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ARTICLE

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Determination of tetanus antibodies by a double-antigen enzyme-linked immunosorbent assay in individuals of various age groups

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Abstract In this study, tetanus immunity was determined in 549 randomly chosen individuals of various age groups in Ankara, Turkey. Antibody levels in sera of the individuals were measured using a double-antigen enzyme-linked immunosorbent assay. Overall, 66.5% (95%CI, 62.4–70.4) of the population studied was found to have basic protection (≥0.01 IU/ml) against tetanus. Protective levels of tetanus antibodies declined progressively with age. The rate of protection in children and adolescents (aged <20 years) exceeded 90%, while only 16.3% (95%CI, 8.9–26.2) of those over 60 years of age were protected. Females over 60 years of age were less immune than males of the same age group (p=0.034). Although the rates of protection in children and adolescents are regarded as satisfactory, the rates among adults are low. Preventive measures against tetanus should therefore focus on scheduled booster immunization for adults as well as children.

Introduction

Tetanus is a rare disease in most industrialized countries, but it is still an important public health problem in many parts of the developing world [1, 2]. Protection from tetanus is achieved by immunization with tetanus toxoid. In our country, administration of tetanus toxoid has been a part of the childhood immunization program since 1962. In 1985, the Expanded Immunization Program was established and is currently administered by the Ministry of Health of Turkey. The national policy of immunization for tetanus includes three doses of combined vaccine against diphtheria, pertussis, and tetanus given at 2, 3, and 4 months of age, followed by a fourth injection at 16–24 months of age. At 7 and 14 years of age, additional boosters are given against diphtheria and tetanus. Thereafter, booster doses are advised for every 10 years. However, booster immunizations after adolescence are not routinely performed. According to the data published by World Health Organization (http://www.who.int/vaccines/globalsummary/timeseries/TSincidenceByCountry.cfm?country=Turkey), the average number of total tetanus cases for our country, including neonatal tetanus, has been about 92.8 cases per year for nearly the last 24 years. More than half of the tetanus cases reported since 1990 in our country occurred in individuals older than 45 years of age [3].

Antibodies that develop after vaccination protect the individual from tetanus. A tetanus antitoxin titre of 0.01 IU/ml determined by a toxin neutralization test or a double-antigen enzyme-linked immunosorbent assay (ELISA) is accepted internationally as an approved level of protection [4, 5]. The level of 0.01 IU/ml as determined by an indirect ELISA may not be equivalent to the same level of antitoxin determined by the standard toxin neutralization method [4]. Furthermore, tetanus cases have been reported in individuals who had the protective level (0.1 IU/ml) of the antibody as measured by indirect ELISA [6, 7]. Therefore, in recent studies, a tetanus antibody level lower than 0.15 IU/ml in an indirect ELISA is considered nonprotective [8–12].

Although vaccination is the main method for preventing tetanus, antibody titers decrease gradually with age and may fall below the protective level, especially in people who do not receive boosters. For this reason, determination of the immune status of subjects in different age groups may aid the development of vaccination schedules to provide continuous immunity.

Conventional indirect ELISAs have been widely used to determine tetanus antibody levels in human serum samples. However, false-positive results are sometimes encountered

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in indirect ELISAs when assaying antibodies in human sera. These false-positive reactions might be related to the presence of serological cross-reactions, autoimmune diseases, or rheumatoid factors [13–21]. Another reason for false-positive reactivity observed in indirect ELISAs is the interference of nonspecific antibodies [22]. Falsely increased tetanus antibody titres detected by conventional indirect ELISAs may also be observed due to the presence of nonspecific or low-avidity antitetanus antibodies in human sera [23, 24].

In an attempt to avoid false-positive reactions that might be encountered in conventional indirect ELISA systems, we used an in-house double-antigen ELISA system, recently developed for measuring antibodies to tetanus and diphtheria [25], to assess antitetanus antibody levels in human serum samples. The principle of the double-antigen ELISA depends on the binding of tetanus antibodies to the antigen-coated solid phase with one of its Fab arms. The solid-phase-bound specific antibody is then traced with enzyme-labelled antigen conjugate via binding to the second Fab arm of the antibody.

In the present study, by using this double-antigen ELISA system, we aimed to determine antitetanus antibody levels in various age groups in an urban population in Ankara, Turkey.

Materials and methods

Study population

This study was performed on residual sera obtained for other diagnostic purposes at the Gazi University Hospital in Ankara, the capital of Turkey. Sera were collected between January 15, 2004 and July 15, 2004. All sera included in the study were derived from an urban population in Ankara, Turkey. Age, sex, and district records of the subjects from whom samples were obtained were taken from database records of our hospital. Samples representative of different districts of Ankara were randomized according to their data file record numbers and then included in the study. Serum specimens were stored at –70°C until use. All samples were grouped according to the relevant age intervals for postassay evaluations. Each age category was designed to contain about 80 samples. Specific data regarding vaccination history was not obtained.

Tetanus toxoid and antitoxin

Tetanus toxoid, at a protein content of 996 μg/ml, was obtained from the Refik Saydam Hıfzısıhha Institute, Vaccine Development Center, Ankara. The tetanus antitoxin standard used in the study was the standard tetanus antitoxin IgG reagent supplied in a commercially available ELISA kit (Tetanus ELISA, lot no. 172-01T; Genzyme Virotech, Rüsselheim, Germany).

Labeling of tetanus toxoid with biotin

Tetanus toxoid was dialyzed against 0.1 M $NaHCO_3$ pH 7.4 and then labelled with biotin (Sigma, St. Louis, MO, USA) as described elsewhere [26].

Double-antigen enzyme-linked immunosorbent assay

Tetanus antibody levels in serum were measured using an in-house double-antigen ELISA system. The double-antigen ELISA has been evaluated previously against a commercial assay (Tetanus ELISA, lot no. 172-01T; Genzyme Virotech). The commercial kit is an indirect ELISA that is intended for the quantitative detection of IgG antibodies against the tetanus toxoid in human sera. The commercial ELISA was performed according to the instructions of the manufacturer. For assay standardization, we used the ready-to-use forms of the standards included in the commercially available kit. According to the insert included with the commercial kit, the ready-to-use standards, with defined concentrations, are calibrated against the International Standard of the WHO Tetanus Immunoglobulin (human) TE-3.

The double-antigen ELISA system is based on the principle previously described by Kristiansen et al. [25]. In brief, high binding capacity ELISA plates (Costar, no. 3590; Corning Inc., Corning, NY, USA) were coated with 100 μl of tetanus toxoid at 2 μg/ml in 0.05 M carbonate-bicarbonate buffer pH 9.6 by incubating at +4°C overnight. To each well was added 100 μl of sample diluted to 1/100 in PBS containing 1% BSA (PBS-1%BSA) or standards of the commercially available tetanus antitoxin ELISA kit and then incubated at 37°C for 1 h. On each plate, standards of the commercially available tetanus antitoxin ELISA kit were added into corresponding wells to obtain a standard curve, and optical densities of unknown serum specimens were interpolated. The results were expressed as IU/ml. Antibody levels less than 0.01 IU/ml were regarded as insufficient protection, levels of 0.01–1 IU/ml as basic protection, and levels higher than 1 IU/ml as full protection.

For testing the specifity of the reaction, an inhibition test was carried out in two steps using antitetanus toxoid standards and human sera with predefined antitetanus toxoid concentrations. These concentrations were predefined by the in-house antitetanus ELISA after validation experiments with this ELISA were regarded as successful. Diphtheria toxoid was used as the irrelevant antigen. Various concentrations of tetanus and diphtheria toxoids were prepared in PBS-1%BSA and added separately to appropriate wells of tetanus toxoid-coated plates at a volume of 50 μl. Except for the blank well, 50 μl of antitetanus toxoid standard at 0.02 IU/ml was added to the remaining wells. Further steps of the ELISA were performed as described above. For evaluation of the inhibition, three different human sera with predefined antitetanus toxoid concentrations were also used. After adding tetanus or diphtheria toxoids, human sera diluted to 1/100 in PBS-1%BSA were added

to appropriate wells at a volume of 50 µl. Further steps of the ELISA were performed as described above.

Statistical analysis

Regression-correlation analysis was carried out to evaluate the performance of the in-house double-antigen ELISA and the commercially available indirect ELISA. Interassay and intraassay coefficients of variation were calculated and used for quality control of the in-house ELISA. The Student's *t* test was used to compare the levels of tetanus antibodies between the groups. Differences in proportions were evaluated using the chi-square test. A p value of <0.05 was regarded as significant. Associations between the protective tetanus antibody level and variables (age and sex) were assessed by logistic regression analysis. The model included tetanus immunity (≥0.01 IU/ml) or susceptibility (<0.01 IU/ml) as the dichotomous outcome variable. The odds ratios (ORs) of different groups for immunity were drawn from the logistic regression analysis. Statistical analyses were performed using SPSS for Windows version 12.0 and EPI version 6.0.

Results

The analytical sensitivity of the in-house double-antigen ELISA was measured to be less than 0.001 IU/ml. The quality control of the in-house ELISA was performed by analyzing its intraassay and interassay coefficients of variation (CV). For the various concentrations of antitetanus antitoxin tested, the intraassay and interassay CV values ranged between 0.8 and 4.1% and between 2.2 and 8.6%, respectively. The antitetanus antibody content in 75 consecutive human serum samples was measured by both the double-antigen ELISA and the commercial ELISA (Fig. 1), and a significant correlation was observed between these two different systems (correlation coefficient = 0.963, $p<0.01$).

The specificity of the in-house ELISA was analyzed by testing the inhibitory effect of the tetanus toxoid both on the antitetanus toxoid standards and on human sera with predefined antitetanus toxoid concentrations. Tetanus toxoid inhibited the binding activity of the antitetanus toxoid standard beginning at 0.02 IU/ml in a concentration-dependent manner (Fig. 2a). The OD values obtained for the dilutions of tetanus toxoid decreased significantly ($r^2=0.7861$, $p=0.024$) and pointed to an inversely proportional relationship. However, an inhibitory effect was not observed ($r^2=0.2398$, $p>0.05$) for the irrelevant antigen, diphtheria toxoid, even at a concentration of 5 µg/ml (Fig. 2a). To determine whether this specificity was also applicable to human sera, a similar assay procedure was performed using three different sera tested in duplicate. Although tetanus toxoid inhibited the binding activity of antitoxoid antibodies in sera in a concentration-dependent manner, no effect was detected for the diphtheria toxoid, indicating the specificity of the in-house ELISA (Fig. 2b).

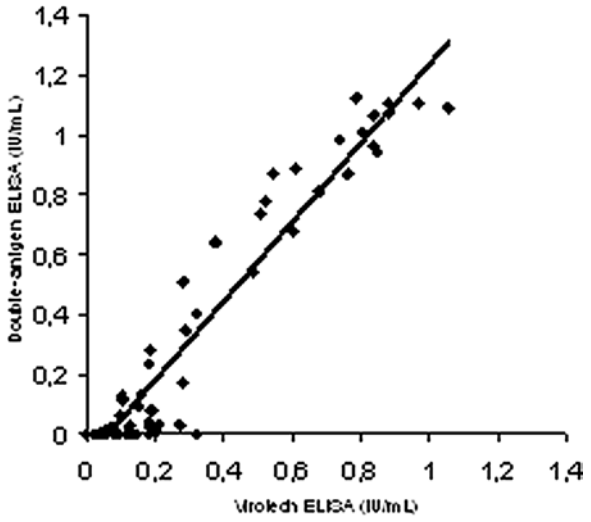

Fig. 1 Comparison of the performance characteristics of the in-house double-antigen ELISA and the commercial (Virotech) indirect ELISA. The two assays were assessed using 75 consecutive serum samples, and a significant correlation between the systems was observed (correlation coefficient = 0.963; $p<0.01$)

A total of 549 sera were included in the study (275 from males and 274 from females). The age of the study population ranged from 6 months to 74 years. Sera were categorized into seven age groups (0–9 years, 10–19 years, 20–29 years, 30–39 years, 40–49 years, 50–59 years, and ≥60 years) and according to sex. Each age group included 74–80 individuals. Age and sex distribution and tetanus immune status of the 549 individuals studied are summarized in Fig. 3. Overall, 66.5% (95%CI, 62.4–70.4) of the study population was found to have basic protection against tetanus. Males showed a higher proportion of protection (69.8%; 95%CI, 64.0–74.2) than females (63.1%; 95%CI, 57.1–68.9).

When antibody levels were examined according to age group, it was observed that as age increased, the percentage of subjects with basic protection decreased from 96.1% (95%CI, 89.0–99.2) in the age group 0–9 years to 16.3% (95%CI, 8.9–26.2) in the age group ≥60 years. The highest proportion (98.6%; 95%CI, 92.7–99.0) of protection was observed in the age group 10–19 years. On the other hand, the highest proportion of individuals with insufficient protection was found in the age group ≥60 years. The ratio of females with protective levels of tetanus antibodies decreased significantly after 30 years of age ($p=0.025$). A similar statistically significant decrease was also observed for females aged 40 years and above ($p=0.032$). For women over 60 years of age, the proportion of individuals considered to have protection against tetanus was 7.5% (95%CI, 1.6–20.4).

Although the decrease in the proportion of males with protective antibody levels after 30 years of age was not found to be significant, a statistically significant decrease was observed after 40 years of age ($p=0.031$). Moreover, the proportion of males with protective antibodies was significantly higher than that of females in the age group ≥60 years ($p=0.034$).

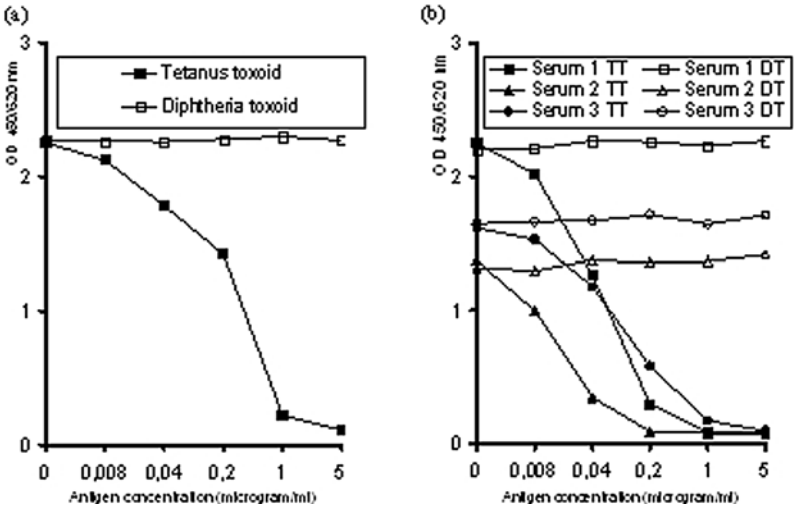

Fig. 2 Specifity testing of the in-house ELISA by inhibitory reaction. Tetanus toxoid inhibited the binding activity of (**a**) 0.02 IU/ml of antitetanus toxoid standard and of (**b**) various human sera containing different levels of antitetanus toxoid antibodies in a concentration-dependent manner. However, inhibition was not observed when a nonspecific antigen, diphtheria toxin, was used even at a high concentration of 5 μg/ml. Data are the means of triplicate wells (TT, tetanus toxoid; DT, diphtheria toxoid)

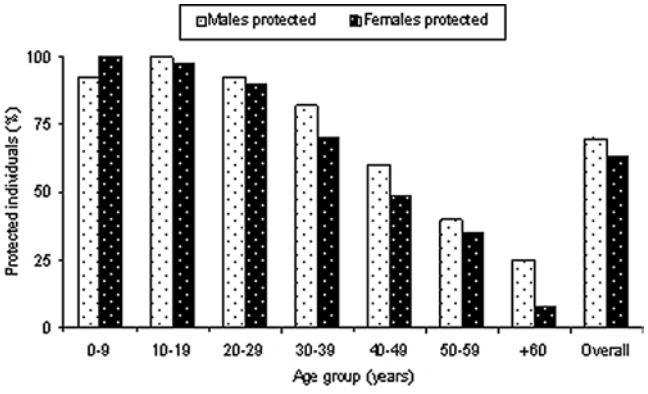

Fig. 3 Distribution of tetanus immunity by age and sex. Each age category contains 74–80 individuals. The rate of protective immunity was found to be significantly higher in males (n=275) than in females (n=274) (OR=1.64; 95%CI, 1.03–2.61), and protective levels of tetanus antibody (≥0.01 IU/ml) were inversely associated with increasing age (OR=0.91, 95%CI, 0.89–0.92)

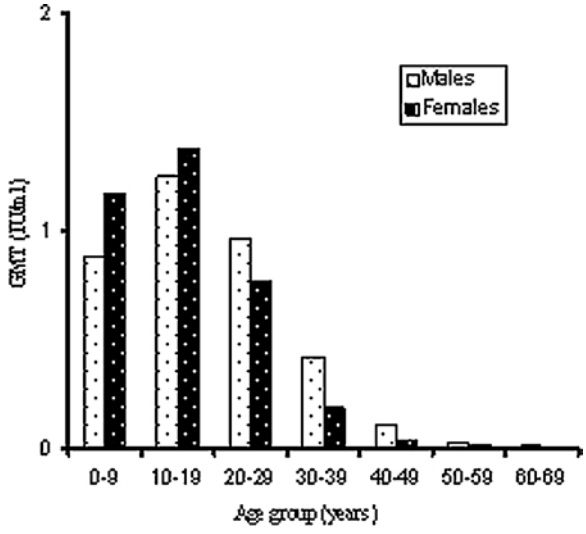

Fig. 4 Geometric mean antibody titres (GMTs) for tetanus in various age groups in 549 subjects. Except for the individuals under 20 years of age, GMTs were higher among males, although the mean differences between sexes were not statistically significant. GMTs reached a peak in subjects aged 10–19 years in both sexes and then decreased, reaching a minimum in the age group ≥60 years

Geometric mean antibody titres (GMTs) for tetanus among individuals are shown in Fig. 4. Except for the individuals under 20 years of age, the GMTs were found to be slightly higher among males in all other age groups, although the mean differences between sexes were not statistically significant. Mean antibody titres decreased significantly from 1.48 IU/ml in the age group 20–29 years to 1.07 IU/ml in the age group 30–39 years for males (p=0.004). A similar significant decrease (from 1.32 IU/ml to 0.79 IU/ml) for these age groups was also observed for females (p=0.002). GMTs reached a peak level in subjects aged 10–19 years in both sexes and then decreased, reaching a minimum in the age group ≥60 years.

Logistic regression analysis indicated that the rate of protective immunity was significantly higher in males (n=275) than in females (n=274) (OR=1.64; 95%CI, 1.03–2.61) and that the protective levels of tetanus antibody (≥0.01 IU/ml) were inversely associated with increasing age (OR=0.91; 95%CI, 0.89–0.92). Protective immunity rate for individuals ≥50 years (n=160) was significantly lower than in those aged <50 years (n=389) (OR=13.5; p<0.001; 95%CI, 8.7–21).

Discussion

The double-antigen ELISA system is a relatively new method that allows detection of antigen-specific antibodies in serum specimens originating from different species [25]. An important feature of the double-antigen ELISA is that this method is proven to correlate well with the in vivo toxin neutralization test, even in low-titre sera [25]. Antibody levels in many sera with low amounts of specific antibody (<0.2 IU/ml) are grossly overestimated in the indirect ELISA [25]. In addition, the double-antigen ELISA system theoretically avoids possible nonspecific reactions caused

by human antimouse antibodies or rheumatoid factors that might be present in human sera. The use of double-antigen ELISA methodology for the detection of tetanus antibodies has been recently reported [5, 27–29].

Our results, based on the double-antigen ELISA methodology, indicate that an important proportion of the adult population in Ankara, Turkey, is not sufficiently protected against tetanus. Overall, 66.5% of the population studied was found to have basic protection (≥0.01 IU/ml) against tetanus. This in in accordance with previous studies reported for Ankara, Turkey, in which the overall protection rate was documented as 68% [30].

In our study, the overall prevalence of protection against tetanus was 26.9% among adults over 50 years of age. This is lower than prevalence rates reported for other provinces of Turkey [31]. Our finding of protected individuals, however, is subtantially lower than rates reported from developed countries. Heath et al. [10] found that 52% of individuals aged 49 years and over were protected against tetanus in Australia. In two other studies from the UK, protection rates among adults over 60 years of age were reported as 50–53% [32, 33]. Similar rates for those aged 50 and above and 60 and above have been reported from Germany and Greece [34–36].

Another finding was the decline in immunity against tetanus with increasing age. This is in accordance with the findings of other studies reported for our country [37, 38]. This phenomenon of decreasing rates of immunity to tetanus among adults is consistent with reports from other countries, despite variations in the methods used for testing the antibody concentration and despite the use of different vaccination and booster schedules [9, 34, 35, 39, 40].

In our study, GMTs reached a peak in the age group 10–19 years, which can be attributed to the national vaccination program in Turkey, which allows six doses of tetanus toxoid to individuals until the age of 14. The GMTs for children in our study population were similar to those reported from developed countries [41]. Except for children and adolescents (aged <20 years), we found higher GMTs in males than in females, although the differences in means were not statistically significant. Similar findings were also reported for other countries [32, 41]. Higher antibody titres in males aged 20 and above may be attributable to factors such as vaccination of men during military service and the fact that men are more frequently exposed to injuries that lead to prophylactic tetanus vaccination. In our study, the mean antibody titres started to decline after the third decade of life. This decline in relation to the age group 30–39 years was statistically significant for both males and females. A significant decrease in mean titres was also seen after the age of 40. Similar trends have also been reported from other countries [32, 41].

The prevalence of protection against tetanus among males has been reported to be higher than that among women in various studies [9, 10, 34, 38, 40]. In accordance with these findings, we observed that men are better protected than women. Although young adults of both sexes are well protected, significant differences were found in the age groups 40–49 years and ≥60 years. Only 16.3% of the individuals ≥60 years had protective antibody levels, and a significant difference between men (25.0%) and women (7.5%) was observed in this age group. Most of the women ≥60 years were found to be susceptible to tetanus infection. The proportion of protected individuals decreased continuously as age increased (from 91.2% in the age group 20–29 years to 16.3% in the age group ≥60 years).

In our country, tetanus vaccination became mandatory for all newborns in the early 1960s. The high levels of protective immunity in our study in individuals under 20 years of age is attributable to the Expanded Immunization Program that was established in Turkey in 1985. The low prevalence of antibody levels against tetanus in the elderly might be related to the fact that adults born before 1960 did not receive any scheduled tetanus vaccination in their childhood. Insufficient routine booster immunizations after adolescence may contribute to the decline in the level of protective immunty observed in the elderly in our country.

In conclusion, simple and reliable serological techniques should be used to determine the level of immunity to tetanus. The double-antigen ELISA is a reliable method for community-wide surveys of tetanus immunity. As our study showed, the levels of immunity to tetanus are satisfactory among children, but it is difficult to conclude the same for adults. A high proportion of the adult population was not sufficiently protected against tetanus. The levels of tetanus antibody declined with age, and a significant difference was found between males and females in terms of protection against tetanus, especially in the older age groups. According to these results and other reports from our country, we suggest that an adult vaccination program be developed to establish adequate protective immunity levels in the elderly.

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