Effect of infant immunisation with meningococcus serogroup C–CRM197 conjugate vaccine on diphtheria immunity and reactogenicity in pre-school aged children

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Abstract

The majority of Men C conjugate vaccines given in the UK use CRM197, a mutant diphtheria toxoid, as their protein carrier. We studied the effects of prior immunisation with Men C–CRM197 conjugate vaccine on immunity to diphtheria in 193 children before and after a booster dose of Men C at 4 years. Baseline diphtheria antibodies were higher in children given four previous doses of Men C (P < 0.0001) and tended to be higher following boosting in those who had received three or four doses. This enhanced immunity was not associated with increased reactogenicity.

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1. Introduction

Conjugate vaccines against Neisseria meningitidis serogroup C (Men C) were introduced into the United Kingdom’s (UK) routine immunisation schedule in November 1999. These vaccines have been demonstrated to be safe and immunogenic in British infants when administered at 2, 3 and 4 months of age [1–3]. The enhanced immunogenicity of conjugate vaccines depends on the chemical conjugation of capsular polysaccharides of organisms such as Haemophilus influenzae type b (Hib) or Men C with immunogenic proteins such as diphtheria or tetanus toxoids. The conjugation of protein component enables the recruitment of T cell help to assist the usually T cell independent immune response to polysaccharide antigens. In this way, antibody levels deemed protective are induced in even young infants. In addition, populations of memory cells are formed following the primary immune response, which are readily reactivated to produce high levels of functional antibody on re-exposure to vaccine antigens [4].

Introduction of a new vaccine such as this always has a potential impact on the immunogenicity and reactogenicity of the immunisation schedule [5]. For this reason, it is important to demonstrate that additional antigens do not significantly interfere with those already routinely administered and that protection is not compromised [6]. CRM197, which is the protein carrier for many of the licensed Men C vaccines, has close immunologic correlation with diphtheria toxoid [7]. While the use of immunogenic proteins such as diphtheria and tetanus toxoids is the basis of the success of conjugate vaccines, there is potential for immunologic interference to occur [8].

In this study, we compared diphtheria antibody levels in 4-year-old children with and without a history of Men C–CRM197 conjugate vaccination in infancy. We also looked for differences in their immune response to diphtheria following a booster dose of the same conjugate vaccine. In addition, safety information was collected at 4 years of age in order to see whether any alteration in the
reactogenicity profile of the Men C or DT vaccines would be observed in children who had received multiple doses of Men C conjugate vaccine in infancy. The relationship between immunogenicity and reactogenicity was explored.

2. Methods

2.1. Subject population

The study from which this data derives has been described in detail elsewhere [2,9]. Briefly, parents of 182 children born in Oxfordshire between 1 April and 30 November 1995 who had been immunised in an infant vaccine study with 0, 1, 3 or 4 doses of Men C conjugate vaccine using CRM197 as the protein carrier were approached to take part in a pre-school follow-up phase. Based on their involvement in the infant study, these children were divided into four groups. Group 1 received four doses of Men C at 2, 3, 4 and 12 months. Group 2 received three doses of Men C at 2, 3 and 4 months and one dose of meningococcal A/C polysaccharide vaccine (MPS) at 12 months of age. Group 3 received three doses of hepatitis B vaccine (HBV) at 2, 3 and 4 months and one dose of Men C at 12 months. Group 4 received three doses of HBV at 2, 3 and 4 months and one dose of MPS at 12 months. The General Practice (GP) surgeries of these original participants provided names of healthy age strata matched children on their lists from whom a comparison population was recruited. These controls were divided into two groups (5 and 6) for a lot comparison study.

Children who had received any meningococcal vaccination, other than as part of the infant study, were excluded. Allergy to vaccine components, immune deficiency or chronic illness which could obscure the immunogenicity or safety evaluations were further grounds for exclusion.

2.2. Study procedure

This study was approved by the Central Oxford Research Ethics Committee and conducted in accordance with the International Conference on Harmonisation Good Clinical Practice guidelines. Children were visited twice at home. Parents indicated their informed consent in writing at the first visit. All children received a single dose of Men C conjugate vaccine at 4 years of age. Blood samples were taken for assessment of vaccine immunogenicity immediately before and 30 days (range: 28–42 days) following immunisation. Safety data were collected following Men C administration. After study completion, children received the pre-school diphtheria and tetanus (DT) booster through their GP surgery. Parents were also asked to complete a further reactogenicity diary for the week following receipt of this vaccine.

2.3. Vaccines

Chiron Men C conjugate vaccine, lot MMHPN002 (Chiron Corporation, USA) was administered to subjects in Groups 1–4. Controls were randomly assigned to receive either MMHPN002 or Chiron Men C Lot PB0175 (Chiron Vaccines, Italy). Each 0.5 ml dose of Men C contained 10 μg of Neisseria meningitidis group C oligosaccharide, conjugated to 12.5–25.0 μg of diphtheria toxoid (CRM197), adsorbed on aluminium hydroxide. One dose of study vaccine was administered to each subject. No concomitant vaccines were given.

All children had received three prior doses of diphtheria vaccine, in combination with tetanus, pertussis, H. influenzae type b (Hib) and oral poliomyelitis vaccines at 2, 3 and 4 months according to the routine UK infant immunisation schedule. The majority of Hib conjugate vaccines used in the UK use tetanus as their protein carrier (PRP-T). None had received pre-school DT booster immunisations prior to the administration of Men C conjugate vaccine.

2.4. Antibody assays

Diphtheria antibodies were measured in the Immunology research laboratory of the Churchill Hospital, Oxford. One hundred microlitres of diphtheria toxoid (Evans Medical, UK) was diluted 1:10,000 to approximately 1 μg/ml in 0.05 M carbonate coating buffer pH 9.6 (Don Whitley Scientific, UK), then added to the wells of Immulon 1 flat-bottomed microtitre plates (Dynatech Lab Inc. 3355, UK). The plates were sealed with wide tape (ICN biomedical, UK) and left at 4°C overnight. One well was left uncoated with antigen as the substrate blank. The following day plates were washed six times with PBS-0.05% Tween (PBS-T) (Merck, UK) and either used immediately in the ELISA or stored at −20°C for up to 3 months. The ELISA was performed at room temperature. A standard curve with seven separate dilutions of Gammagard starting at 1:50 (Baxter, UK) was prepared and run in duplicate on each plate. The highest concentration of Gammagard was given a value of 1 EU/ml, which corresponded to a value of 1 IU/ml diphtheria antibody. This had been previously calibrated against the NIBSC standard for diphtheria antibodies (Sesaric, NIBSC, personal communication). Calibration of the standard curve was checked against the NIBSC reference every 3 months to assess any drift. Serum samples obtained at Visit 1 were serially diluted at concentrations of 1:50, 1:100, 1:200 and 1:400 in PBS-T and 100 μl of each sample added in duplicate to the plate which was then incubated at 20°C for 2 h. Visit 2 samples were similarly diluted, but at concentrations of 1:800, 1:1600, 1:3200 and 1:6400. The plate was washed six times in 100 ml of PBS-T of 1/1000 dilution. Goat anti-human IgG conjugated to alkaline phosphatase (Sigma, Dorset, UK) was then added and the plate was allowed to incubate for 1 h at 20°C. After this time, plates were again washed in PBS-T six times before 100 μl of phosphatase substrate (Sigma, Dorset, UK) was added to the wells and the reaction allowed to continue until the OD of the second standard reached approximately 1.0. The reaction was stopped with 50 μl of NaOH and
the plate left for 5 min before reading on an ELISA reader (Dynatech, UK). The antibody concentration of the samples was calculated using a log-linear standard curve. Because of the poor correlation between functional (neutralisation) assays and ELISA at low diphtheria titres, the lower limit of the assay was 0.1 EU/ml.

2.5. Sample size and statistics

The primary aim of the study was to explore the relationship between the number of doses of Men C conjugate vaccine given in infancy and immunity to diphtheria, both before and after a further dose of Men C vaccine at 4 years of age. In addition, we studied the effect of the different infant immunisation schedules on subsequent local and systemic reactions to a pre-school booster dose of Men C. We also looked for evidence of a correlation between immunogenicity and reactogenicity and assessed the relative risk of a local reaction to subsequent DT vaccination occurring in subjects who experienced a local reaction following Men C vaccine at 4 years.

Geometric mean concentrations (GMCs) of diphtheria antibody before and after Men C immunisation were compared between groups using analysis of variance (ANOVA). Where comparisons were made between only two of the groups, the Scheffe adjustment for multiple comparisons was used. Regression analysis was used to look for a linear dose response relationship between prior doses of Men C and antibody concentrations. The odds of children in each group experiencing local and systemic reactions in the week following Men C were calculated and a test for trend of odds in relation to number of prior doses of Men C was performed. Regression analyses were used to explore a possible relationship between immunogenicity and reactogenicity. Risk ratios were calculated to describe the relative risk of a local reaction occurring following DT vaccine, in children with or without such prior reactions to Men C. All statistical analyses were performed using STATA version 7.0 [10].

3. Results

3.1. Subject characteristics

Ninety-five children who had been involved in the infant study were re-enrolled to take part in the pre-school phase. Their prior vaccination experience and progress through the study is summarised in Table 1. A total of 1184 children were invited by letter to participate in this study for the first time, and 103 were ultimately recruited. The 20% positive response rate elicited from parents was higher than usual using this method, which we have been required to adopt due to ethical constraints on approach to subjects. Eighty percent of the exclusions noted in Table 1 were due to receipt of the pre-school diphtheria booster prior to study commencement. Two children were inappropriately enrolled into group 4—one had received BCG fewer than 60 days before immunisation and the other had withdrawn from the infant study before the 12-month Men C immunisation. Three subjects terminated the study prematurely, two withdrawing consent and the third lost to follow-up. One reactogenicity diary was missing for a child in the control group who otherwise completed the study according to protocol.

No significant difference in age, weight or height was seen between groups. A non-significant gender imbalance, also present in the infant phase of this study, was noted. Ninety-five of children taking part were Caucasian, which is representative of the Oxfordshire population.

3.2. Immunogenicity

Immunogenicity results, with the number of children from whom pre- and post-vaccination blood samples were available, are shown in Table 2. Fig. 1 places these results in context, additionally showing diphtheria GMCs for the same children by vaccine group at 2, 5, 12 and 13 months of age in the infant study. The proportions seronegative (<0.1 EU/ml) and with protective antibody concentrations (≥0.1 EU/ml) are also shown [11]. Children who had received four doses

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Subject flowchart</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Subject involvement in study</strong></td>
<td></td>
</tr>
<tr>
<td>Infant study participants</td>
<td>Control group</td>
</tr>
<tr>
<td>Number approached</td>
<td>178 completed initial study; group 1 (58), group 2 (60), group 3 (30), group 4 (30)</td>
</tr>
<tr>
<td>Reasons for non-participation</td>
<td>28 moved out of study area, 12 exclusions, 8 refusals, 35 no reply</td>
</tr>
<tr>
<td>Study group 1</td>
<td>Study group 2</td>
</tr>
<tr>
<td>Enrolled</td>
<td>31</td>
</tr>
<tr>
<td>Received vaccine</td>
<td>31</td>
</tr>
<tr>
<td>Did not complete study protocol</td>
<td>1 withdrawal of consent before immunisation</td>
</tr>
<tr>
<td>Completed study</td>
<td>31</td>
</tr>
</tbody>
</table>
Table 2

<table>
<thead>
<tr>
<th>Vaccination group</th>
<th>Diphtheria GMC pre-vaccination (95% CI)</th>
<th>Proportions in immune categories pre-vaccination (EU/ml)</th>
<th>Diphtheria GMC post-vaccination (95% CI)</th>
<th>Proportions in immune categories post-vaccination (EU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Men C × 3; Men C; Men C</td>
<td>0.35 a (0.27, 0.46)</td>
<td>&lt;0.1–6%; ≥0.1–94%</td>
<td>9.00 b (6.26, 12.92)</td>
</tr>
<tr>
<td>Group 2</td>
<td>Men C × 3; MPS; Men C</td>
<td>0.18 (0.14, 0.23)</td>
<td>&lt;0.1–13%; ≥0.1–87%</td>
<td>8.55 c (5.10, 14.34)</td>
</tr>
<tr>
<td>Group 3</td>
<td>HBV × 3; Men C</td>
<td>0.20 (0.12, 0.31)</td>
<td>&lt;0.1–19%; ≥0.1–81%</td>
<td>2.82 b, c (1.52, 5.26)</td>
</tr>
<tr>
<td>Group 4</td>
<td>HBV × 3; MPS; Men C</td>
<td>0.10 a (0.08, 0.14)</td>
<td>&lt;0.1–49%; ≥0.1–51%</td>
<td>4.55 (3.47, 5.95)</td>
</tr>
</tbody>
</table>
| Group 5 and 6 | None; none; Men C | Geometric mean concentration (GMC) of diphtheria antibody given by group in EU/ml, 95% confidence intervals in parentheses.

3.3 Reactogenicity

The only difference in local reactions observed between groups following immunisation was a significant reduction in reported tenderness in children who had received more doses of Men C vaccine in infancy (P = 0.004). All other local (Table 3) and systemic reactions (data not shown) were recorded with equal frequency between groups.

Unfortunately, only 46 diary cards (24%) were returned following DT administration. The sample that did return them, however, was reasonably representative in terms of prior doses of Men C administered (Table 4). No significant increase in the risk of local reactions following DT was seen for children who had experienced a previous local reaction to Men C at 4 years.

3.4 Relationship between immunogenicity and reactogenicity

No significant relationship was observed between pre-existing immunity to diphtheria and local reactions to Men C vaccine. The presence of erythema at the injection site was significantly related to higher post-immunisation antibody levels [coefficient: 1.43, standard error: 0.71, r²: 0.024 (P = 0.05)] in a simple regression model. No such relationship was observed between a greater immune response and either tenderness or induration associated with the vaccination site, or any other systemic response to immunisation (data not shown).

4. Discussion

With the escalating availability of new vaccines in the developed world, infant immunisation schedules have become increasingly complex, with subsequent potential for immunologic interference. Infant studies of Men C conjugate vaccines have provided reassurance that no such effects were seen immediately following an accelerated infant primary course given at 2, 3 and 4 months [1,2]. In this study,
we have demonstrated enhanced persistence of immunity to diphtheria at 4 years of age in children who received four doses of Men C in infancy. Further, a trend to higher diphtheria antibody responses following a booster dose of Men C was observed following at least three prior doses. Similar boosting of immunity to diphtheria and tetanus has been observed with administration of conjugate vaccines using these carrier proteins [12]. Conversely, it is also possible that immunologic interference may occur [8]. One recent study in Belgium demonstrated impaired tetanus immunity in infants, persisting into early childhood, following receipt of a primary course of Hib vaccine conjugated to tetanus toxoid [13].

It is acknowledged that standardisation of diphtheria assays across the UK has been difficult, with many potential technical factors introducing variation in results in inter-laboratory comparisons. If a sample does not fall within the linear part of the standard curve, small differences in optical density may be interpreted as large differences in EU/ml, leading to discrepancy in reporting. Consequently inter-laboratory CVs of 20–30% have been reported by the National External Quality Assessment Service (Griffiths, personal communication). In this study, nationally recognised standards were used to calibrate antibody measurements. All of the ELISAs were performed by a single operator, who used positive and negative controls for each assay. Where these controls were beyond the range of two standard deviations of the mean, the sample was discarded. Consequently, the CVs observed in our laboratory were within the range expected of this assay.

We live in an era where ‘vaccines are increasingly the focus of controversy rather than compliments’ [14]. Ensuring

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**Table 3**

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>Vaccines given</th>
<th>Number of prior doses</th>
<th>Tenderness</th>
<th>Erythema</th>
<th>Induration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2, 3, 4 months; 12 months; 4 years</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>Men C × 3; Men C; Men C</td>
<td>4</td>
<td>31</td>
<td>Any—12 (39%)</td>
<td>Any—12 (39%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pain on movement—0 (0%)</td>
<td>&gt;25 mm—3 (10%)</td>
</tr>
<tr>
<td>Group 2</td>
<td>Men C × 3; MPS; Men C</td>
<td>3</td>
<td>31</td>
<td>Any—11 (35%)</td>
<td>Any—8 (26%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pain on movement—1 (3%)</td>
<td>&gt;25 mm—1 (3%)</td>
</tr>
<tr>
<td>Group 3</td>
<td>HBV × 3; Men C; Men C</td>
<td>1</td>
<td>16</td>
<td>Any—7 (44%)</td>
<td>Any—6 (38%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pain on movement—1 (6%)</td>
<td>&gt;25 mm—0 (0%)</td>
</tr>
<tr>
<td>Group 4</td>
<td>HBV × 3; MPS; Men C</td>
<td>0</td>
<td>114</td>
<td>Any—69 (61%)</td>
<td>Any—35 (31%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pain on movement—2 (2%)</td>
<td>&gt;25 mm—9 (8%)</td>
</tr>
<tr>
<td>Groups 5 and 6</td>
<td>None; none; Men C</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

P-value for trend 0.0044 0.71 0.75
Table 4
Relative risk of local reactions to DT given prior Men C reactions

<table>
<thead>
<tr>
<th>Number of prior Men C doses</th>
<th>Number returning DT diary cards per group</th>
<th>Any tenderness post-DT</th>
<th>Pain on movement post-DT</th>
<th>Any erythema post-DT</th>
<th>Erythema &gt;25 mm post-DT</th>
<th>Any induration post-DT</th>
<th>Induration &gt;25 mm post-DT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>23/114</td>
<td>14/23 (61%)</td>
<td>0/23 (0%)</td>
<td>14/23 (61%)</td>
<td>7/23 (30%)</td>
<td>12/23 (52%)</td>
<td>7/23 (30%)</td>
</tr>
<tr>
<td>1</td>
<td>6/16</td>
<td>4/6 (67%)</td>
<td>0/6 (0%)</td>
<td>4/6 (67%)</td>
<td>2/6 (33%)</td>
<td>0/6 (0%)</td>
<td>0/6 (0%)</td>
</tr>
<tr>
<td>2</td>
<td>10/31</td>
<td>6/10 (60%)</td>
<td>0/10 (0%)</td>
<td>3/10 (30%)</td>
<td>0/10 (0%)</td>
<td>4/10 (40%)</td>
<td>0/10 (0%)</td>
</tr>
<tr>
<td>Total</td>
<td>46/192</td>
<td>28/46 (61%)</td>
<td>0/46 (0%)</td>
<td>25/46 (54%)</td>
<td>10/46 (22%)</td>
<td>22/46 (48%)</td>
<td>9/46 (20%)</td>
</tr>
</tbody>
</table>

The acceptability of vaccines is crucial to achieving levels of immunisation uptake required to guarantee population immunity. This study demonstrated no increase in local or systemic reactions following a dose of Men C vaccine at 4 years of age in children who had received as many as four prior doses of the same vaccine. No significant increase in the risk of subsequent reactions to the pre-school DT booster in children with a local reaction to Men C at 4 years was observed in a small subset of children for whom this information was available. While we recognise that the low rate of return of the second diary card may have introduced sampling bias, reported rates of reactions to DT were similar to those previously described in this age group [15,16].

The relationship between diphtheria antibody titles and local reactions to immunisation in adult studies has been variously reported. Investigators involved in the development of diphtheria vaccines in the 1940s noted that adults with natural immunity to diphtheria, denoted as an antibody level of >0.01 IU/ml, experienced more local reactions following diphtheria vaccination than those who were not immune [17]. A similar but non-significant trend was observed in Swedish car factory workers [18]. No such relationship was observed more recently in a study of young British adults given a low dose diphtheria preparation, in which all subjects had diphtheria antibody levels of <0.1 IU/ml at study entry [19]. Post-immunisation specific antibody levels were shown to be higher in Danish adults suffering from local reactions after a booster dose of diphtheria than in those with no reactions, and higher still in those experiencing systemic reactions [20]. The present study has shown an association between post-vaccination antibody levels and erythema at the injection site in pre-school aged children administered a diphtheria toxoid conjugate vaccine, but no association with local induration or tenderness, or any systemic side effects.

The introduction of diphtheria vaccines in the 1940s in England and Wales was associated with a dramatic reduction in the number of notifications of disease, and subsequent mortality. In 1940, more than 45,000 cases of diphtheria were reported in England and Wales, with nearly 2500 deaths; by 1951, this figure had fallen dramatically to 37 cases with only 6 deaths [21]. Optimism in the 1980s regarding the potential for elimination of diphtheria from Europe was, however, short-lived. Outbreaks in a number of the Newly Independent States of the former Soviet Union highlighted the ongoing threat of this disease [22]. One factor in this resurgence was a breakdown in the provision of childhood immunisation in many of these countries. Further, many cases occurred in adults, in whom post-vaccination antibody levels had waned in the absence of natural boosting through exposure to the organism [23]. Sero-epidemiologic studies of British adults, conducted in response to these epidemics, have shown that only 30% of adults over 60 years of age have titles of antibody to diphtheria deemed protective [24]. Ensuring safe and effective immunisation against the ‘forgotten’ vaccine preventable diseases remains an important priority. This study provides...
reassurance that the introduction of Men C conjugates in the UK should in no way compromise this protection.

Acknowledgements

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