Diphtheria Antitoxin Levels in US Blood and Plasma Donors

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Plasma samples from 500 blood donors were titrated for diphtheria antitoxin (DAT) by the toxin neutralization (TN) test. Only 1.6% of donors had <0.01 IU/mL DAT, the minimum protective level against diphtheria; 15% had levels between 0.01 and <0.1 IU/mL, indicating basic protection, and 83.4% had levels ≥0.1 IU, indicating full protection. Three hundred fifty samples were studied by ELISA for diphtheria toxoid IgG antibodies to assess the utility of the assay as a quick, convenient method for evaluating diphtheria immunity. Although the correlation between TN and ELISA titers for the 350 samples was high (r = .90), there was no correlation (r = .07) for samples with antitoxin titers <0.1 IU/mL, the level of special interest for serosurveys for protection. Titration of 62 immune globulin samples (prepared from 1957 to 1994) showed that DAT levels in Massachusetts blood donors increased concurrently with increased use of tetanus-diphtheria vaccine in the state.

The incidence of diphtheria in developed countries declined rapidly after introduction of a vaccine in the 1940s and 1950s. During the early 1980s, when its incidence was lowest, there was optimism that indigenous respiratory diphtheria might be eliminated in Europe [1]. However, the recent diphtheria epidemic in eastern Europe, particularly in Russia and Ukraine [1, 2], and lack of protective levels of diphtheria antitoxin (DAT) in a large proportion of the adult population in some European countries [1–5] have caused concern that diphtheria outbreaks could occur in developed countries [6]. Some have speculated that the US adult population may also have low DAT levels, but data are lacking because there have been few recent serosurveys. A recent workshop [6] emphasized the importance of epidemiologic studies of DAT levels in adults and the need for a rapid diagnostic method for diphtheria. The lack of large amounts of potent and safe therapeutic DAT in the United States is also a concern.

The toxin neutralization (TN) test in animals or cell cultures is the most reliable test for determining DAT levels in sera, because it measures functional antibodies that neutralize diphtheria toxin [3, 4]. Other assays, mainly the ELISA and the passive hemagglutination test, can also be used to determine antibodies to diphtheria toxin [3]. These assays are rapid, easy to perform, and particularly useful for determining diphtheria antibody levels in large numbers of samples for serosurveys or potentially for screening plasma samples for preparing a human diphtheria immune globulin (DIG). However, these assays have not been reliable for antibody levels <0.1 IU/mL, which are near the protective threshold [3]. We measured antibodies to diphtheria toxin in plasma donors in three states by the TN test in Vero cells [7] and by ELISA to assess donor immunity to diphtheria and to evaluate the feasibility of
preparing a DIG by screening plasma from adults. To evaluate temporal trends in DAT levels in US plasma donors, we also evaluated 62 immune globulin preparations dating from 1957 to the present. We also examined the potential utility of ELISA for evaluating immunity to diphtheria and for screening plasma donors to prepare DIG.

Materials and Methods

Reagents. Crude diphtheria toxin (>30 years old) was used for the TN test in Vero cells, and chromatographically purified diphtheria toxoid (purity $>1500$ limits of flocculation [Lf]/mg protein nitrogen) was used to coat ELISA plates (both provided by B. Rost, Massachusetts Public Health Biologic Laboratories). Standard DAT at 6 IU/mL was obtained from the Center for Biologics Evaluation and Research, Food and Drug Administration (Bethesda, MD). Human plasma samples were used as a reference standard and as internal controls for ELISA and were calibrated in weight-based units (microgram per milliliter) for diphtheria toxoid IgG antibodies by the method of Zollinger and Boslego [8]. Medium 199, fetal bovine serum, and other cell culture reagents were from BioWhittaker (Walkersville, MD).

Plasma samples and immune globulins. Plasma samples from 500 healthy plasmapheresis donors from Westgate Plasma Center, Bryan, Texas (n = 202), DCI Plasma Center, Gallup, New Mexico (n = 131), and Bowling Green Biologicals, Bowling Green, Kentucky (n = 167) were provided by J. Rios (DCI Plasma Center). Sixty-two conventional and specific immune globulin preparations included licensed preparations (16.5% IgG) from retained samples from our production dating to 1957, commercial preparations (5% IgG) from various manufacturers, and tetanus and varicella zoster immunoglobulins (16.5% IgG) from our production. Immune globulin preparations from our laboratories were made from plasma from whole blood donations from Massachusetts donors. All plasma samples were inactivated at 56°C for 30 min before testing.

TN test. The TN test was done in Vero cells at a diphtheria toxin dose of Lcd/1000 as described elsewhere [7]. An Lcd/1000 is the limit of cytopathic dose at 0.001 IU/mL DAT (the minimum diphtheria toxin concentration required to produce a cytopathic effect on Vero cells in 4 days, when the toxin solution is mixed with an equal volume of standard DAT at 0.001 IU/mL). In brief, the plasma samples and standard antitoxin (in duplicate on each plate) were diluted in 2-fold steps in 96-well flat-bottom plates in 50-µL volumes using medium 199 as a diluent. Diphtheria toxin diluted to the Lcd/1000 level was added to appropriate wells in 50-µL volumes. After gentle mixing, plates were incubated 1 h at room temperature, and 10,000 Vero cells were added to each well. Appropriate toxin and cell controls were included on each plate. After plates were incubated 5–7 days at 37°C in a 5% CO₂ incubator, they were assessed microscopically for cytopathic effect due to toxin. The unitage of unknown samples was calculated from the standard antitoxin titration.

Plasma samples were tested twice, once at low DAT levels (starting at 0.006 IU/mL) to evaluate donor immunity to diphtheria, and again at high levels (starting at 1.25 IU/mL) to screen donors for DIG. Immune globulin samples were tested in duplicate beginning at 0.15 IU/mL.

ELISA for diphtheria toxoid IgG antibodies. Samples from 350 donors were tested individually for diphtheria toxoid IgG antibodies by ELISA as described elsewhere for animal sera [9] with minor modifications. Briefly, 96-well flat-bottom Immulon II plates (Dynatech Laboratories, Chantilly, VA) were coated with diphtheria toxoid (100 µL at 0.5 µg/mL in PBS, pH 7.2) overnight at room temperature. After every step, plates were washed three times with PBS containing 0.05% Tween 20. Plasma samples and reference and internal control plasma samples on each plate were diluted in 2-fold steps using PBS with 0.1% Brij 35 (Sigma, St. Louis) and 0.5% bovine serum albumin (PBB) as a diluent. After plates were incubated 2 h at room temperature, 0.5% nitrophenyl phosphate (Sigma) in diethanolamine-MgCl₂ buffer was added to each well. After 30 min, plates were read at A₄₅₀ on an ELISA reader (Titertek Multiskan; ICN Biomedicals, Costa Mesa, CA). The diphtheria toxoid IgG antibody concentrations of unknown samples were calculated from a standard curve of the reference plasma generated by the four parameter logistic equation [10].

Antibody concentrations of unknown samples with titration curves parallel to the reference plasma were estimated from the geometric mean concentration calculated from dilutions giving 20%–80% of the maximum absorbance. Antibody concentrations of unknown samples with nonparallel titration curves were arbitrarily estimated from the dilution falling closest to 33% of the maximum absorbance. For a valid assay, the internal control plasma showed values within the specified limits. The reproducibility of the ELISA was determined by calculating coefficients of variation (CV) for tests done on the same day on different plates and on different days. One internal control (mean diphtheria toxoid IgG antibodies, 3.95 µg/mL) tested over 9 months showed a CV of 5.3% for plates tested the same day and a CV of 11.7% for assays done on 14 different days. The other internal control (mean diphtheria toxoid IgG antibodies, 14.34 µg/mL) tested over >2 years had CVs of 9.2% and 26.4%, respectively, for variations between plates and between assays done on 31 different days.

Statistical analysis. Titers of 350 plasma samples determined by ELISA and TN test were analyzed by Pearson's correlation coefficient individually and after they were divided into three categories by DAT levels: <0.1, 0.1–2.5, and $>2.5$. DAT levels of donors from different plasma centers were tested for significance by Student's t test.

Results

US plasma donor DAT levels. Most donors (98.4%) had protective levels against diphtheria ($>0.01$ IU/mL). Of these, 75 donors (15%) had DAT levels between $0.01$ and $<0.1$ IU/mL (indicating basic protection against the disease), and 83.4% exhibited full protection ($>0.1$ IU/mL). Eighty-five donors had $>1.0$ IU/mL DAT, 9 had $>2.5$ and $<5.0$ IU/mL, and 7 had $>5$ IU/mL. Mean ± SD DAT levels (IU/mL) of donors from 3 plasma centers in Texas, New Mexico, and Kentucky, respectively, were $0.71 ± 1.09$ (n $= 202$), $0.40 ± 0.75$ (n $= 202$), and $0.03 ± 0.22$ (n $= 202$).
Figure 1. Distribution of tetanus (T) vaccine (A) and tetanus and diphtheria (Td) vaccine (B) for adult immunization by Massachusetts Public Health Biologic Laboratories (1955–1994) and diphtheria antitoxin levels in normal human immune globulin samples (16.5% IgG), 1957–1992 (C). Regression line \( y = -0.0003x^2 + 1.19x - 1180 \) was drawn by nonlinear second order polynomial regression.

131), and 0.68 ± 0.79 (n = 167). There were no significant differences between donor DAT levels by center (P > .01).

**Immune globulin DAT levels.** DAT levels in 1957–1992 immune globulin samples (16.5% IgG) were 5–16 IU/mL (figure 1C). Levels calculated for 1% IgG (about equivalent to normal human plasma) were 0.30–0.99 IU/mL, similar to the mean DAT level of the 500 plasma donors (0.62 IU/mL) tested in this study. DAT levels of immune globulin samples prepared
before 1967 were <10 IU/mL, whereas levels in samples after 1967 were >10 IU/mL, except for one, which was 8 IU/mL (figure 1C).

Figure 1 shows the distribution of adsorbed tetanus toxoid and tetanus and diphtheria (Td) adult vaccine supplied by the Massachusetts Public Health Biologic Laboratories, which provided all of the tetanus and diphtheria vaccines used in the state. From 1961 to 1967, tetanus toxoid was replaced with the Td vaccine because of concern about low adult diphtheria immunity. Although tetanus toxoid distribution was resumed in 1968, the use of Td vaccine continued to increase, and the tetanus vaccine was completely replaced with Td vaccine in 1990.

The antitoxin levels of 12 commercial immune globulin samples for intravenous administration containing 5% IgG were 3–12 IU/mL (data not shown).

Correlation between TN test and ELISA. Diphtheria antibody levels determined by TN test and ELISA in 350 plasma samples had a strong correlation ($r = .80, P < .001$). However, there was poor correlation between these tests for samples with antitoxin levels <0.1 IU/mL ($r = .07, P > .2$) or ≥1.0 IU/mL ($r = .32, P < .01$) (figure 2). Samples with antitoxin levels ≥0.1 and <1.0 IU/mL were moderately correlated ($r = .61$).

Discussion

Most adult plasma donors (98.4%) in our study had protective levels of antibodies against diphtheria (≥0.01 IU/mL). This differs from findings in the United Kingdom, where 37.6% of blood donors are susceptible to diphtheria [5]. Immunity to diphtheria among adults in several European countries is low [1, 3, 4]. These differences may be due to use of Td vaccine in the United States: This vaccine contains small amounts of diphtheria toxoid (<2 Lf) for booster immunization. The Td vaccine was developed in the United States in the 1950s for booster immunization of adults [11] to maintain immunity against tetanus and diphtheria and was licensed in 1955 [12]. Although the immune globulin preparations made from Massachusetts blood donors have had high DAT levels since 1957, these levels increased in the mid-1960s (figure 1C), coinciding with the widespread use of adult Td vaccine (figure 1B). Immune globulin preparations from commercial manufacturers

Figure 2. Correlation between diphtheria antibody levels determined by toxin neutralization test in Vero cells (IU/mL) and ELISA (IgG antibodies in µg/mL). Regression line ($y = 0.643x + 0.883$) drawn by linear regression.
also had high DAT levels (similar to those in Massachusetts), indicating similar levels of immunity to diphtheria in plasma donors elsewhere in the United States. The mean DAT levels in 500 plasma donors (0.62 IU/mL) was consistent with levels in immune globulin preparations made after 1967.

Specific immune globulins are prepared by screening donors for naturally occurring high titered antibodies or by stimulating donors with antigens to elicit high levels of antibodies. Screening of plasma donors for high titered antitoxin may not be a viable option because only 7 donors (1.4%) had antitoxin levels >5 IU/mL, the titer needed to produce a potent human DIG. Thus, efficient preparation of DIG will require boosting plasma donors with a highly purified diphtheria toxoid. Thus, we are planning to evaluate alum phosphoryl tetanus and well-trained staff. We, therefore, evaluated a standardized and reproducible ELISA for quantitation of diphtheria toxoid IgG antibodies. Analysis of 350 plasma donors revealed a strong correlation between the ELISA and the TN test in Vero cells (r = .80), when the full range of titers was included. However, when the two tests were evaluated in the titer ranges most critical for seroepidemiologic surveys of susceptibility or for screening plasma donors, these tests had poor correlation (figure 2). Our results thus confirm that ELISA for titration of antibodies to diphtheria toxoid at levels <0.1 IU/mL is not reliable [3, 13].

The minimum protective levels of diphtheria and tetanus antitoxins in serum are estimated to be 0.01 IU/mL [3, 5, 14]. Our data allow an estimation of the concentration of IgG antibody that corresponds to this protective level. Although the IgG diphtheria antibody of individuals may vary in its antitoxic activity due to its epitope specificity and its affinity, the mean ratio between IgG antibody and DAT (in micrograms and international units per milliliter, respectively) for 350 plasma donors was 9. This indicates that 0.09 μg is equivalent to 0.01 IU. This closely resembles the antitoxin activity of IgG antibody to tetanus, where 0.10 μg/mL is equivalent to the protective level of 0.01 IU/mL [15]. This also resembles the estimated minimum protective level of antibody to Haemophilus influenzae type b (0.15 μg/mL) [16].

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References