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RIVM report 120000 001

Local tolerance and general toxicity of tetravalent pneumococcal conjugate vaccine

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This investigation has been performed by order and for the account of the Ministery of Health, Welfare and Sports, Inspectorate for Health Protection, Commodities and Veterinary Public Health, within the framework of project 120000, Development of a pneumococcal conjugate vaccine.

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Abstract

In order to evaluate the safety of a tetravalent pneumococcal conjugate vaccine, the final product was tested in 24 female rats, 6 weeks of age, by duplicate intramuscular injections. Parameters for general toxicity were body weight gain, food consumption, general clinical parameters and hematology. Local tolerance was evaluated by histopathology of injection sites and regional (iliac and popliteal) lymph nodes. The latter were also weighed.

Results indicated no effect of the test vaccine on clinical parameters, body weight gain, food consumption and hematology. The weight of the regional lymph nodes was slightly increased (though without statistical significance), but not as dramatically as in the reference control vaccine (DPT-polio). Local reactions after adjuvant or complete vaccine were mild compared to the reference control vaccine (DPT-polio).

Provided a protective immune response is conferred, the responses after pneumococcal conjugate vaccines in rats as observed in this study, are acceptable because:
1. The widely accepted local reaction after DPT-polio injection is more severe.
2. The administered doses in this local tolerance and general toxicity study are 1/4 of the DPT-polio human dose and 1/2 of the pneumococcal vaccine human dose, which are great overdoses on body weight basis for the animals used in this study.

Contents

Samenvatting

Om de veiligheid van een tetravalent pneumococcen conjugaat-vaccin te testen werd het eindproduct dat bedoeld was voor gebruik in een klinische trial, toegediend aan 24 vrouwelijke ratten van 6 weken oud middels twee intramusculaire injecties. Parameters voor algemene toxiciteit waren: groei, voedselopname, algemene klinische parameters en hematologie. Lokale tolerantie werd beoordeeld aan de hand van histopathologisch onderzoek van de injectieplaatsen en de regionale lymfknopen. Deze lymfknopen werden eveneens gewogen. Er werden geen effecten van het pneumococcen conjugaat-vaccin gevonden op de groei, voedselopname, klinische en hematologische parameters. Het gewicht van de regionale lymfknopen was weliswaar gering maar niet statistisch significant toegenomen; voorts was de toename niet zo dramatisch als na toediening van het referentie controle vaccin (DKTP).

De lokale reacties in rattten na toediening van adjuvans of het complete vaccin waren mild in vergelijking tot het effect van toediening van het referentie controle vaccin (DKTP). De reacties in ratten na toediening van het hier gebruikte peumococcen conjugaat-vaccin worden acceptabel beschouwd - op voorwaarde dat toediening leidt tot een beschermende immuniteit - omdat:

1. De alom geaccepteerde locale reacties na DKTP injectie sterker zijn.
2. In deze studie naar lokale tolerantie en systemische toxiciteit de toegediende dosis van DKTP 1/4 van de in de mens gebruikte dosis is en de toegediende dosis van het pneumococcen vaccin 1/2 van de in de mens gebruikte dosis is, hetgeen in beide gevallen - voor de dieren gebruikt in deze studie-grote overdoseringen zijn op basis van lichaamsgewicht.

1 Introduction

For the safety evaluation of a new tetravalent pneumococcal conjugate vaccine, a local tolerance test is mandatory (Committee for Proprietary Medicinal Products, CPMP, 1997). Furthermore, it is recommended to study the general toxicity parameters.

We adopted a similar protocol as used for testing a closely related product (Meningococcus-B outer membrane vesicle Vaccine; study 199800078). Two intramuscular injections were given to young rats with a 1-week interval, and the results for local and general toxicity were compared to those from DPT-polio, a widely used and accepted paediatric vaccine, and with the respective adjuvants or saline controls. More and prolonged exposures were considered inappropriate because of possible interference of immune effects with potentially toxic effects.

The study was performed under number 9800669; all relevant information and specimens are saved in the LPI-archives under this number.

2 Materials and Methods

2.1 Animals

Twenty four female Specified Pathogen-Free Rivm:WU rats , 6 weeks of age, were obtained from the Institute's breeding colony. They were assigned at random to 4 experimental groups, each consisting of 6 animals. Housing condition were as follows: Animals were housed individually in macrolon cages, room temperature 20-24^{0}C, with a light regime of 12-12, relative humidity 60-75%, food (RMH.GS) and tap water were provided *ad libitum*.

2.2 Vaccines

The tetravalent pneumococcal conjugate vaccine, final lot 80234, was used as test vaccine. One vial of the test vaccine contained lyophilised powder, consisting of 10.8µg of conjugated type 6B polysaccharide, 3.6 µg of conjugated type 14 polysaccharide, 3.6 µg of conjugated type 19 polysaccharide, 3.6 µg of conjugated type 23F polysaccharide, 25 µg of conjugated tetanus toxoid, 0.7 mg tris (hydroxymethyl)aminomethane and 51 mg sucrose. For use, the powder was reconstituted with 0.6 ml reconstitution fluid charge nr. 70368A, containing 1.5 mg/ml $AlPO_4$ at pH7.

As reference DPT-polio lot 184B was used, routinely produced for pediatric vaccination in the Netherlands, containing per dose 15 Lf (limit of flocculation) diphteria toxoid, 16 opacity units Bordetella pertussis bacteria, 5 Lf tetanus toxoid, 40 units D polio type 1, 4 units D antigen polio type 2, 7.5 units D polio type 3 and 1.5 mg $AlPO_4$ as adjuvant.

As an additional (adjuvant) control mock vaccine LVR035GMP was used, while as negative control physiological saline was injected. The saline (0.9% NaCl) was purchased from SVM, batch 9803843.

The intended human dose is 0.5 ml for the pneumococcal conjugate test vaccine and 1 ml for DPT-polio.

2.3 Experimental protocol

The animals arrived on September 1, 1998 (Day 1) and were allowed to acclimatise for two days. On Day 3 (September 3, 1998) the animals were weighed and injected intramuscularly in the right *M. biceps femoris* with saline (group 1), DPT-Polio (group 2), mock vaccine (group 3) Pneumococcal conjugate vaccine (group 4). Injection volume was 0.25 ml. The animals were inspected daily for clinical abnormalities and food consumption was monitored twice weekly. Six days after the first injection the animals were weighed and given a second injection in the left *M. biceps femoris*. Two weeks after the first injection (September 17, 1998) the animals were weighed, euthanized (bleeding from abdominal aorta under CO_2 narcosis), inspected grossly for abnormalities of abdominal and thoracic organs, injection site and regional lymph nodes; the latter were also weighed.

At exsanguination, blood was collected in EDTA coated tubes for hematology. Hematological parameters included white blood cell count (WBC) and differentiation, hemoglobin (Hb), hematocrit (Ht), All hematology parameters were determined in a Technicon H1-E multispecies hematology analyzer (Bayer Diagnostics, Miles Inc., Tarrytown, NY, USA).

Gross abnormalities, injection site and regional lymph nodes were sampled and fixed in neutral buffered formalin (4% formaldehyde) for histopathological examination.
Histological sections (5µ paraffin) were prepared and stained with haematoxylin and eosin according to the laboratory's routine procedures.

2.4. Statistics

Results are presented as mean ± SD, unless indicated otherwise. Data on general toxicity were analysed with student's t-test (two-tailed), using Microsoft Excel version 5.0. Hematological data and data on body weight were analysed by one factor analysis of variance (ANOVA), using Microsoft Excel version 5.0. Dixon analysis was performed for testing homogeneity of variance using STATA program, release 2.05.

(Histo-)pathology data were entered and tabulated in Pathos 4.3 data acquisition system (Pathology Operating Systems Ltd., Harrogate, England).*

(Histo-)pathological findings were analyzed statistically with the Fisher's exact probability test (two-tailed, unless indicated otherwise). Level of significance was set at $p < 0.05$.

2.5. Retention of records, samples and specimens

All raw data and other information relevant ot the quality and integrety of the study will be retained. They will be filed in the archives of the Laboratory of Pathology and Immunobiology (LPI) after termination of the study. Wet specimens and paraffin blocks will be stored for a period of 5 years and slides for a period of 15 years. At the end of the storage period the materials will be discarded.

* Regarding the original raw data in Pathos, the organ/tissue is the left if no body side is added; the abdominal lymph node is in fact the left iliac lymph node.

3 Results

3.1 Clinical observations

During the experimental period no mortalities occurred, and no signs of illness, pain, abnormal locomotion or any other discomfort was noticed.

3.2 Body weight

Body weight gain is shown in figures 1 and 2. In the graph it is evident that body weight gain was almost similar over the groups, with a an indication of a slightly lower weight gain in the animals injected any vaccine (group 2, 3 and 4), but none of the values reached statistical significance.

3.3 Feed intake

Cumulative feed consumption showed an equal pattern over the groups without indication for difference between any of the groups (see Figure 3).

3.4 Necropsy

At necropsy all animals appeared in good condition without gross changes in internal organs. Gross lesions were observed only at the injection sites and the draining lymph nodes, thus directly related to the i.m. injection. See below and table 1.

3.4.1 Injection sites

Injection site lesions were found only in the *m. biceps femoris* or, occasionally, in the *fascia lata*. In the saline control group (1) injection sites were not detected, while in group 2 (DPT-Polio) the injection sites were nearly always visible as nodules. Sometimes the content was clearly necropurulent (yellow-white). In group 3 (mock vaccine) injection sites were also nearly always visible as nodules though occasionally the aspect was different from the necropurulent lesions as observed in group 2 (DPT-Polio). In group 4 animals (pneumococcal conjugate vaccine) half of the number of the injection sites were macroscopically detected and 5 out of 6 detected injection sites had a white colour.

3.4.2 Lymph nodes (incl. weight)

As regional draining stations, iliac and popliteal lymph nodes were sampled. Enlargement of iliac lymph nodes was found in all animals from group 2 (DPT-Polio), while this occurred only occasionally in other treatment groups. Lymph nodes were also weighed; these figures (see below) are generally considered more reliable in quantitative sense than gross observations on these minute organs.

Lymph node weights (Iliac) are shown in figure 4a. The reference vaccine (DPT-Polio) showed a significant increase in iliac lymph node weight compared to saline control and test vaccine. The adjuvant alone did significantly differ from saline, the contribution of the antigen to lymph node weight (which signifies intended immune stimulation) is limited compared to the adjuvant alone (group 4 vs. 3). Only the weight of the left (side of the second injection) iliac lymph node in the test vaccine group was significantly increased compared to saline control.

Popliteal lymph node weights are shown in figure 4b. In the popliteal lymph node no significant increase in weight had occurred after injection of neither the reference vaccine, nor the test vaccine.

3.5 Haematology

In the DPT-Polio vaccine treated group a slight, though not statistically significant increase in WBC was noted. This increase was associated with a statistically significant increase in absolute and relative numbers of neutrophilic granulocytes (see Figures 5, 6 and 7).

3.6 Histopathology

Histopathological data are listed in table 2.

Group 1: No histological changes were seen in saline injected animals, except for one animal in which a minimal focus of muscular regeneration and for another animal in which a minimal granuloma was observed in the left injection site.

Regional lymph nodes were unaffected.

Group 2: DPT-Polio treated animals showed marked lesions at the injection sites (figure 8). These lesions essentially were pyogranulomatous i.e. consisting of large areas of swollen histiocytes (= "granuloma"), often with focal areas of lytic necrosis of these histiocytes and intragranulomatous presence of neutrophilic granulocytes (= "pyo-"). Surrounding muscles showed evidence of necrosis, regeneration and fibroangioblastic tissue. Peri-granulomatous infiltrates of neutrophilic granulocytes and lymphoid cells were usually present. In the right injection site, occasionally plasma cells were present.

Lymph nodes were enlarged, especially the iliac lymph nodes, due to lymphoid hyperplasia in both B and T cell areas.

Group 3. The injection sites of the mock vaccine group were recognised as granulomas. These were usually smaller than after DPT-Polio injection. Histiocytes were swollen with bluish granular material, interpreted as phagocytised adjuvant. Indeed, there was usually diffuse cell death among these macrophages; sometimes this cell death was extensive. Still, cell death in these granulomas was not directly associated with intragranulomatous presence of neutrophilic granulocytes.

For those samples in which no or relatively small reactions were observed, additional recuts were prepared. Lesions detected in recuts were often smaller than the lesions detected

macroscopically and could be an underestimate of the real lesion. One of the histopathological lesions of the injection sites at the right was so small that it was interpreted as "no equivalent sample".

In contrast to the DPT-polio group, lymphoid hyperplasia in the iliac lymph nodes was limited. The politeal lymph nodes were slightly affected.

Group 4: The injection sites of the pneumococcal conjugate vaccine showed the same type of lesions as in the corresponding (adjuvant-only) group 3 (see Figure 9), although one left injection site had minimal lytic necrosis with intragranulomatous presence of neutrophilic granulocytes.

Histiocytic cell death and perigranulomatous influx of neutrophilic granulocytes were slightly increased in severity compared to group 3 animals. Differences in lymphoid infiltrate were not obvious. Corresponding lymph nodes were frequently enlarged due to lymphoid hyperplasia comparable to group 3 in both severity and distribution.

4 Discussion

In this study on general and local toxicity of tetravalent pneumococcal conjugate vaccine a dual i.m. injection in young rats was considered most appropriate, more injections or higher dose are considered irrelevant for this aspect (Speijers et al. 1988a). As reference materials the saline (negative control) and the widely accepted and used paediatric vaccine DPT-polio (positive control) were used. To study the relative contribution of the active vaccine component, also an adjuvant control group was used.

Parameters for general toxicity were clinical behaviour, food consumption and body weight gain, haematology as well as gross pathology (and histopathology, if indicated). In none of these, significant differences were observed, except for the neutrophilia observed in the positive control (DPT-polio). Gross- and histopathology of injection sites and regional lymph nodes were used as parameters for local toxicity and indicated significant differences. In addition regional lymph node weight was recorded. The results indicated grossly visible lesions after DPT-polio injection, while these were considerably less conspicuous after the pneumococcal conjugate vaccine and adjuvant control administration. Also lymph node weights were significantly increased in the DPT-polio group. The local reaction after DPT-polio injection was severe enough to result in enlargement and weight increase (though not statistically significant) of the politeal lymph node, normally not involved as draining lymph node of the *m. biceps femoris*. The unilateral popliteal lymph node weight increase can result from influx of exsudate from more distal parts of the leg which are normally drained by the politeal lymph node, and/or a secondary reaction upon vaccine antigens.

Lymph node weights were only slightly and often not statistically significantly increased in other groups. The contribution of the antigen component in the pneumococcal conjugate vaccines to the severity of local reaction and general toxicity parameters was marginal compared to adjuvant alone. From these data an initial indication for the mild local response could be concluded, while the immune response, on the basis of lymph node changes, was clearly present though limited compared to DPT-polio. However, main antibody or CTL induction could have been located elsewhere, e.g. in the bone marrow or spleen. Similar DPT-polio-induced lesions were previously reported by Speijers et al (1988a and 1988b). In contrast to a previous experiments with Men-B OMV (study 199800078), in which - despite the cautious injection procedure - occasionally the injection appeared to be located in the *m. gastrocnemius* instead of *m. biceps femoris*, or injection sites were reported as missing, in in the present study more attention was given to the injection technique and lesions were found only in the *m. biceps femoris* or, occasionally, in the *fascia lata*, thus resulting in lower number of missing lesions.

Histopathology of the injection sites revealed granulomatous responses consisting of accumulation of macrophages with conspicuous granular slightly basophilic cytoplasm, interpreted as a result of phagocytosis of the injected adjuvant, resulting in a so-called non-immunological granuloma. Such lesions are considered foreign body reactions, at least using aluminium salts as adjuvants (Turk 1992, Pineau et al 1992). This was concluded since hardly any response was seen in the saline control, and the response in the adjuvant-only groups was almost similar to those found in the pneumococcal conjugate group. In the latter a mild influx of inflammatory cells (with predominancy of lymphoid cells) was observed suggestive of an

immunological reaction based on antigens present in the granuloma. In these granulomas diffuse cell death (mainly apoptotic in nature) could be seen, which was clearly different from the more extensive local lytic necrosis with influx of neutrophilic granulocytes in the DPT-polio group. This was probably a result of poor circulation in combination with excessive phagocytosis. The positive control (DPT-polio) showed a different pattern with focal lytic necrosis, more pronounced invasion of neutrophilic granulocytes and lymphoid cells. These reactions signify a more pronounced local toxicity of this vaccine, together with signs of a perceivable immune response, which was also considered responsible for the regional lymph node enlargement. The severity of the local inflammatory reaction correlates well with the observation of neutrophilia after DPT-polio injection only.

Statistically significant differences in Hb, Ht, WBC and parameters on red blood cells after DTP-polio injections, as reported by Speijers et al (1988), were not observed.

In performing a risk assessment of these vaccines it must be emphasised that both a granulomatous reaction and a lymph node stimulation are designed effects; the acceptability should be on the basis of optimal balance of a maximal protective immune response on one hand and the least tissue reaction practically achievable on the other hand. Provided a protective immune response is conferred, the responses after pneumococcal conjugate vaccines in rats as observed in this study, are considered acceptable because:

1. The widely accepted reaction after DPT-polio injection is more severe.
2. The administered dose in this study and the human dose differ for DPT-polio and pneumococcal vaccine, being 1/4 and 1/2 of the human dose, respectively, both being a great overdose on body weight base for the animals used in this study.

5 Conclusion

Pneumococcal vaccine tested in this protocol was devoid of general or local adverse effects. Lesions found are considered intentional effects implicit to vaccination, mainly due to the application of the adjuvant.

6 Deviations from the protocol

From 01 09 98 onwards drs. P. Dortant acted as study director while dr. P.W.Wester acted as deputy director of this study.

Clinical data were not documented for animal # 18 on September 4, 1998.

In addition to the original protocol the following hematological parameters were measured: red blood cell count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), relative distribution range of erythrocytes (RDW), relative distribution range of Hb content (HDW), platelet count (PLT), mean platelet volume (MPV) and number of large unstained cells (Luc).

Data were not indicative for any toxicological effect of either of the vaccins administered.

The left popliteal lymph node of animal nr. 9 was lost during tissue processing.

Due to clotting the blood sample of animal nr. 10 could not be analysed.

References

1. Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines. Committee for Proprietary Medicinal Products (CPMP), CPMP/SWP/465/95. London, 1997
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3. Speijers GJA, Danse LHJC, Beuvery EC, Strik JJTWA, Vos JG. Local reactions of the saponin quil A and a quil A containing ISCOM measles vaccine after intramuscular injection in rats: a comparison with the effect of DPT-polio vaccine. Fund. Appl. Toxicol. 1988a, 10, 425-430.
4. Speijers GJA, Danse LHJC, Beuvery EC, Derks HJGM, Vos JG. Local reactions of Zwittergent-containing meningococcal vaccine after intramuscular injection in rats: comparison with the effect of diphteria-pertusssis-tetanus-polio vaccine. Vaccine 1988b, 6, 419-422.
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6. Pineau A, Durand C, Guillard O, Bureay B, Stalder JF. Role of aluminium in skin reactions after diphteria-tetanus-pertussis-poliomyelitis vaccination: an experimental study in rabbits. Toxicol. 1992, 73, 11-125.
7. Wester P. Personal communication on the Report on study 199800078: Local tolerance and general toxicity of hexavalent Meningococcal PorA OMV Vaccine.(in preparation).

Statement of GLP-compliance:

Undersigned hereby declare that the report constitutes a true and faithful account of the methods and results of this study. The study was conducted at the Central Laboratory Animal Facility (CDL) and the Laboratory of Pathology and Immunobiology (LPI) of the National Institute of Public Health and the Enviroment (RIVM) according the current OECD Good Laboratory Practice Principles.

drs.P.Dortant
study director

Signature : .....

Date : 21/12/99

dr.H.van Loveren
principal investigator haematology

Signature : .....

Date : 21.12.99

Participants:
Necropsy was performed by A.Timmerman, R.Vlug and P.Dortant. Hematological analyses were performed by A.Fluitman. Histological sections were prepared by H.Loendersloot. Hematological data were evaluated by H.van Loveren, all other data were evaluated by P.Dortant. From 01 09 98 onwards drs.P.Dortant acted as the study director and dr.P.W.Wester as deputy study director.

Quality assurance statement

GLP inspections were performed on the items mentioned below. The results of the findings were reported to the study director and the management.

	date(s) of inspection(dd-mm-yy):
Study design	:14-08-1998
Inspection at CDL	:08-09-1998 & 17-09-1998
Record review including raw data	:16-12-1998

The results as presented accurately reflect the raw data.

A.N. de Klerk Signature:
Quality Assurance Officer
Laboratory of Pathology and Immunobiology Date: 25/10/1999

Experiment nr.:9800669
AAP nr. :199800669
PAT nr. :9800669

At request of the projectleader dr.ir. P.Hoogerhout (LVR/RIVM) individual data were not included in this report, but remain stored in the LPI-archives and are available at appeal.

Appendix 1 Tables and figures

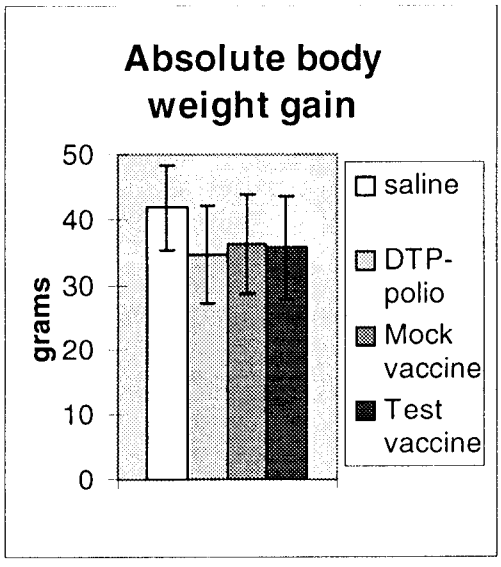


Figure 1

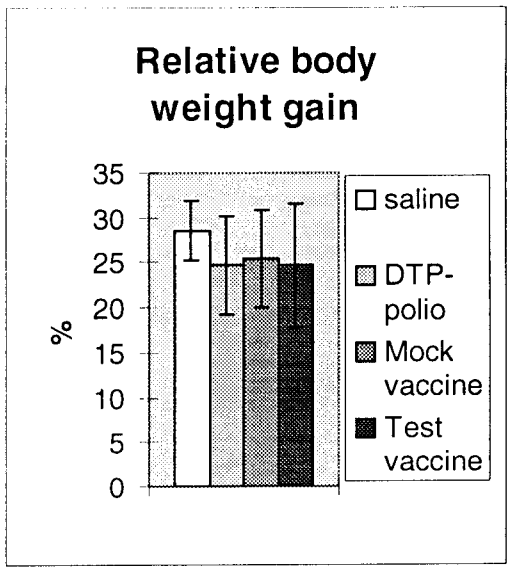


Figure 2

Figures 1 and 2. Absolute (grams) and relative (%) body weight gain during the experimental period.

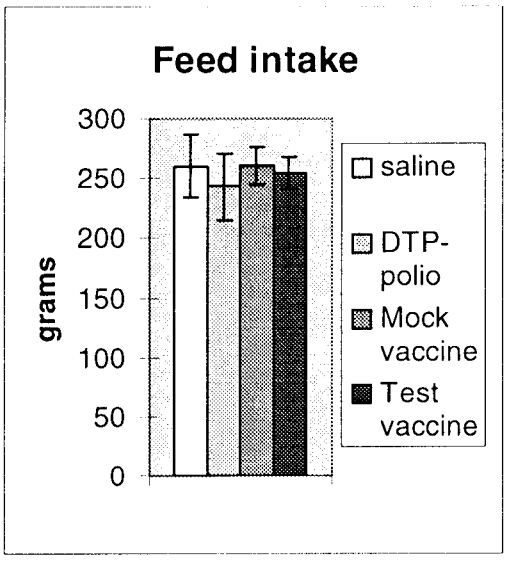
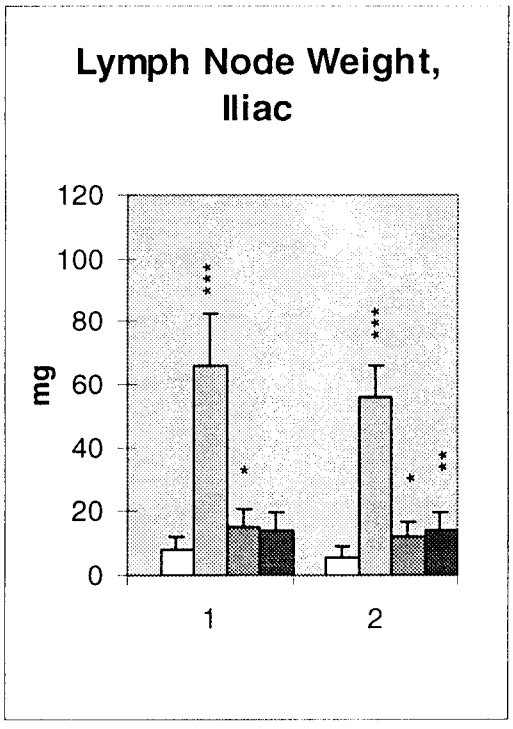


Figure 3.Feed intake during the experimental period (grams).



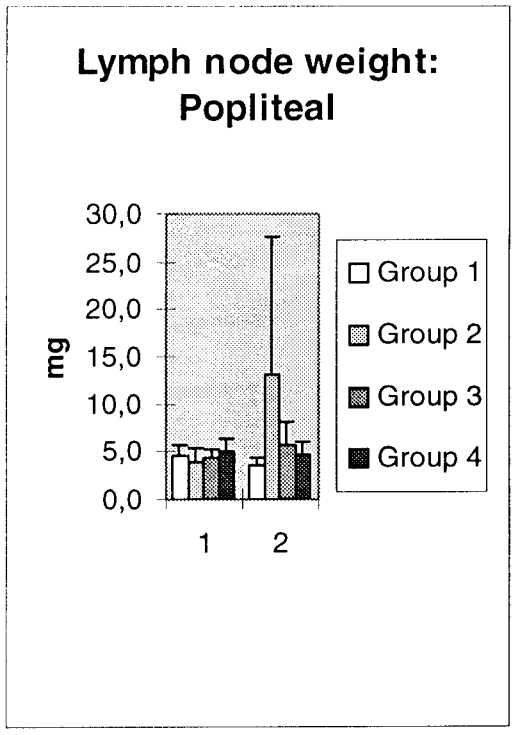


Figure 4a

Figure 4b.

Figures 4a and 4b. Lymph node weights.X-axis: 1= first injection (= right) side, 2= second injection (= left) side. Group 1 is saline control group, group 2 is DPT-polio (reference-) vaccine group, group 3 is Mock vaccine (solvent only) group, group 4 is test vaccine (tetravalent pneumococcal conjugate) group.

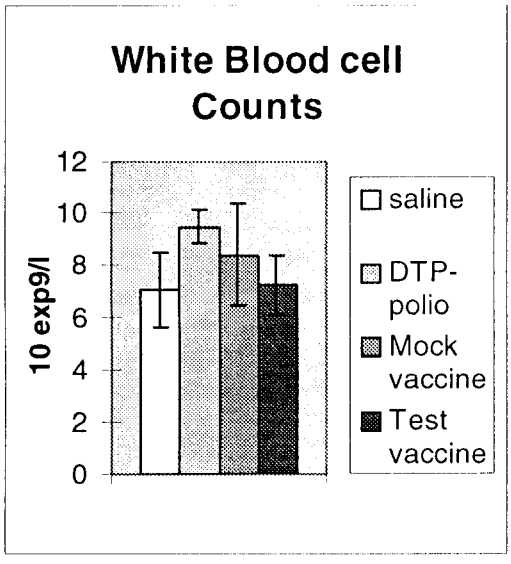


Figure 5

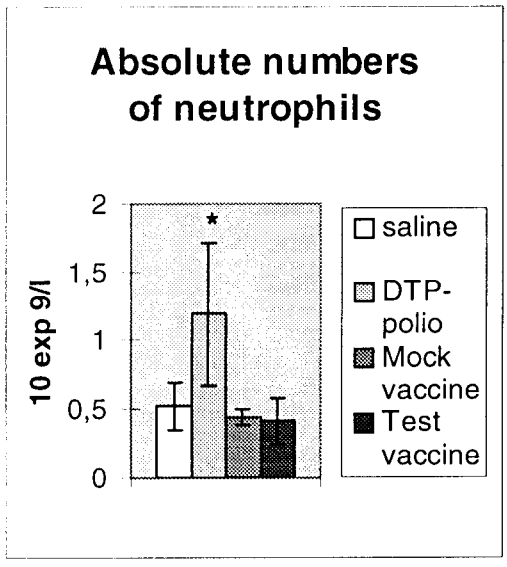


Figure 6

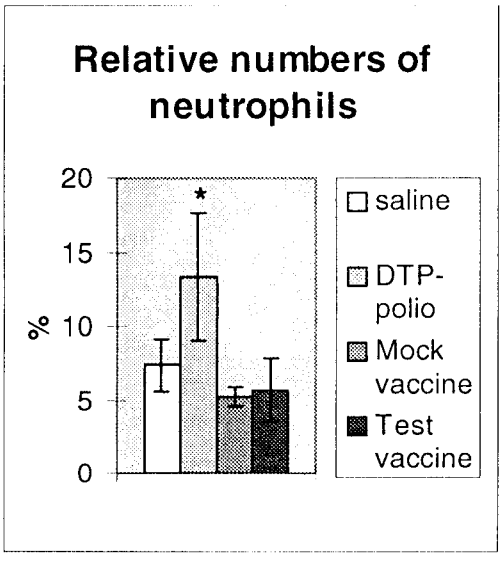
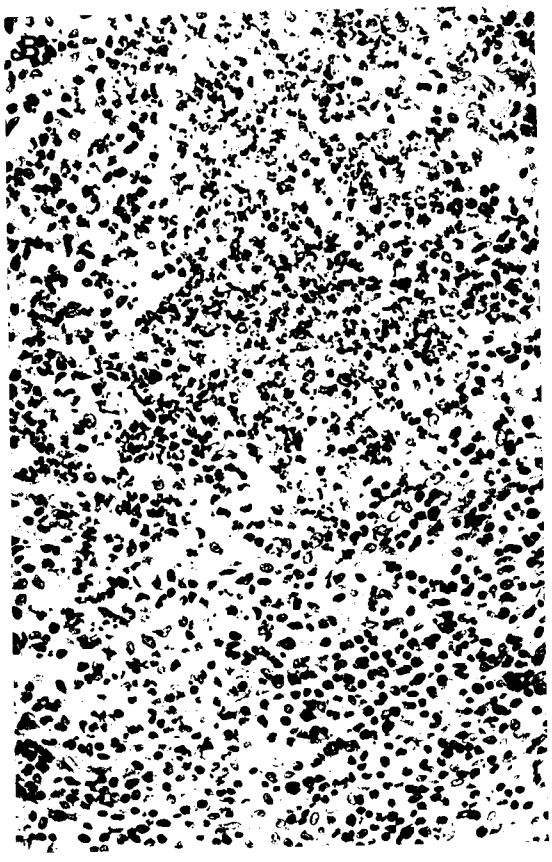


Figure 7

Figures 5, 6, and 7. White blood cell count, absolute and relative number of neutrophilic granulocytes.


A


C



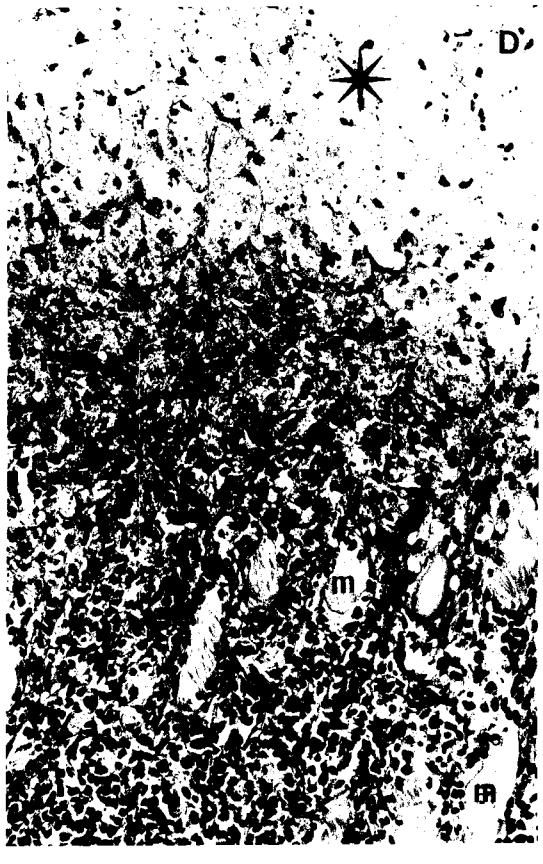

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Figure 8. Micrographs of representative intramusculair lesions 2 weeks after injection of 0.25ml DPT-Polio (A & B) or the teravalent pneumococcal conjugate vaccine tested (C & D).

A: Relatively large spherical lesion containing much cellular debris (pale area in lesion), mainly consisting of necrotic musle fibers and polymorph nuclear cells (HE, x 22).

B: Detail of Figure 8 A. The inflammatory reaction after DTP-Polio injection consisting of neutrophilic granulocytes and mononuclear cells (merely pale stained macrophages) in approximately equal numbers (HE, x 220).

C: Relatively small, branching lesion with pale area, consiting of injected material and some apoptotic macrophages (HE, x 22).

D: Detail of C. The inflammatory infiltrate surrounding intact muscle fibers (indicated with "m") consists of mainly mononuclear cells. In the injected material (indicated with asterix) remnants of apoptotic macrophages are visible as small dark dots (HE, x 220).

Table 1. Group incidence necropsy

Kill type: All

	LEFT				RIGHT			
Group:	1	2	3	4	1	2	3	4
Sex:	F	F	F	F	F	F	F	F
Vaccin type:	Saline	DPTP	Mock	Pneu.	Saline	DPTP	Mock	Pneu.
Number:	6	6	6	6	6	6	6	6

Tissue Observation

Injection site	LEFT				RIGHT			
Not remarkable	6	1	1	2	6	1	1	4
Lesion detected	0	5	5	4	0	5	5	2

Iliac lymph node	LEFT				RIGHT			
Not remarkable	6	0	5	5	6	0	5	5
Enlarged	0	6	0	1	0	6	0	1

Popliteal lymph node	LEFT				RIGHT			
Not remarkable	6	3	6	5	6	4	6	6
Enlarged	0	3	0	0	0	2	0	0

Table 2. Group incidence: histopathology - Summary

	LEFT				RIGHT			
Group:	1	2	3	4	1	2	3	4
type:	Saline	DPTP	Mock	Pneu.	Saline	DPTP	Mock	Pneu
Number:	6	6	6	6	6	6	6	6
Tissue Observation								
Injection site	**LEFT**				**RIGHT**			
Number examined	6	6	6	6	6	6	5	6
Not remarkable	4	0	0	0	6	0	0	0
No equivalent sample	0	0	0	0	0	0	1	0
Regeneration minimal	1	0	0	0	0	0	0	0
Granuloma	1	1	0	0	0	2	0	1
minimal	1	1	0	0	0	1	0	0
slight	0	0	0	0	0	0	0	1
marked	0	0	0	0	0	1	0	0
Pyogranulomatous reaction	0	5*	0	1	0	4@	0	0
minimal	0	0	0	1	0	0	0	0
severe	0	5	0	0	0	4	0	0
Granuloma with focal necrosis	0	0	6**	6**	0	0	5*	5*
minimal	0	0	0	0	0	0	1	1
slight	0	0	2	0	0	0	2	0
moderate	0	0	4	6	0	0	2	3
marked	0	0	0	0	0	0	0	1
Lymphoid infiltrate	0	5*	6**	6**	0	6**	5*	6**
minimal	0	2	1	1	0	0	1	2
slight	0	1	5	5	0	4	4	2
moderate	0	2	0	0	0	2	0	2
PMN infiltrate	0	6**	1	4@	0	4@	1	4@
minimal	0	1	1	3	0	1	1	3
slight	0	2	0	1	0	2	0	1
moderate	0	2	0	0	0	1	0	0
marked	0	1	0	0	0	0	0	0
Iliac lymph node	**LEFT**				**RIGHT**			
Number examined	6	6	6	6	6	6	6	6
Not remarkable	6	0	1	0	6	0	0	1
Lymphoid hyperplasia	0	6**	5*	6**	0	6**	6**	5*
minimal	0	0	0	1	0	0	1	0
slight	0	0	4	1	0	0	1	2
moderate	0	0	0	3	0	0	4	2
marked	0	3	1	1	0	1	0	1
severe	0	3	0	0	0	5	0	0
Popliteal lymph node	**LEFT**				**RIGHT**			
Number examined	6	5	6	6	6	6	6	6
No sample	0	1	0	0	0	0	0	0
Not remarkable	6	0	2	1	6	1	2	1
Lymphoid hyperplasia	0	5**	4@	5*	0	5*	4@	5*
minimal	0	0	2	3	0	4	0	0
slight	0	2	2	2	0	1	4	3
moderate	0	1	0	0	0	0	0	2
marked	0	1	0	0	0	0	0	0
severe	0	1	0	0	0	0	0	0

* or @ :p< 0.05 two-tailed or one-tailed, respectively; ** p< 0.01 two-tailed; all Fischer exact test

Appendix 2 Mailing list

1. Dr.F.Schuring, algemeen directeur Inspectie Inspectie Gezondheidsbescherming, Waren en Veterinaire Zaken, Den Haag
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5. Depot Nederlandse Publikaties en Nederlandse Bibliografie, Den Haag
6. Directie RIVM
7. Dr.Ir.G.de Mik, directeur sector 3/4
8. Prof.Dr.B.van der Zeijst, directeur sector 1
9. Prof.Dr.J.G.Vos, hoofd LPI
10. Dr.L.van Alphen, hoofd LVR
11. Dr.Ir.P.Hoogerhout, LVR
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19. Drs.A.Vroege, SVM

20-22. Auteurs

23. SBD/Voorlichting & Public Relations
24. Bureau Rapportenregistratie
25. Bibliotheek RIVM

26-40. Bureau Rapportenbeheer

41-42. Reserve exemplaren LPI