Abstract

The main objective of this study was to investigate the booster antibody response in individuals with initially high levels of diphtheria antitoxin. Sixty individuals eligible for the routine booster by the age of 18 years each received a single dose of 5 Lf of diphtheria toxoid in diphtheria-tetanus vaccine. A double antigen ELISA was used for the assessment of the antibody levels. Chaotropic disruption in paired ELISA was used to test antibody avidity. The ratio between initial and maximum antibody concentrations after 1 month was >10 times higher and after 6 months still four times higher in those with initial antibody levels <1 IU/ml. In individuals with initial antibody levels $\geq$ 1 IU/ml a two-fold decrease was observed after 6 months compared to the initial levels. Thus, vaccination of individuals with initial long-term protection against diphtheria (antibody levels $\geq$ 1 IU/ml) is unnecessary and should be avoided.

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1. Introduction

Diphtheria was a major threat in the pre-vaccine era until widespread vaccination was implemented. The resurgence of diphtheria in the Former Soviet Union countries (FSU) in 1990 was the first large diphtheria epidemic that has been registered in a country with a longstanding childhood immunisation programme [1]. The epidemic in the FSU started in 1990 and spread rapidly from 1991 to 1994. The epidemic peaked in 1995, and a steady decrease was then seen during the following years. Throughout the epidemic 70% of cases were reported among people aged 15 years or older [2].

In order to increase protection in the population in the FSU countries several changes of the vaccination schedule in childhood were made and urgent extraordinary immunisation of adults was performed. About 30 million doses of adult formulation of diphtheria toxoid were administered to adults only in 1994 [3]. By January 1998, more than 99 million adults had received at least one extra dose during the previous 8 years [2]. These measures increased the vaccination coverage and the number of protected individuals, but at the same time, many were boosted despite being well protected. Thus, protection among most children and many adults reached very high levels. In many individuals, the antibody concentrations exceed the level considered to give long-term protection with 50 times and in some age groups the full protection achieved 100% [4].

Primary vaccination elicits both protective immunity and memory. It is well known that immunological memory provides a swift and strong immune response upon re-exposure, as well as long-term protection even in individuals with very
low and undetectable antibody levels [5]. At the same time, little is known about the influence of specific IgG on post-
secondary booster immune responses. We expected young Russian adults to have high levels of
diphtheria antitoxin. The aim of this investigation was to study the dynamics of the booster response in individuals with
initially high antitoxin levels to detect the immune response of boosting on high antibody levels.

2. Materials and methods

2.1. Subjects

One hundred and eighty-two healthy Russian men eligible for a routine booster of dT (diphtheria-tetanus vaccine) were
included in the study. A 6-month complete follow up was possible for only 60 of them. The remaining 122 dropped out
because of reasons unrelated to vaccination (military service assignments). The study was approved by the Ethic Com-
mittee of the Research Board of the North State Medical University, Arkhangelsk, Russia.

The mean age of the participants was 18.7 ± 1 (S.D.) years. The previous vaccination status was documented for
36.6% of participants. The mean time since the last vacci-
nation was 4.3 ± 3.6 (S.D.) years. The minimum time was
6 months. The majority had received three booster doses of
diphtheria toxoid after completing the primary vaccination.
All participants received a single dose of dT, “Biomed”, Rus-
sia (five flocculation units of diphtheria toxoid, 5 units of
tetanus toxid).

The Russian routine immunisation schedule in general consisted of primary vaccination three or two doses during
the first year of age and boosters given at 2, 6–7, 11–12, 16 and than every 5 years [6]. Last decade the schedule was
changed several times.

The sample collection was conducted during May to
November 2001. Blood samples were taken before the
booster doses were given and after 1 week, and then 1–3 and 6 months after. Sera were separated from clotted blood
and stored at −20 °C until tested.

2.2. Serological methods

2.2.1. The double antigen ELISA

The double antigen ELISA (DAE) described by Kris-
tiansen et al. [7] was used for the assessment of the an-
tibody levels. Briefly, two-fold dilutions of tested and ref-
erence sera (international standard for diphtheria antitoxin,
WHO) were incubated overnight in antigen-coated 96-well
microtiterplates (Nunc-Immuno plates MaxiSorp cat. no.
349454, NUNC, Denmark). Next day, 1-h incubations with
biotin-labelled antigen (0.5 μg/ml) and streptavidin - horse
radish peroxidase were performed. Reactions were visualised
by using O-phenylene diamine as chromogen in citrate buffer
0.2 M 1.25 × 10⁻³% H₂O₂. A log–log reference curve was
used and the results were expressed as International Units per
millilitre (IU/ml). To minimise variation samples taken from
the same persons were performed on the same plate.

An antibody level of <0.01 IU/ml was considered as un-
protective, between 0.01 and 0.1 IU/ml as relatively protec-
tive, ≥0.1 as protective [8], and antibody levels ≥1 IU/ml as
providing a long-term protection [9].

The DAE results for 21 sera were compared to the in vitro
neutralisation test measurements (Vero cells) [10]. A good
correlation was shown (R²=0.83) (data not presented).

2.2.2. Avidity index

For the description of the functional activity of antibody
and its possible changes during the course of the immune
response, the relative avidity of the antitoxin was measured.
For avidity index (AI) assessment denaturing enzyme-linked
immunosorbent assay was used [11,12]. The method was
based on DAE. All sera were performed in four parallels,
two of which were treated with urea 8 M for 1 h in darkness
in room temperature. The other two were incubated in PBS
1% BSA. The checkerboard titration was performed to es-
idue peak concentration influence on the antibody concentra-
tion decline. “Back transformation” and original data scale
were used where possible.

Avidity index were found to be normally distributed. The
statistical analysis was performed without transformations.
The mean, standard deviation, median and quartiles were
computed to describe the distribution and variability. Paired
t-test was used to compare the means.

3. Results

3.1. Antibody levels

The initial antibody levels were high in all individuals
(Table 1). Nobody was considered to be unprotected, six per-
sons were relatively protected, and the other 54 individu-
Significant increase of the antibody levels occurred already after the first week and the highest levels were reached 1 month after the vaccination (Table 1). The increase to maximum concentration averaged seven-fold. After 5 months the antibody levels decreased significantly from the maximum values but the difference between initial and final concentrations was not significant.

The study population was divided into two groups to investigate if very high initial antibody levels influenced the antibody response to booster. The “low” group included participants with initial antibody level lower than 1 IU/ml, and the “high” group individuals with initial antibody level higher than or equal to 1 IU/ml. We found significant differences in antibody levels between the two groups only before the booster vaccination and 1 week and 3 months after (Table 2).

In both groups, the peak antitoxin concentration was reached within 1 month. The ratio between initial and maximum concentration was more than 20 times higher in the “low” group (Table 2). However, the ratio between maximum and final (6 months after) concentration was around five-fold for both groups and also for the whole sample. In the “low” group, we observed a four-fold increase in the antibody levels 6 months after booster. In the “high” group, however, we found a significantly lower mean level compared to the mean level before boosting.

We found a significant relationship between the initial antibody levels and the increase within 1 month \((R^2 = 0.5, p < 0.001)\) using linear regression. However, no relationship was revealed between the initial levels and the subsequent decline. The antibody levels measured after 1 month had a mild influence on the antibody levels 6 months after the booster and also on the decline in the antibody levels \((R = 0.2, R^2 = 0.27, p < 0.001)\).

3.2. Avidity index

Very slow and statistically not significant increase from initial \((0.42 \pm 0.10)\) to 3-month \((0.47 \pm 0.12)\) avidity index was found. During the subsequent 3 months, a statistically significant decline was observed to the index \(0.40 \pm 0.10\). No significant difference in avidity index was found between the “high” and the “low” group in the course of the immune response. There was no correlation neither between AI and GMC at any point of examination nor between AI and changes in antibody levels in the course of booster response.

### Table 1

Diphtheria antitoxin levels measured before and five times after the booster

<table>
<thead>
<tr>
<th>Time after the booster</th>
<th>Number of subjects</th>
<th>GMC*</th>
<th>Range</th>
<th>Ratio (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Day</td>
<td>60</td>
<td>0.73</td>
<td>0.01–17.35</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>1 Week</td>
<td>56</td>
<td>2.71</td>
<td>0.04–75.0</td>
<td>3.7 (2.3–6.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1 Month</td>
<td>46</td>
<td>5.07</td>
<td>0.14–148.10</td>
<td>7.0 (3.8–12.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2 Months</td>
<td>43</td>
<td>3.94</td>
<td>0.17–241.7</td>
<td>4.4 (2.5–7.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3 Months</td>
<td>56</td>
<td>3.11</td>
<td>0.04–95.81</td>
<td>4.6 (2.9–7.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>6 Months</td>
<td>60</td>
<td>1.10</td>
<td>0.01–19.68</td>
<td>1.4 (0.9–2.4)</td>
<td>0.158</td>
</tr>
</tbody>
</table>

a Geometric mean concentration.

b Ratio GMC for the GMC of day 0.

c Confidence interval.
d For comparison with level at day 0.

### Table 2

The dynamic of diphtheria antitoxin levels after the booster in groups with initially high (≥1 IU/ml) and low (<1 IU/ml) antitoxin levels

<table>
<thead>
<tr>
<th>Time after the booster</th>
<th>“Low” group</th>
<th>“High” group</th>
<th>Group ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Day</td>
<td>0.21</td>
<td>1</td>
<td>2.94</td>
</tr>
<tr>
<td>1 Week</td>
<td>1.77</td>
<td>4.40</td>
<td>4.60</td>
</tr>
<tr>
<td>1 Month</td>
<td>4.44</td>
<td>22.80</td>
<td>5.88</td>
</tr>
<tr>
<td>2 Months</td>
<td>3.53</td>
<td>12.95</td>
<td>4.36</td>
</tr>
<tr>
<td>3 Months</td>
<td>2.24</td>
<td>10.65</td>
<td>4.66</td>
</tr>
<tr>
<td>6 Months</td>
<td>0.85</td>
<td>4.1 (2.1–7.7)</td>
<td>1.35</td>
</tr>
</tbody>
</table>

a Ratio GMC for the GMC of day 0.
b Level compared to day 0.
c Ratio GMC for the “low” group compared to the “high” group.
d The difference between the “low” and the “high” group was statistically significant.
4. Discussion

The main purpose of booster immunisation is to maintain sufficient protection against a disease. The frequency and number of boosting with most vaccines is still a matter of discussion. Rare boosters or absence of routine booster pro-
grammes does not provide protective immunity in an adult population [13]. On the other hand, short intervals between doses will result in unnecessary vaccination of protected individuals [4]. It cannot, however, always be avoided if the benefit for population at risk outweighs the possible disadvantages. There is no doubt that a routine vaccination schedule has to provide protection in the target population. The protection has to be maintained at a minimal sufficient level, which mainly is provided by high immunisation cover-
age. Additional boosters often reach the same individuals and cause a hyperimmunisation, but influence the coverage little. During an epidemic the increasing risk of the infection pro-
vides at the same time opportunity for natural immunisation and reduces the necessity of boosters. Immunised individ-
uals who are infected usually develop carriage or very mild disease.

Little is known about the influence of specific IgG on the booster immune response. Suppressive influence of diphther-
ia antitoxin on primary immune response has been observed in humans [14], and has been demonstrated in mice models [15]. A tendency of a decreasing booster effect with increas-
ing preboosting antitoxin levels was found by Fel’diblum et al. [16] and Ronne et al. [17].

In the present study, the vaccinees with initially long-term protection with antibody levels ≥1 IU/ml responded to the booster dose by doubling the antibody levels after 1 month. However, after 6 months they had significantly lower anti-
body levels than before the booster dose was given. Individ-
uals with lower initial protection (antibody levels <1 IU/ml) had a 20-fold increase after 1 month and reached approxi-
mately the same maximum levels as those with high levels. After 6 months, the GMCs were significantly higher than the initial ones. Thus, vaccination of individuals with initial long-term protection against diphtheria seems unnecessary and should be avoided since the boosting has a suppressive effect on the antibody levels.

An inverse influence of pre-vaccination antitoxin levels on booster response has also been found in other studies [13,16–18]. Because of much lower initial concentrations found in these studies, the relation was not as strong as demonstrated in our study. We experience confirmed the gen-
eral knowledge of booster response kinetics. First, a rapid in-
crease to the maximum concentration within 1 month is seen then keeping the maximum for a few weeks and, thereafter, a fast decline followed by a subsequent slower decline. In our study, the average antibody level increase (seven-fold) was much lower than demonstrated in other studies [13,17,19], but corresponded well to findings when the participants had cor-
responding antibody levels before boosting (0.1–1.0 IU/ml) [13].

We have chosen the threshold level at 1 IU/ml, since it is assumed to provide a long-term protection. Unexpectedly, it divided the sample in half. Similar distribution was reported by Olander et al. [20] for a group of Finnish adults (mean age 26.1 years).

There was no significant difference in the peak concentra-
tion between the “low” and “high” group. This indicates that frequent boosters could result in an increase of the antibody level in individuals until they reach the maximum antibody levels. More stimulation will not change this peak-level sig-
ificantly. This explains the independence of the maximum concentration from the pre-vaccination antitoxin level [20]. At the same time, fast decline from the maximum level may have a “standard” rate independent of antibody levels. We found a weak influence of maximum concentration on the fast decline but considered it immunologically insignificant although it was statistically significant. Simonsen et al. [21], though, found the peak values decisive for the duration of immunity. Even if our conclusion is true only for high and very high concentrations, it seems important to measure the antibody levels 3–6 months after booster in order to assess the booster response and make some predictions regarding long-term protection of the target population.

We assessed the antitoxin concentration by double antigen ELISA. This method was proven to correlate well with the in vitro neutralising test [7]. Poor correlation of antibody levels measured by ELISA has been found mainly for low values [10], but none of the participants in this study had low antibody levels. Hence, this method was appropriate for the study.

Some problems regarding external validity were caused by a high number of dropouts in the follow up period. The reason for this was not connected to any medical causes. The loss of follow up happened randomly in the study, as the antibody levels were unknown to the participants, their superiors, and the investigators until well after sample collecting was over.

We used estimation of relative avidity (the avidity index, AI) as a supplementary characteristic of post-vaccination an-
tidiphtheria immunity. For assessment of relative avidity, we used the method of the antibody–antigen bond disruption by a chaotropic agent. These methods are widely used for differ-
ent antigens, however, to our knowledge has never been used with diphtheria toxoid. The study design did not allow us to establish a scale for low, intermediate or high relative avid-
ity for diphtheria antitoxin, since only boosted adults were included.

We found minor statistical changes in the index during the course of booster response. We believe this apparent increase of AI in well-boosted individuals to be of little biological sig-
nificance, although it has been shown in a cell culture model that the affinity continued to increase after three-repeated antigen stimulation [22]. We fail to find any connections be-
tween avidity index and antibody levels. Pollen et al. [23] de-
scribing similar findings assumed independence of antibody levels and avidity. Our findings support the theory taking into consideration the nature of affinity maturation.
For statistical guidance.

Clinical Research, Haukeland University Hospital, Norway) for skilful technical assistance. This should be taken into account when planning a routine vaccination schedule. On the other hand, the lowest threshold of antidiphtheria antibody levels providing sufficient long-term booster response remains to be established.

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References