

Diphtheria Immunity of a West German Population by Measurement of Antitoxin Antibodies with Enzyme-linked Immunosorbent Assay

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Immunology & Infectious Diseases 5, 10-14, 1995

Summary

Diphtheria antitoxin antibodies were measured by solid-phase enzyme-linked immunosorbent assay (ELISA) on the basis of a calibrated antibody standard. Protective antibodies were expressed as International Units per ml (IU/ml). The method of quantitation employed is easily performed under routine conditions. Sensitivity proved to be 0.09 mIU/ml, and direct measurement of antitoxin concentrations ranging from 0.12 to 8.30 mIU/ml was carried out by using the standard curve. With a routine assay dilution of 1:100, serum concentrations from 12 to 830 mIU/ml were covered.

Sera from 2418 men and women in the age range of 2 - 70 years served to evaluate diphtheria immunity. Generally, immunity to diphtheria was at a higher level in the younger persons than in the older ones. Mean value of antitoxin titres for men in all age groups was higher than in the respective age groups of women. If diphtheria antitoxin titres >0.1 IU/ml were supposed to be protective, satisfactory including long-lasting immunity was found only in 20.5% and no immunity (≤ 0.01 IU/ml) in 32.1%, while 47.4% of the collected specimens exhibited low immunity (>0.01 to 0.1 IU/ml).

Individuals with insufficient immunological protection have a high risk of infection. In order to avoid epidemic spreading, compulsory immunizations programs are helpful. The need for booster vaccinations is easily supported by ELISA measurement of the respective antibodies.

Introduction

Diphtheria is caused by *Corynebacterium diphtheriae*. Diphtheria infections have become rare in developed countries. This is mainly due to postnatal vaccination programs carried out according to expert recommendations (1, 2). Furthermore, bacteriological examinations have also shown a disappearance of toxigenic diphtheria strains, at least in Germany (3), and natural immunity will not be acquired. In any case, reliable immune protection can be only expected from periodic booster immunizations.

The minimum protective titre is thought to be > 0.01 IU/ml (4, 5), but the true value of protection is not exactly known. Thus, a ten-fold higher diphtheria antitoxin concentration is recommended (6-9); > 0.1 IU/ml may provide a satisfactory level.

Outbreaks of diphtheria in Eastern Europe, and the recently increased numbers of disease cases in Western Europe show that this infectious disease has not disappeared (8-13). There may be various reasons for this. In any case, serological surveys on diphtheria immunity will contribute to the decision as to whether and what vaccination strategies are to be adopted. For such prevalence studies, routine methods of wide applicability are needed.

Antitoxin immunity is traditionally assessed with functional methods such as the intradermal neutralization test in guinea pigs and rabbits (14, 15) or clinically by the Schick test (16, 17). *In vitro* assays have also been proposed including tissue culture neutralization assay (18), passive hemagglutination (19), quantitative immunoelectrophoresis (20) and enzyme immunoassay (21). Even if a trend towards enzyme-linked immunosorbent assays (ELISA) can be observed, the procedures described were mostly designed for research and not for application under routine conditions.

It is supposed that diphtheria antitoxin ELISAs may be an alternative method for the rapid estimation of antitoxin antibodies in diagnostic laboratories as well as for epidemiological studies. Thus, the authors developed an ELISA analogous to a previously described tetanus antitoxin assay for seroprevalence studies. It proved useful for this purpose (22). The assay is calibrated against the standard WHO preparation. Various experiments demonstrated appropriate sensitivity and specificity. Then, diphtheria immunity in male and female persons from Germany was reviewed by a seroepidemiological study on the age-dependent distribution of diphtheria antitoxin titres.

Methods and Materials

Subjects and sera

Serum samples were collected in the years 1992 - 1994 from normal volunteers and from patients aged 2 - 70 years. Patients had been submitted for reasons other than diphtheria to various local hospitals in different areas of Germany. The study on age-dependent distribution of diphtheria antitoxin comprised a total of 2418 specimens from men and women.

Antigen

Diphtheria toxoid of high purity (2900 limit flocculation units per ml, 1730 Lf/mg N and 10.5 g of protein per l) was obtained from Behringwerke Marburg, Germany.

Antitoxin

A human serum pool from 16 normal children aged 9 - 18 years with a complete diphtheria immunization history was prepared for use as local reference serum and substandard in our ELISA test. Calibration was performed by Behringwerke Marburg, Germany, using intradermal neutralization tests in guinea pigs and a horse standard preparation (diphtheria antitoxin standard, 9 IU/ml, Behringwerke Marburg, Germany; International Standard for Diphtheria Antitoxin, 10 IU/ml, Statens Seruminstitut, Copenhagen, Denmark).

Conjugates

Alkaline phosphatase-labeled goat anti-human IgG was purchased from Dianova, Hamburg, Germany and used at a dilution of 1:5000.

ELISA procedure

ELISA was performed analogously to a previously described tetanus antitoxin ELISA (22). Briefly, microtitre plates were coated with purified diphtheria toxoid at a dilution of 1:6400. Plates were incubated for 45 min with patient sera at a dilution of 1:100 (measurement range 12 to 830 mIU/ml) or 1:1000 (measurement range, 120 to 8300 mIU/ml). Each microtitre plate contained the sera of the standard curve and all patient sera as duplicates, (positive control sera as well as negative controls). Anti-IgG antibodies conjugated with alkaline phosphatase served as second antibodies. This incubation period (45 min) was followed by a reaction with the substrate (*p*-nitrophenylphosphate, Merck, Darmstadt, Germany) for 20 min. All reagents were used in standard volumes of 100 µl, and all reactions were carried out at 37°C in a water bath. The optical density at 405 nm was measured after the addition to each well of 50 µl of stop solution (1 M NaOH) with an ELISA reader. The amount of diphtheria antitoxin was calculated by a curve fit programme based on linear regression of measured standards and their defined values.

Intra- and inter-assay studies were performed using serum samples of high, medium, and low antitoxin levels. Intra- and inter-assay experiments were done in replicates of 15 and 17 runs, respectively. Sensitivity of the ELISA was defined by the lowest measurable standard concentration which can be discerned from the zero standard, i.e. 3 SD above zero-standard.

For the evaluation of matrix effects, bovine serum albumin (5%), fetal calf serum, normal goat serum, normal horse serum, normal rat serum and hemoglobin (at levels up to 250 mg/dl) were spiked with serum of high antitoxin titre. Linearity of the ELISA was proved by dilution experiments. Serum specimens with elevated concentrations of diphtheria antitoxin were assayed at various dilutions covering the working range of the standard curve.

Results of diphtheria antitoxin antibodies were associated with predefined titre levels (7, 11, 23, 24), i.e. (a) no immunity (≤ 0.01 IU/ml); (b) low immune protection ($> 0.01 - 0.1$ IU/ml); (c) satisfactory immune protection ($> 0.1 - 1.0$ IU/ml); (d) long-lasting satisfactory immune protection (> 1.0 IU/ml).

Neutralization test

In parallel with the ELISA procedure, some low and high titred human sera were checked with neutralization tests in cultures of VERO cells (18, 25). Briefly, serial dilutions of diphtheria antitoxin standard and human sera were pipetted in volumes of 100 µl each into the wells of a microtitration plate. Then, 50 µl of diphtheria toxoid (0.001 Lf/ml) and 50 µl of VERO cell suspensions (2.5×10^5 cells/ml) were added. Sealed plates were incubated at 37°C for 6 days. Antitoxin titre in serum specimens was calculated by observation of cytopathological effects and by comparison with the diphtheria antitoxin standard.

Statistical methods

Antitoxin measurements and personal data according to age groups and sex were transferred onto dBASE computer files. Statistical tests (Wilcoxon test, chi-square distribution) were employed for analysis of data.

Results

The ELISA technique used provided reproducible results. Sensitivity proved to be 0.09 mIU/ml, and antitoxin concentrations ranging from 0.12 to 8.30 mIU/ml could be measured directly from the standard curve. With a routine assay dilution of 1:100, serum concentrations from 12 to 830 mIU/ml were covered. Intra- and inter-assay variations reflecting the precision of the test are shown in Table 1. No significant matrix effects were noted when samples were spiked with serum proteins from different species or with high amounts of hemoglobin. A series of dilution experiments revealed excellent recovery (Table 2). Diphtheria antitoxin titres obtained with ELISA correlated well with the results of neutralization tests.

Table 1. Comparison of the intra- and inter-assay variations using sera of various diphtheria antitoxin concentrations

Controls	Mean (mIU/ml)	Intra-assay (CV (%))	Interassay CV (%)
Low	507	8.27	14.24
Medium	361	9.88	11.15
High	33	6.02	12.28

Table 2. Dilution parallelism, recovery of diphtheria antitoxin from diluted within-range specimens

Specimen dilution	Expected value (mIU/ml)	Observed value (mIU/ml)	Recovery (%) of expected value
1:100	9.95	8.75	88
1:1000	0.99	0.96	97
1:2500	0.39	0.35	90
1:5000	0.20	0.19	95
1:7500	0.13	0.11	85

Tables 3-5 show details of sex and age with regard to predefined immunity stages. Sufficient diphtheria immunity, i.e. antitoxin titres > 0.1 IU/ml, was found in only 20.5% of the collected specimens, and low immunity (> 0.01-0.1 IU/ml) occurred in 47.4%. No immunity (\leq 0.01 IU/ml) was detected in 32.1% of all persons. Antitoxin titres varied with respect to age and sex in that with increasing age antibody titres decreased in general, and the number of persons with deficient immunity increased concomitantly. Within the 2-5 years age group, only 3.8% did not have measurable antitoxin antibodies, while this was the case in 24.3% of the over 60-year-old group. Finally, the mean value of antitoxin titres for men in all age groups was higher than in the respective age group of women. The medians of diphtheria antitoxin titres were regularly lower than the respective mean values.

Table 3. Distribution of defined diphtheria antitoxin titres with regard to sex

Specimens	Diphtheria antitoxin titres (IU/ml)			
	≤ 0.01	> 0.01–0.1	> 0.1–1.0	> 1.0
Male persons (n = 1302)	400	579	312	11
percent	30.7	44.5	24.0	0.8
Female persons (n = 1116)	375	566	172	3
percent	33.6	50.7	15.4	0.3

Table 4. Age distribution of defined diphtheria antitoxin titres in male subjects

Antitoxin (IU/ml)	Number (and percent in parentheses) of men with diphtheria antitoxin titres in age group (years)								
	2–5	6–10	11–14	15–20	21–30	31–40	41–50	51–60	> 60
≤ 0.01	5 (5.5)	22 (15.2)	24 (25.4)	28 (19.9)	58 (33.9)	97 (51.6)	71 (39.5)	21 (15.8)	74 (46.8)
> 0.01–0.1	46 (50.5)	66 (45.5)	29 (30.5)	62 (44.0)	71 (41.5)	76 (40.5)	92 (51.1)	80 (60.1)	57 (36.1)
> 0.1–1.0	37 (40.7)	56 (38.6)	41 (43.2)	49 (34.7)	41 (24.0)	14 (7.4)	15 (8.3)	32 (24.1)	27 (17.1)
> 1.0	3 (3.3)	1 (0.7)	1 (1.0)	2 (1.4)	1 (0.6)	1 (0.5)	2 (1.1)	0 (0.0)	0 (0.0)
Mean of values	0.260	0.182	0.221	0.171	0.113	0.043	0.060	0.079	0.052
Median of values	0.09	0.07	0.08	0.05	0.02	0.01	0.02	0.04	0.02

Table 5. Age distribution of defined diphtheria antitoxin in titres in female subjects

Antitoxin (IU/ml)	Number (and percent in parentheses) of women with diphtheria antitoxin titres in age group (years)								
	2–5	6–10	11–14	15–20	21–30	31–40	41–50	51–60	> 60
≤ 0.01	16 (16.7)	26 (21.8)	10 (11.9)	10 (24.4)	41 (31.8)	57 (39.9)	80 (54.1)	44 (34.6)	91 (39.7)
> 0.01–0.1	37 (38.6)	55 (46.2)	49 (58.3)	18 (43.9)	65 (50.4)	75 (52.4)	62 (41.9)	76 (59.9)	120 (56.4)
> 0.1–1.0	42 (43.7)	37 (31.1)	25 (29.8)	13 (31.7)	23 (17.8)	10 (7.0)	6 (4.0)	7 (5.5)	9 (3.9)
> 1.0	1 (1.0)	1 (0.9)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)
Mean of values	0.185	0.132	0.108	0.090	0.087	0.039	0.023	0.033	0.021
Median of values	0.09	0.04	0.05	0.021	0.019	0.013	0.01	0.018	0.013

Discussion

Standard methods for estimating levels of diphtheria antitoxin are the neutralization test in guinea pigs (14) or in rabbits (15). Apart from these *in vivo* methods, *in vitro* procedures have been proposed. Of these, passive hemagglutination is widely used (19, 23, 26, 27). Neutralization tests in tissue culture may well be employed but require tissue culture facilities (18). All these methods, however, suffer from the drawback of complex handling and standardization as well as being time-consuming to perform. Their application is confined to specialized laboratories. The rapid development of ELISA techniques for the measurement of all kinds of antigens and antibodies seems to be a useful alternative to *in vivo* assays and a promising approach to quantifying diphtheria immunity (21).

The ELISA method described here proved suitable for the routine laboratory. Small as well as large specimen numbers were readily measured. For the latter purpose, devices for microprocessor controlled robotic sampling and dilution could be easily adapted. The assay was sensitive and easy to perform. Appropriate calibration by use of a WHO standard preparation was

an important step enabling reproducibility of the results. The assay sensitivity covered a wide range of diphtheria antitoxin concentrations which was found to be sufficient for routine administration. Good correlation was found in a series of experiments with low, medium and high titred antitoxin sera in both ELISA and neutralization tests. This supported the reliability of the antibody concentration measured by ELISA.

The data have shown that only 20.5% of the persons investigated had definitive immunity to diphtheria. Indeed, 0.6% of this population had very high antitoxin titres greatly in excess of 1.0 IU/ml. In a further 47.4% of the subjects, a low but nevertheless demonstrable immunity could be detected. Altogether, 32.1% of all subjects did not have adequate immunity.

In general, mean antitoxin titres and, consequently, diphtheria immunity decreased steadily with increasing age. This is in agreement with other reports (3, 7, 8, 23, 24, 28-35). To a large extent, children also exhibit significant deficiency in diphtheria immunity within 5-6 years after their primary immunization (27).

However, the 51- to 60-year-old age group exhibited better immune protection by virtue of higher antitoxin titres than the 41- to 50-year-old group. This observation was highly significant (probability of error in the Wilcoxon test and chi Square distribution $< 0.1\%$). It might be that these persons have maintained long-lasting natural immunity, probably acquired from past diphtheria epidemics in Germany in the 1940s (30); differences in vaccination characteristics compared with other age groups seem unlikely.

Generally, women exhibited lower antitoxin titres than men. This finding was also observed in the Danish population in the age range of 30 - 70 years. It was thought to be due to routine vaccination of military personnel (33). Comparable results were reported for Sweden (8). This, however, cannot hold true in this study because sex differences occurred in all age groups including children, and vaccination of military persons with diphtheria toxoid is not routine in Germany. Hence, this phenomenon remains unclear. An objection that the observed sex differences might come from systemic troubles inherent to the ELISA principle appears very unlikely because no such findings have been reported until now in the large field of other ELISA applications.

The reasons for the recrudescence of diphtheria are the decreasing immunity due to relaxation of endeavours for appropriate vaccination and the introduction of toxigenic pathogens, especially from developing countries and from the East. In the absence of antigen stimulation by circulating toxigenic diphtheria bacteria strains or without regular booster vaccinations, the protective antibody titres fall below the protective threshold. Unprotected persons then do not only have a high individual risk, but also once more enable spreading of diphtheria on an epidemic scale.

Actually, it is not usual to vaccinate adults against diphtheria. However, if the introduction of toxigenic strains cannot be prevented, vaccination is required. This is in agreement with other authors who propose primary immunization or re-vaccination of all adults with non-protective antitoxin titres. Such a procedure is supported by the observation that immunization of adults with licensed diphtheria toxoid is safe and effective for the generation of protective antibody titres (36-38). Apart from primary or booster vaccinations, a regular seroimmunological surveillance is important.

After immunization in childhood, appropriate re-vaccinations are omitted for various reasons. Fear of adverse reactions in the course of diphtheria booster vaccination bears much of the

responsibility Another aspect is that the disease and its complications have been put out of mind. Also, re-vaccinations are simply forgotten and physicians do not take opportunities to administer the booster vaccinations required. It was concluded from titration patterns of specimens which were obtained from children at two different time periods that the administration of a booster dose should be considered about 12 years after the last injection (39).

In consideration of a rise of diphtheria together with the obvious decrease of diphtheria immunity, one can deduce that compulsory immunization programmes are necessary. Each medical consultation (whether in private practice or in hospital) is an opportunity to verify the diphtheria immune status and to complete necessary vaccinations. In order to promote public health awareness, comprehensive information about the importance of immunizations, the diseases they prevent and the recommended vaccination schedules are necessary. Finally, serological review of diphtheria immunity is a criterion to monitor the effectiveness of vaccination and can be regarded as a part of public health care.

Acknowledgement

We gratefully acknowledge the helpful suggestions of Mrs. Milcke-Ungeheuer (Behringwerke Marburg, Germany) who provided us with diphtheria toxoid of high purity and who also calibrated a human serum pool.

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