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Lack of significant differences in immunity against diphtheria between populations of Eastern and Western regions of Poland

Diphtheria is a bacterial disease in which the clinical manifestation results from the action of an extracellular substance (exotoxin) produced by Corynebacterium diphtheriae. This disease is acquired through respiratory droplets and close personal contact. The incubation period is usually 2 to 5 days. Diphtheria is a disease affecting the tonsil, the pharynx, the larynx and the nose. The lethality of diphtheria is almost entirely due to extracellular diphtheria toxin, so immunity to this disease depends primarily on antibodies directed against diphtheria toxin. Specific antitoxin, is mainly of the IgG class. Antibody may be induced by diphtheria toxin during infection, carrier state, or by diphtheria toxoid immunization. These antibodies are identical and cannot be distinguished by any existing technique (15).

During the past years there has been an increase of the incidence of diphtheria in countries which were formerly a part of the Soviet Union (3). In recent years there has been an increase of the incidence of diphtheria in countries to the east of Poland: the Russian Federation (13), Belarus and Ukraine (12).

Several seroepidemiological studies have shown low levels of antibodies able to neutralise diphtheria toxin among adults, even when immunity to diphtheria among children and teenagers in the same population has been demonstrated in cases reported in Poland and it is high (full). It is believed that a circulating diphtheria antitoxin level of 0.01 IU/ml determined by the neutralization test in animals or in cell cultures provides immunity against the disease. In some studies which used *in vitro* techniques, a level of 0.1 IU/ml was considered protective (2,4). The estimation of specific antitoxin IgG levels in the population is important to monitoring the potential threat of infection.

The aim of the study was to evaluate immunity to diphtheria in healthy individuals from two geographically different regions of Poland. In this study, serum diphtheria antitoxin IgG levels in two group of hospitalized patients in Western (Zielona Góra) and Eastern (Lublin) regions of Poland was evaluated.

MATERIAL AND METHODS

Diphtheria antitoxin level was tested in the total of 1,236 blood samples by enzyme immunoassay (toxoid ELISA). The serum samples analyzed in this study were obtained from hospitalized patients (without upper respiratory tract symptoms) from Western (n= 707) and

Eastern (n= 529) regions. Samples from patients with cancer, autoimmune diseases, primary or secondary immunodeficiencies or acute infections, or those who had received immunosuppressive medication were excluded.

Enzyme-linked immunosorbent assay (ELISA). Anti-diphtheria toxin antibodies in all samples were determined by enzyme immunoassay using the Toxoid ELISA test written previously by Walory et al. (10). The absorbance was measured at 492 nm using Power Wave X (Bio-Tek, USA) plate spectrophotometer. The optical densities measured in the wells with test serum dilutions were compared with reference antitoxin dilutions. Only those OD values which fell within the range of the linear part of the standard curve were used to calculate titres. The results were expressed in international units per ml of serum (IU/ml). The standard for human diphtheria antitoxin (1.5 IU/ml, NIBSC, UK) calibrated antitoxin titre. For calibration, as a reference method, the neutralization test of toxin on Vero cells was used (10).

Statistical analysis (5,14). The antitoxin concentrations obtained were classified according to the following categories of protection: no protection (<0.1 IU/ml), protection (0.1-1.0 IU/ml), high protection (>1.0 IU/ml) 1,6. The data were analyzed using the following computer programs: Excel 7,0, Statistica Pl. for Windows. The arithmetic (AMT) and geometric mean titre (GMT) was calculated to characterize the central tendency. Differences between two groups were compared by the Student's t- test.

RESULTS

The prevalence of antibodies to diphtheria toxin was studied in sera samples of 1236 individuals, ranging from newborns to persons over 65 years of age. The AMT diphtheria antitoxin concentrations for Western (W) and Eastern (E) regions were respectively 0.43 and 0.60 IU/ml. The differences were statistically significant at $p<0.0001$. Differences for the age

Table 1. Statistical parameters of diphtheria antibody levels by age groups in Western and Eastern region of Poland

Age groups	< 2month		2m.-18 years		> 19 years		Total		Risk group*	
Region	Western region	Eastern region	Western region	Eastern region	Western region	Eastern region	Western region	Eastern region	Western region	Eastern region
Arithmetic Mean-AMT	0.26	0.32	0.76	0.72	0.29	0.44	0.43	0.60	0.23	0.33
Geometric mean-GMT	0.17	0.19	0.46	0.37	0.17	0.22	0.23	0.30	0.14	0.17
Median	0.25	0.27	0.52	0.45	0.18	0.20	0.22	0.37	0.13	0.17
Standard deviation	0.19	0.28	0.79	1.04	0.35	0.67	0.56	0.92	0.25	0.54
Minimum	0.01	0.02	0.018	0.01	0.006	0.008	0.006	0.008	0.006	0.008
Maximum	0.61	0.96	5.29	6.9	3.7	5.32	5.29	6.9	1.79	3,81
Number of persons	26	23	211	322	470	184	707	529	136	128

* Number of persons in age groups, where percentage non-protected persons were over 30% total non-protected evaluated population. For Western region was from 30 to 64 years old age, for Eastern region from 25 to 64 years old age

group > 19 years (W=0.29 IU/ml; E=0.44 IU/ml) and the risk group (W=0.23 IU/ml; E=0.33 IU/ml) were statistically significant respectively at p=0.0043 and p=0.036. Differences for age group < 2 months of age were statistically insignificant, p=0.4. The distribution of AMT and GMT diphtheria antitoxin titres for each of the different age groups for the evaluated regions is presented in Table 1.

Table 2 shows the distribution of persons in different categories of protection and percentage of diphtheria antibody levels by age groups. In the Eastern group there were insignificantly fewer individuals (p=0.29), who completely lacked immunity to diphtheria (< 0.1 IU/ml), 113/529 (21.3%) in comparison to Western region 170/707 (24%). Also, no significant differences were found in the age groups > 19 years, < 2 months of age and the risk group, p= 0.54; p= 0.56 and p=0.66, respectively. Significant differences between regions were observed in the age group 2 months-18 years of age for persons lacking protective immunity against diphtheria (W=7.6%; E=16%; p=0.0001) and for persons long-term protected (W=28%, E=15%; p=0.0004). The majority of non-protected individuals (>60%) were adults >19 years of age. In protected individuals no significant differences in mean, median and GMT of diphtheria antitoxin levels were observed among sexes. The best protected group consisted of persons aged from 6 months to 29 years. None of the individuals over 50 years of age was protected for more than five years (titre >1.0 IU/ml). The mean antitoxin titre in person from 25 to 75 years age, as well as in children of 1 month old was reduced significantly compared to the other age groups.

Table 2. Distribution of non-protected and protected persons against diphtheria by age groups in Western and Eastern regions of Poland

Age groups	< 2month		2m.-18 years		> 19 years		Total		Risk group	
Region	Western region	Eastern region	Western region	Eastern region	Western region	Eastern region	Western region	Eastern region	Western region	Eastern region
Persons < 0.1 IU/ml	8	9	16	52	146	52	170	113	94	46
Percentage (%)	30.8	39	7.6	16	31.0	28.2	24.0	21.3	40	36
Persons 0.1-1.0 IU/ml	18	14	136	221	303	119	457	354	140	78
Percentage (%)	69.2	61	64.4	69	64.5	64.7	65	67	57	60.9
Persons > 1.0 IU/ml	-	-	59	49	21	13	80	62	8	4
Percentage (%)	-	-	28	15	4.5	7.1	11	11.7	3.0	3.1
Total (N)	26	23	211	322	470	184	707	529	236	126

DISCUSSION

Many properties of diphtheria toxin *in vivo* and *in vitro* are utilized to determine the activity of diphtheria antibodies. For ethical, economic and practical reasons there is a growing interest in *in vitro* techniques. The enzyme-linked immunosorbent assay (ELISA) involves

binding of antigen to the wells of a microtitre plate. Exotoxins (or toxoids), which have a highly lipophilic moiety in their molecule, coat the tubes efficiently (9). Results of the toxoid-ELISA test have been found to be highly reproducible (1). When the antibody level is above 0,1 IU/ml, the results of the ELISA test correspond well with results of the *in vivo* neutralization test in guinea pigs (6) and the *in vitro* neutralisation test in tissue culture (7,10). When the antibody titre is low, the results of the ELISA test correspond poorly with results of the neutralisation tests. A titre of 0,001 IU/ml with the neutralization test can be 10 to 100 times higher (0.01 to 0.1 IU/ml) with the toxoid-ELISA test (6,7). Protection to diphtheria based on antitoxin levels can be classified in three groups (6,7,8) no protection (< 0.1 IU/ml), probable protection for less than one year (0,1-1,0 IU/ml) and lasting protection for more than five years (> 1.0 IU/ml).

Comparison of protection status in both regions shows lack of statistically significant differences, despite meaningful geographical differences of the evaluated regions. Complete lack of humoral protection before falling ill was found 24% in Western region population and 21% in population of Eastern Poland. The majority of the populations examined had antibody levels indicating relative protection for less than one year (W=65%, E=67%), while only 11–12% were long–term protected – for more than five years. Also, no significant differences were found in the age group > 19 years, < 2 months of age and the risk group. Significant differences between regions were observed in the age group 2 months – 18 years of age for persons lacking protective immunity against diphtheria (W=7.6%; E=16%) and for persons long-term protected. Differences probably result from different realization of booster doses immunization against diphtheria at 6, 14 and 19 years (Td). The national recommendations for immunization against diphtheria consists of primary vaccination at 2–3 months, 4.5 and 6 months (with a combined vaccine against diphtheria, tetanus and pertussis (DTP), followed by the fourth dose at 19–24 months and booster doses at 6, 14 and 19 years (Td). In this study it has been shown that children immunized by the primary vaccine program with DTP still had protective diphtheria antibodies approximately 16 years later. In this study the groups with the lowest level of diphtheria antibodies were found to be about of 1 month of age and 25–65 year-olds. Our data compares well with the results of previous studies in Poland, in which the age group with the lowest level of diphtheria antibodies was of 40–50-year-olds (4) or 30–64-year-olds (11).

Outbreaks of diphtheria in the Russian Federation (13), Belarus, Ukraine (12) have drawn attention to the reappearance of a vaccine-preventable disease.

CONCLUSION

Higher overall rate of protection in population of the Eastern region of Poland in comparison to the Western region has been revealed. The insignificant difference is probably due to the higher frequency of contacts with a natural reservoir of bacterium and Russian epidemics. We suggest routine booster immunization in people older than 25 years in regions with high rate of ongoing migration.

According to current and previous data from Poland and other European countries, one can conclude that due to inadequate immunity to diphtheria, recommendations for routine booster vaccinations of adults should be implemented in Poland, particularly in the border regions of the country as well as in other countries with considerable immigration from high risk areas.

REFERENCES

1. Camargo M. E. et al.: Immunoenzymatic assay of antidiphtheric toxin antibodies in human sera. J Clin. Microbiol., 20, 772, 1984.
2. Cellesi C. et al.: Immunity to diphtheria in a sample of adult population from central Italy. Vaccine,7, 417, 1989.
3. Centers for Disease Control: Diphtheria epidemic – new independent states of the former Soviet Union, 1990-1994. Morbidity and Mortality Weekly Report , 44, 177, 1995.
4. Galazka A. et al.: Immunity against diphtheria in adults in Poland. Epidemiology & Infection, 103, 587, 1989.
5. Harlow E. et al.:Antibodies. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1988.
6. Knight P.A. et al.: Studies on the correlation of a range of immunoassays for diphtheria antitoxin with the guinea-pig intradermal test. Dev. Biol. Stand., 64, 25, 1986.
7. Melville-Smith M. et al.: Estimation of Corynebacterium diphtheriae antitoxin in human sera: a comparison of an enzyme-linked immunosorbent assay with the toxin neutralization test. J. Med. Microbiol.,25, 279, 1988.
8. Scheibel I. et al.: Duration of immunity to diphtheria and tetanus after active immunization, Acta Pathol. Microbiol. Scand., 67, 380, 1966.
9. Swenson S.B. et al.: An enzyme-linked immunosorbent assay (ELISA) for the determination of diphtheria toxin antibodies., J. Immunol. Methods,17, 249, 1977.
10. Walory J. et al.: Comparison of four serological methods for detection of diphtheria antitoxin antibody. J. Immunol. Methods, Nov 1, 245, 1-2, 55, 2000.
11. Walory J. et al.: W. The prevalence of diphtheria immunity in healthy population in Poland, Epidemiology & Infection,126, 225, 2001.
12. World Health Organization: Expanded programme on immunization. Diphtheria epidemic. Weekly Epidemiological Record, 34, 253, 1994.
13. World Health Organization: Expanded programme on immunization. Outbreak of diphtheria. Weekly Epidemiological Record, 19, 134, 1993.
14. World Health Organization: Laboratory methods for the testing for potency of diphtheria (D), tetanus (T), pertussis (P) and combined vaccines, 1992, WHO Technical Report Series No. BLG/92.1, Geneva 1992.
15. World Health Organization: The Immunological Basis for Immunization / Module 2, Diphtheria 1993.

SUMMARY

Incidents of diphtheria in countries which were formerly part of the Soviet Union (Ukraine, Russia and Belorus) resulted in the need to evaluate thoroughly the effectiveness of preventive vaccination in Poland, especially in the border regions of the country where the biggest migration of population can be observed. The aim of this work was a comparison of the immunity to diphtheria in two geographically different regions of Poland – eastern (Lublin) and western (Zielona Gora) ones. It showed immunoprophylaxis to diphtheria that was implemented on these areas. Diphtheria antitoxin level (IgG) was determined with application of the ELISA method in 1236 (529/707) people. No significant differences were found in the level of antibodies in the groups < 2 years of age and > 19 years of age in people below the protective titre (0.1 IU/ml). The difference occurring in the interval between 2nd and 18th year of life (in western Poland 7.6% and in eastern Poland 16%) may result from different implementation of the vaccination programame in these regions (booster doses). Recommendations for vaccination to diphtheria in people over 25 years of age should be implemented especially in the frontier regions of Poland adjoining countries threatened with diphtheria occurrence.

Brak istotnych różnic w odporności przeciwbłoniczej pomiędzy populacjami wschodniej i zachodniej Polski

Przypadki błonicy w krajach byłego Związku Radzieckiego (Ukraina, Rosja, Białoruś) stworzyły konieczność dokładnej oceny skuteczności szczepień ochronnych w Polsce, szczególnie na terenach przygranicznych, gdzie obserwuje się największą migrację ludności. Celem pracy było porównanie stanu odporności przeciwbłoniczej dwóch różnych geograficznie obszarów przygranicznych Polski - wschodniego (Lublin) i zachodniego (Zielona Góra). Dało to obraz prowadzonej na tych terenach immunoprofilaktyki przeciwbłoniczej. Oznaczenie przeciwciał przeciwbłoniczych (IgG) przeprowadzono metodą ELISA u 1236 (529/707) osób. Nie stwierdzono istotnych różnic w poziomie przeciwciał w grupach < 2 lat i > 19 lat u osób poniżej miana ochronnego (0,1 IU/ml). Różnica występująca w przedziale między 2 a 18 rokiem życia (w zachodniej Polsce 7,6%, wschodniej 16%) może wynikać z różnej realizacji na tych terenach programu szczepień (dawek przypominających). Powinno być zalecane szczepienie przeciwko błonicy u osób powyżej 25 roku życia, szczególnie na obszarach przygranicznych, sąsiadujących z krajami zagrożonymi występowaniem błonicy.