 Hib anti-PRP antibodies

Antibodies directed against Haemophilus influenzae type b capsular polysaccharides are detected by the HbO-HA Elisa, and expressed in µg/ml. The tests were done according to SOP LVM/M1100. These methods have been described by Kaythy (12).
The concentration of specific antibodies in two-fold dilutions of the (inactivated) blood samples can be determined by comparing the optical densities of a series of dilutions of the test sample to those of a reference serum (US standard lot 1983, Center for Biological Evaluation and Review, Food and Drug Administration, Bethesda, USA).

2.5.2 Pertussis antibodies

Pertussis Agglutinating antibodies were determined according to SOP LPO/14C-29
The method of this test has been described by Manclark (13).
Pertussis toxin specific IgG antibodies were determined by Elisa according to SOP LBA/M-205. Results are expressed in arbitrary units, a percentage of the concentration of a RIVM standard serum. The purified Pertussis toxin used for coating was prepared according to SOP LBV/13C-PER-03.
IgA antibodies against Bordetella pertussis were determined by Elisa using sonicated whole cells of 3 different Bordetella pertussis isolates for coating according to SOP LBA/M-205. Results are expressed in arbitrary units, a percentage of the concentration of a RIVM standard serum. The antigens for coating were prepared according to SOP LBV/13C-PER-01.
The methods for both tests have been described by Nagel et al. (14,15).

2.5.3 Poliovirus antibodies

Neutralising antibodies against poliovirus strain 1, 2 and 3 were titrated by micro-neutralisation assay according to SOP 17C-IPV11-WP1/2, using a twofold dilution range of the serum samples: 1-12 (1/2 - 1/4096). The results are expressed as 2 log reciprocal titres, e.g. $1/32 = 2^{-5}$ is expressed as 5. In short, inhibition of viral growth by the test serum is compared to a reference serum with a known neutralising antibody titre by assessment of the cytopathogenic effect of virus replication in VERO-cells. The test has been described by Albrecht (16).

2.5.4 Diphtheria and tetanus antibodies

Anti diphtheria and tetanus antibodies were determined by the toxin-binding inhibition (ToBi) test according to the SOP LPO 14C-23 as described elsewhere (17). This test is based on the inhibition of the binding of the toxin to antitoxin-coated ELISA plates by preincubation of toxin with two-fold dilutions of the samples.

2.6 Adverse events evaluation

The observations of adverse events following the administration of vaccines in this study will be reported separately.

2.7. Data handling and validation

All data of participants relevant for the study have been recorded on Case Report Forms (CRF) and listings of results from antibody measurements. Antibody titres were obtained later, but have been regarded as an integral part of the final CRF. Clinical and serological data have been entered in a LVO database, for storage and analysis.

Operational data, such as dates of vaccinations and blood samplings were entered in a computerised system by the research team in Rotterdam, in addition to UTN and treatment group.

Results of the antibody titrations have been handed over to LVO, either on forms or on diskettes, after a formal acceptation of the integrity and plausibility of the data. Written antibody results have been entered in a computer by a company, specialised in data entry (UPC, Nieuwegein). Results on diskettes were transformed to fit in the LVO database, the 'Serologie Informatie Systeem (SIS)', an in house developed run-time application of Dbase IV® as a system for storage of serological results (18). For further statistical analysis the data have been imported in a Microsoft Access database. After each step, checks were made to ensure that the correct data were used for final reporting.

2.7.1 Study monitoring

During the clinical stages of the study, monitoring visits were made to approximately 10% of all study sites (CB's) by a physician-monitor assigned by the RIVM to guard protocol adherence. Study progress at each of the two study sites was reviewed on a regular basis in meetings with executives of the participating organisations.

2.7.2 Study Audit

A RIVM-KRZ GCP-audit was done on September 2 nd 1994 at the end of the clinical stages of the study, regarding study facilities of the principal investigator at the SKZ in Rotterdam and the RIVM study monitoring activities.

2.8 Data editing

2.8.1 Default choices for serological values beyond measuring range

To facilitate statistical analysis, serological values under (<) or exceeding (>) the measuring ranges were attributed a default value according to the table below.

antibody test	serology value	default choice
anti Hib PRP antibodies	< 0.03	0.001 μg/ml
pertussis agglutination	< 2	1.00 (titre)
	> 5120	5500
pertussis IgA	<	1.00 (%)
pertussis IgG	<	1.00 (%)
poliovirus neutralisation	<	1 (2^n)
	>	13
anti D and T antibodies	< 0.01	0.01 IU/ml
	>	50.00 IU/ml

2.8.2 Protocol adherence

After entering all data in a computer database, a final assessment of protocol adherence was done. All data from a child have been excluded from analysis in the following situations:
- children with missing records of a correct intake procedure, including the informed consent.
- children with doubtful fulfilment of inclusion and exclusion criteria, specified in the study protocol.
- children with missing data forms, or apparently faulty filled forms.
- children with more extended vaccination intervals than specified in the study protocol: longer than 18 weeks between the first and the third DPT-IPV dose, and/or more than 57 weeks between the first and the fourth dose of DPT-IPV.

In case of the situations listed below children were excluded from analysis from the moment that the protocol violation had occurred.
- children that did not adhere to the study regimen, i.e. switched from one group to another.
- children that did receive vaccines other than the lots specified in the study protocol.

In a number of cases the study protocol had not been followed strictly to the letter. Formally these children had to be excluded. This situation was discussed with the RIVM Bureau for Quality and Regulatory Affairs (KRZ), and it was decided that these children could be included in the final data set for analysis, after careful evaluation that by inclusion there would be no selection for one of the study groups (as compared with the strict settings in the study protocol). This inclusion was done in the situations listed below:
- children born in January 1993 were included. The protocol restricted the inclusion to children born in February and March 1993 only.
- children with blood samplings and vaccinations outside the narrow intervals specified in the protocol.

2.9 Statistical analysis

The aim of the study was:
1. to assess the immunogenicity and adverse events following the newly introduced simultaneous vaccination with DPT-IPV and Hib-PRP-T in the National Immunisation Programme.
2. to study the possibility of combined administration of both vaccines, mixed in one syringe just before injection.
3. to investigate whether interference between vaccine antigens occurred, such as reported before in Chile (regarding pertussis antigens) and later in Israel and Canada (with regard to tetanus toxoid).

The analysis consists therefore of a descriptive and an analytic part. The antibody results are given in the tables, the description in the (a.) part, and the analysis in the (b.) part of these tables.

The data obtained are described by individual line listings of the serological results per UTN and order of blood sample. This list is not included in this report. In this report the antibody titres are described per antigen by study group and by order of blood sample.

For description of data, Epi Info 6.02® software was used (19). The distribution of antibody levels over a range of concentrations, including the MIT values, as well as the median concentrations are given in the table (a.) section of this report. These descriptive data are relevant to assess the consequences of the results of this study for protection against disease, especially if MIT values are known. This is of particular importance for the evaluation of the simultaneous vaccine administration, as chosen in the National Immunisation Programme.

The analytical part is done to answer the two other questions for which the study was initiated. Serological data from children in group A were used as controls for group B and

C data up till the second blood sampling. Thereafter the study regimen from group A was so much different from group B and C (because a separate Hib vaccination was introduced in group A) that group A could no longer serve as a pure control for groups B and C. Because of this a direct comparison would be more complicated, especially with regard to Hib and tetanus antibodies. However, adverse events (which will be reported separately) could be evaluated in children from group A also after the 4th DPT-IPV, providing useful control data for the group B and C children that received DPT-IPV and Hib at the same time.

For analysis the software programme SAS 6.08 was used. The full rationale of this analysis is described by De Bruijne (20). In brief, analysis of variance was done to maintain maximum power for comparison of potentially different study groups. In a pre-analysis, a difference between antibody levels to several antigens obtained in children studied in Apeldoorn versus the 'Rotterdam cluster' was found. This may be due to several factors, yet unidentified. The analytic method used for comparison of the vaccination groups was therefore balanced for the contribution of data obtained from each of the study sites. By this approach mean antibody levels are estimated and expressed as LS mean values. Results on antibody responses to Hib, pertussis IgG and IgA, and the diphtheria and tetanus toxoids are processed after logarithmic transformation of the antibody values. The LS mean values are obtained by backtransformation of the logaritmically expressed results.

3 RESULTS

3.1 Study population

A total of 635 children were enrolled in the study (table 1). These children have been recruited from an approximated total population of 1350 in the Rotterdam cluster and 160 in Apeldoorn.
For the analysis of the antibody responses, samples were excluded according to the criteria mentioned in paragraph 2.8.2. "protocol adherence", leaving 543 children evaluable at the onset of the study, and 475 (87% of 543; 75% of 635) at the last blood sampling.
The study populations at the two study sites were comparable as to male/female distribution and allocation of vaccination groups (table 2). There were 300 male and 243 female participants (ratio: 1.23). The evaluable numbers of participants per group, blood sampling and study site are given in table 3.

3.2 Antibody response

The serological response observed in all groups is described in the (a) section of the tables. Distribution of antibody values over several levels as well as median values are given. In this study, the levels listed below are considered as discriminating levels of a certain degree of protection against clinical infection. For pertussis antibodies such minimum immune titre (MIT) is not known.

Hib	: 0.15 and 1.0 μg/ml
Pertussis aggl.	: MIT not known
Pertussis IgA	: MIT not known
Pertussis IgG.	: MIT not known
Polio	: 3 (1/8) neutralising titre
Diphtheria	: 0.01 and 0.1 IU/ml
Tetanus	: 0.01 and 0.1 IU/ml

In the literature diphtheria and tetanus antitoxin titres ≥ 0.01 IU/ml are often considered to be protective, although clinical infections in individuals with these titres have been reported. Because in our opinion higher minimum immune titres (0.1 IU/ml) give a more reliable indication of protection against disease, we prefer this higher value.
With regard to Hib antibodies, in the literature two values of minimum immune anti-PRP titres are accepted (and often concurrently reported) to estimate protection against invasive Hib disease: 0.15 μg/ml is considered indicative of immediate, short term protection and 1.0 μg/ml is considered indicative of long term protection.

In the analytic section (b) of the tables, only the results from the first two blood samples obtained from participants in group A were used as a control for the DPT-IPV titres in the other 2 immunisation groups. The results are given as LS mean titres, as estimates for the true geometric mean titre. These values are mainly used for comparison of the three treatment groups. For description of the data, we prefer the results of the (a) sections.

3.2.1. Antibody response to Hib PRP-T (Table 4)

In both study sites pre immunisation median antibody values of 0.08-0.09 μg/ml are comparable for the three immunisation groups; 32% - 35% of the children had antibody levels ≥ 0.15 μg/ml. One month after completion of the primary vaccination series and one month after the booster vaccination there is no statistically significant difference between groups B and C. As expected, there is a clear difference with the unvaccinated control group A, in which the antibody levels are decreased in second blood sample. After vaccination at 6, 7 and 13 months of age the control children have very high antibody levels. These levels are higher than found in groups B and C, but acquired at an older (immunologically more mature) age. The point estimates for antibody levels in group C were just lower than in group B, although there was no significant difference.

In group B treated according to the current immunisation schedule in the RVP, after the 4th dose of separately given DPT-IPV and Hib vaccines, all of 156 children had antibody levels above 0.15 μg/ml, and 98% over 1.0 μg/ml. In group C, these values were 99% and 96%, respectively.

3.2.2. Antibody response to pertussis antigens (Table 5)

In none of the evaluated antibody responses (pertussis agglutinin antibodies, pertussis IgA, pertussis IgG-anti-LPF) significant differences between the three vaccination groups could be demonstrated in the second blood sample, after completion of the primary series. In the subsequent blood samples of the children no effective increase in IgA or IgG antibody titres could be demonstrated.
Comparison of antibody responses in groups A, B and C in blood sample 2 did not show interference of simultaneous or mixed administration of DPT-IPV and PRP-T vaccines. In addition, in groups B and C there was no significant difference in blood samples 3 and 4.

In general the antibody titres to pertussis antigens were much lower in the participants than in patients with clinical pertussis, especially the IgA and IgG-anti-LPF response.
In a routine diagnostic setting the combination of results of these two tests is converted to a numeral lab-code. A difference of 3 or more between two consecutive serum samples is regarded as significant evidence for a recent infection with B.pertussis. In 40 participating children such a rise in IgA and IgG-anti-LPF antibody levels was found, of which 30 occurred between the third and fourth blood sample. Theoretically, these children could have had an intercurrent infection with B.pertussis. These children were evenly distributed over the three study groups (group A: 5/122, group B: 13/158 and group C: 12/155; $p=0.16$) and over the study sites. The pertussis agglutinating antibody response was strongly associated with the lab-code for IgA and IgG-anti-LPF response ($p<0.00001$). Although the IgA and IgG-anti-LPF responses were not much influenced by vaccination, the pertussis agglutinating antibody response was indeed also vaccination induced, as many of the children that did not have evidence of a possibly intercurrent infection, as described above, showed a response with agglutinating antibodies characteristic of the dynamic pattern following a series of vaccinations.

3.2.3. Antibody response to poliovirus (Table 6)

In each phase of the study all children had detectable neutralising antibodies against poliovirus types 1, 2 and 3. Differences between the three groups were not found. The usual dynamics of a vaccine induced immune response is seen in the three groups, with high antibody titres seen one month after the primary series of three vaccinations, followed by a decline in the third blood sample taken just before the fourth dose of DPT-IPV. In this blood sample about 5%, 10% and 25% of the children had neutralising titres below 3 (<1/8) to the three types of poliovirus. But almost all (97.5% in group A, 98% in group B, and 99% in group C) responded to revaccination with the 4th dose of DPT-IPV with titres >3, often at very high levels.

3.2.4. Antibody response to diphtheria toxoid (Table 7)

After the primary series there is no significant difference in the levels of anti-diphtheria toxoid antibodies between the three study groups, with >90% having antibody levels >0.1 IU/ml, and all but 2 children had levels >0.01 IU/ml. The third and fourth blood samples in groups B and C showed the characteristic dynamics; the antibody titre has decreased just before the DPT-IPV revaccination, followed by an increase: all children in groups B and C had antibodies >0.1 IU/ml, while in group A 5 children had antibodies below 0.1 IU/ml, but still detectable.

3.2.5. Antibody response to tetanus toxoid (Table 8)

After vaccinations all children had antibody levels >0.01 IU/ml, the level required for protection. All the more, after the four DPT-IPV vaccinations all children had antibodies >0.1 IU/ml. However, two significantly differences between study groups were found:
First, children in group A had higher antibody titres in the second blood sample than children in groups B and C. The estimated mean values were 1.95 IU/ml (group A), 1.30 IU/ml (group B) and 0.88 IU/ml (group C).
Second, in the third and fourth blood sample children in group B had significantly higher antibody levels than children from group C. Before booster immunisation, 8 of 157 children in group B (5%) and 21 of 158 children in group C (13%) had anti-tetanus antibody levels <0.1 IU/ml, but in all children antibodies were >0.01 IU/ml. In blood sample 4, all children in the study were protected with levels >0.1 IU/ml. In blood samples 3 and 4 GMTs are significantly higher in group B than in group C.

4 DISCUSSION

In the present study the immunogenicity of DPT-IPV and Hib PRP-T vaccines was investigated, given in two ways of administration (simultaneous but separate injections, versus mixed injections) and according to two schedules. The study was done in two study sites: Apeldoorn and Rotterdam and included 543 evaluable infants. The results of the antibody assays surprisingly showed a consistent pattern with higher antibody titres in the Rotterdam study site as compared to the Apeldoorn study site. This observation could be caused by a difference in blood sampling material (in Rotterdam heparinized plasma and in Apeldoorn serum was collected), or blood processing (in Rotterdam blood was centrifuged and plasma was stored the same day at -70°C, in Apeldoorn blood samples were mailed to RIVM and processed there upon arrival). In addition other factors may be important, such as differences in maintaining the study schedule (at intake Apeldoorn children were older than Rotterdam children; the Apeldoorn children kept their vaccination intervals better at a 4 weeks rhythm during the primary series of vaccinations; the second blood sample was taken in Rotterdam at about 2 weeks post 3rd vaccination, while in Apeldoorn this interval was about 4 weeks). Altogether it is not clear which of these factor either alone or in combination could be responsible for the observed difference between the antibody levels in both study sites.
The phenomenon of higher titres in heparinized plasma was not experienced before using both materials in any of the assays used in this study. Moreover, it could not be reproduced in a subsequent experiment (data not shown).
Although the data obtained are clearly heterogeneous, having two distinct populations, the differences between the three study groups can be addressed together, without loss of statistical power, by using the analysis of variance technique, weighing and compensating data from both clusters and providing estimators for mean values.

The results indicate that DPT-IPV and PRP-T vaccines can be given in either way, inducing sufficient antibodies to diphtheria, tetanus, pertussis, poliovirus and Hib in nearly all recipients after a complete series of four vaccinations of both vaccines. The good immunogenicity of the Hib PRP-T vaccine reported here (with 97% of children with anti-PRP levels >0.15 μg/ml after the primary vaccinations) parallels the disappearance of invasive Hib infections among vaccinated children in the Netherlands (10, 11).
In our study we found lower mean anti-tetanus antibody levels after the 3rd dose of the mixed vaccines (group C; blood sample 2, 3 and 4). In addition we observed a statistically significant reduction in the proportion of infants in this group with protective titres ≥0.1 IU/ml in blood sample 3. When the internationally accepted minimum immune titre of 0.01 IU/ml is applied in our study, all children achieved protective anti-tetanus antibody levels in blood samples 2, 3 and 4. In addition, it should be realised that the risk to acquire tetanus in this short period at this age is extremely low. With regard to the lower anti-tetanus response in the mixed administration group, it should be noted that in the studies by Dagan et al. and Gold et al. a MIT ≥ 0.01 IU/ml was considered protective (see paragraph 3.2) (21,22).

Mixing of different vaccines in one syringe requires investigation of immunogenicity and safety which should be as good as separate administration of these vaccines (23-26). By others it was demonstrated that the combined administration of DPT with PRP-T vaccine

could induce interference with antibody response between different components of these vaccines (8,9). The occurrence and magnitude of interference seemed to depend on the manufacturer(s) of the vaccine(s) studied (8,9). Nevertheless in the USA, freeze-dried Hib PRP-T was licensed for mixed administration after reconstitution with DPT or DPT-IPV vaccine of specified manufacturers in 1993 (27). A limited number of studies investigating combined administration of DPT-IPV with PRP-T vaccine has been published (21,22). Dagan et al. (21) demonstrated decreased levels of tetanus antibodies and pertussis agglutinin titres after mixed administration of 3 doses of vaccine. Gold et al. (22) demonstrated reduced GMTs of tetanus antibodies after mixed administration of 3 doses of vaccine due to a smaller proportion of children attaining high titres. They also found a smaller proportion of children attaining high pertussis agglutinin titres and lower GMTs of antibodies against PRP after mixed administration than after simultaneous administration. (23). In neither of these studies the reduced GMT of tetanus antibodies in the mixed administration group resulted in a statistical significant reduction in the percentage of unprotected children (21,22).

We did not find interference with regard to pertussis antigens, as found in Chile (8). This interference with pertussis was one of the main reasons to initiate the present study. The results clearly showed that the anti-pertussis antigen titres were generally much lower than observed in pertussis patients. However, this pattern was already known, especially for IgA antibodies. In general it is known that some whole-cell pertussis vaccines induce also low IgG titres to pertussis toxin (28-30).
The relevance of the presence of pertussis antibodies for protection against infection and disease is not known, although agglutinating antibodies may be the most important indicator for protection. Also the relevance of the difference in level between vaccine and infection induced antibodies is not known (12). We found serological evidence of an intercurrent infection with B.pertussis during the entire study in 40 of 543 (7.4%) participants. It was not known whether these children had experienced clinical symptoms of pertussis infection. These children were equally distributed over the three study groups and were not clustered by study site. Therefore we conclude that these assumed intercurrent infections do not impede a comparison between the three study groups. The prevalence of pertussis in 7% of the participating children is very high and would have generated high notification rates from the study sites and also in patients from other ages. These notifications did not occur. The presumption that these participants have clinically relevant infections is therefore questionable.

Administration of Hib PRP-T vaccine mixed with other vaccines which are routinely administered to infants in the course of the national immunisation programme is potentially advantageous:
- the smaller number of injections is likely to improve acceptance of parents, especially if the vaccination programme will be extended.
- the smaller number of injections will reduce handling time
- the reduced number of injection materials will reduce the costs of immunisation programmes (the cost-benefit analysis on the introduction of the Hib PRP-T vaccine in the RVP was based on the assumption of mixed administration with the current DPT-IPV) (7).
- It may be also important to reduce the amount of wasted material in the immunisation programme.

A decision to introduce combined administration of the DPT-IPV and PRP-T vaccine mixed in one syringe should be made weighing the study results against the afore-mentioned advantages.

5 CONCLUSIONS AND RECOMMENDATIONS

The results of the present study indicate that both the separate and mixed administration of DPT-IPV and Hib PRP-T vaccines induce sufficient antibody levels to diphtheria, tetanus, pertussis, poliovirus and Hib after the complete series of four immunisations. The mixing of both vaccines in one syringe did not result in diminished protection when estimated by internationally accepted minimum immune titres.
We found comparable antibody levels in children who received separate or combined vaccinations with DPT-IPV and Hib PRP-T, with one exception. The tetanus antibody levels in the "combined" group were significantly lower than in the "separate" group, but all children had antibodies above the protective level of 0.01 IU/ml.

It should be discussed whether the observed interference with regard to tetanus antibodies is such strong that mixed administration should be discouraged. This should be done in the light of the the risk to acquire tetanus in the less well protected short period of life, the advantages of mixed administration of both vaccines for the acceptance and the operational facilitation of the immunisation program by mixed administration of these important vaccines.

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Legend to tables

The tables which contain serology results (tables 4-8) have a descriptive section (a.) and an analytical section (b.). Empty cells in the b-section of the serology tables represent values excluded from statistical analysis. Empty cells in the statistical (p or p values) part of a table represent p values > 0.08.

Table 1. Number of evaluable participants

		Evaluable participants per sample			
	total intake	sample 1	sample 2	sample 3	sample 4
Rotterdam	511	432	394	370	354
Apeldoorn	124	111	97	95	82
total	635	543	491	465	436

Table 2. Sex of evaluable participants by study site by immunisation group

Apeldoorn	Male	Female	Total
Group A	18	13	31
Group B	24	17	41
Group C	21	18	39
total	63	48	111
Rotterdam	Male	Female	Total
Group A	86	65	151
Group B	78	62	140
Group C	73	68	141
total	237	195	432

Table 3. Number of evaluable participants per vaccination group

	Group A				Group B				Group C			
Blood sample	1	2	3	4	1	2	3	4	1	2	3	4
Rotterdam	151	137	115	105	140	128	125	125	141	129	130	124
Apeldoorn	31	28	26	18	41	35	35	33	39	34	34	31
total	182	165	141	123	181	163	160	158	180	163	164	155

Table 4. Hib antibodies

a. distribution of Hib antibody levels

	Group A				Group B				Group C			
µg/ml	1	2	3	4	1	2	3	4	1	2	3	4
< 0.15	119	114	3	2	123	5	25	0	123	7	23	1
0.15 - 0.99	55	48	24	0	46	33	71	3	48	40	82	8
≥ 1.00	8	3	114	121	11	125	64	155	9	116	59	146
median	0.08	0.10	2.51	26.3	0.08	3.44	0.62	19.2	0.09	2.37	0.65	18.8
n	182	165	141	123	180	163	160	158	180	163	164	155

b. Estimated geometric mean anti PRP titre (µg/ml), expressed as LS mean

	Group			P values			
Sample	A	B	C	Total*	A&B	A&C	B&C
1	0.11	0.11	0.12				
2	0.10	2.60	2.12	0.0001	0.0001	0.0001	
3		0.60	0.59				
4		15.10	13.79				

* total = p - value comparing group simultaneously

Table 5. Pertussis antibodies

a. distribution of pertussis antibody levels

pertussis agglutinating antibodies (titre)

	Group A				Group B				Group C			
level	1	2	3	4	1	2	3	4	1	2	3	4
0-9	62	25	48	13	64	21	55	7	46	26	58	13
10-79	111	84	76	34	96	92	85	22	114	92	84	32
80-319	9	43	16	43	21	38	19	53	18	29	21	45
320-hi	0	13	0	33	0	12	1	76	2	16	1	65
median	16	32	16	128	16	32	16	256	16	32	16	256
n	182	165	140	123	181	163	160	158	180	163	164	155

pertussis IgA anti-B.pertussis sonicate (%)

	Group A				Group B				Group C			
level	1	2	3	4	1	2	3	4	1	2	3	4
1-4	173	146	134	92	175	142	150	110	167	143	161	108
5-9	5	13	5	18	4	15	3	31	8	12	3	28
10-29	4	3	2	11	2	4	7	15	2	7	0	19
30-74	0	2	0	2	0	1	0	2	2	0	0	0
≥75	0	0	0	0	0	0	0	0	0	0	0	0
median	1	1	1	1	1	1	1	3	1	1	1	2
n	182	164	141	123	181	162	160	158	179	162	164	155

pertussis IgG anti -pertussis toxin (%)

	Group A				Group B				Group C			
level	1	2	3	4	1	2	3	4	1	2	3	4
1-4	110	111	90	59	126	98	95	65	114	102	103	72
5-9	41	35	25	32	35	36	40	41	38	36	30	42
10-29	26	15	23	21	14	20	19	30	19	18	26	27
30-59	4	2	2	7	4	5	2	11	7	5	3	5
60-119	1	0	1	2	2	2	1	6	1	0	1	6
120-239	0	1	0	1	0	1	1	1	0	1	0	0
≥240	0	0	0	0	0	0	2	4	0	0	1	3
median	3	2	3	5	2	3	3	6	3	3	3	5
n	182	164	141	122	181	162	160	158	176	162	164	155

Table 5. Pertussis antibodies (continued)

b. estimated geometric mean pertussis antibody level (LS mean)

		Group			P values			
	Sample	A	B	C	total*	A&B	A&C	B&C
pertussis aggl	1	17	17	22	0.058		0.034	0.044
	2	50	49	44				
	3		19	19				
	4		258	185	0.070			0.070
pertussis IgA	1	1.13	1.06	1.19				0.057
	2	1.55	1.61	1.56				
	3		1.23	1.14				
	4		2.96	2.90				
pertussis IgG	1	3.02	2.60	2.86				
	2	3.04	3.58	3.30				
	3		3.99	3.50				
	4		7.04	5.61				

* total = p - value comparing (A&B)&C simultaneously

Table 6. Poliovirus antibodies

a. distribution of poliovirus neutralising antibody titres

Polio 1	Group A				Group B				Group C			
Titre	1	2	3	4	1	2	3	4	1	2	3	4
0	0	0	0	0	0	0	0	0	0	0	0	0
1-2	15	0	7	0	21	0	7	1	20	0	7	0
3 +	163	163	132	123	159	161	153	157	156	162	156	155
median	5	11	8	12	5	11	7	13	5	11	8	13

Polio 2	Group A				Group B				Group C			
Titre	1	2	3	4	1	2	3	4	1	2	3	4
0	0	0	0	0	0	0	0	0	0	0	0	0
1-2	47	1	13	0	46	0	12	0	39	2	10	0
3+	131	162	126	123	134	161	148	158	137	160	153	155
median	4	9	7	11	4	9	6	13	4	9	7	13

Polio 3	Group A				Group B				Group C			
Titre	1	2	3	4	1	2	3	4	1	2	3	4
0	0	0	0	0	0	0	0	0	0	0	0	0
1-2	74	1	29	2	77	2	30	3	74	5	32	1
3+	104	162	110	121	1 3	159	130	155	102	157	130	154
median	3	9	5	10	3	8	5	12	3	8	5	12

	Group A				Group B				Group C			
	1	2	3	4	1	2	3	4	1	2	3	4
Total n	178	163	139	123	180	161	160	158	176	162	163	155

Table 6. Poliovirus antibodies (continued)

b. estimated mean titre, expressed as LS mean value

		Group			P values			
	Sample	A	B	C	total*	A&B	A&C	B&C
Polio 1	1	37	34	32				
	2	1032	1129	1136				
	3		177	219				
	4		5873	6441				
Polio 2	1	16	16	15				
	2	483	523	477				
	3		84	89				
	4		3399	4194	0.078			0.078
Polio 3	1	9	10	9				
	2	344	282	253				
	3		34	37				
	4		2334	2580				

* total = p - value comparing (A&)B&C simultaneously

Table 7. Diphtheria antibodies

a. distribution of diphtheria antitoxin levels

	Group A				Group B				Group C			
IU/ml	1	2	3	4	1	2	3	4	1	2	3	4
0.01	57	0	6	0	44	0	8	0	44	2	3	0
>0.01-<0.1	75	16	48	4	86	13	49	0	82	8	49	0
≥ 0.1	41	142	83	118	43	145	100	157	43	147	106	153
median	0.03	0.88	0.16	2.15	0.05	0.73	0.16	4.70	0.04	0.77	0.18	4.80
n	173	158	137	122	173	158	157	157	169	157	158	153

b. estimated geometric mean titre (IU/ml), expressed as LS mean value

		Group			P value			
	Sample	A	B	C	Total*	A&B	A&C	B&C
Diphtheria	1	0.03	0.04	0.04		0.050		
	2	0.62	0.57	0.64				
	3		0.13	0.15				
	4		3.73	4.44	0.080			0.080

* total = p - value comparing (A&)B&C simultaneously

Table 8. Tetanus antibodies

a. distribution of tetanus antitoxin

	Group A				Group B				Group C			
IU/ml	1	2	3	4	1	2	3	4	1	2	3	4
0.01	11	0	0	0	4	0	0	0	7	1	0	0
>0.01-<0.1	37	0	1	0	42	1	8	0	32	3	21	0
≥ 0.1	125	158	136	122	127	157	149	157	130	153	137	153
median	0.29	1.90	2.8	10.75	0.27	1.40	0.74	13.4	0.29	0.88	0.41	8.90
n	173	158	137	122	173	158	157	157	169	157	158	153

b. estimated geometric mean titre (IU/ml), expressed as LS mean value

		Group			P value			
	Sample	A	B	C	total*	A&B	A&C	B&C
Tetanus	1	0.22	0.25	0.25				
	2	1.95	1.30	0.88	0.0001	0.001	0.0001	0.001
	3		0.66	0.37	0.0001			0.0001
	4		11.92	7.64	0.0001			0.0001

* total = p - value comparing (A&)B&C simultaneously