

## IgE SYNTHESIS IN MAN

### I. Development of Specific IgE Antibodies after Immunization with Tetanus-Diphtheria (Td) Toxoids<sup>1</sup>

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**Specific IgE and IgG antibodies were measured in 39 adolescent and adult humans preselected to have less than 0.125 antitoxin units (AU) ml of tetanus antitoxin (IgG) irrespective of previous immunization history. Mean antitoxin values (IgG) measured by sheep red cell hemagglutination (SRBC) increased by 2 logs to 1.49 AU/ml sera for tetanus and by 1 log to 0.025 AU/ml sera for diphtheria. Specific tetanus IgE was noted to increase after immunization from a mean  $2.0 \pm 0.2\%$  counts bound to  $3.1 \pm 0.6\%$  counts bound, but was not statistically significant. Diphtheria-specific IgE rose significantly ( $p < 0.05$ ) from a mean  $1.5 \pm 0.2\%$  counts bound to a mean  $4.0 \pm 1.2\%$  counts bound in atopic individuals and from a mean  $1.2 \pm 0.0\%$  counts bound to a mean  $2.2 \pm 0.5\%$  counts bound in non-atopics. These data document the development of tetanus and diphtheria IgE antibodies after booster immunization.**

Since the medical community's recommended use of tetanus toxoid for prophylactic immunization against the sequela of infection with *Clostridium tetani* in 1936, sporadic reports of anaphylaxis and other immediate hypersensitivity reactions from the toxoid have appeared in the literature (1-3). Most of the reactions described before 1953 appear traceable to contamination of the toxoid with protein from the peptide media used in its preparation. Since the introduction of protein-free media for the preparation of tetanus toxoid in 1953, several additional case reports have appeared reporting anaphylaxis after immunization with refined tetanus toxoid (4-11). Review of the literature yielded only one report of an anaphylactic reaction to diphtheria toxoid (12).

Other adverse reactions including local induration, erythema, pyrexia, and malaise after immunization with tetanus and/or diphtheria toxoid have been discussed in numerous reports (9, 13-18). Some of these reactions may be related to hyperimmunization; i.e., either too frequent administration or excessive antigen. In a previous study 63% of a group of normal individuals who had previously been immunized with tetanus and diphtheria toxoids had an immediate wheal and flare reaction to skin testing with intracutaneously administered tetanus antigen (19). As a result of these reports, a prospective study was designed to determine if, after booster immuniza-

tion, antibodies of the IgE class might be found to specific tetanus and diphtheria toxoids.

#### MATERIALS AND METHODS

*Study population and method of immunization.* Thirty-nine individuals were selected from a group of 85 volunteers comprising patients from a private allergy practice and the allergy clinic and medical personnel of Children's Hospital of Pittsburgh. All persons studied had received their primary immunizations with either tetanus toxoid or combined tetanus, diphtheria, pertussis (DPT)<sup>3</sup> vaccine. For many individuals, it was impossible to document the previous immunization schedule, or the number and date of any boosters that had been received. By their own statement, none of the subjects had experienced anaphylaxis, bronchospasm, urticaria, or angioedema from previous immunizations, nor had incapacitating reactions such as severe local induration, erythema, fever, or malaise from prior tetanus immunizations. Eighteen individuals were regarded as atopic on the basis of a personal history of allergen-induced asthma and/or allergic rhinitis. Family history was not considered in assignment to atopic or nonatopic groups. Thirteen of these 18 individuals had previously demonstrated reaginic antibody by cutaneous testing to inhalants and/or foods. The remaining five atopic individuals were not skin tested.

Age range of the study population was 9 to 57 years (mean 29 years). There were 20 females and 19 males. The atopic population consisted of seven females and 11 males with a mean age of 26 years. The nonatopic group consisted of 13 females and nine males with a mean age of 31 years.

All subjects were initially screened by determination of tetanus hemagglutinating antibody (see below). Individuals with antitoxin values higher than 0.125 antitoxin units (AU)/ml were excluded from the study and not immunized. After informed consent was obtained, each individual was immunized by the intramuscular route with 0.5 ml of a commercial alum-precipitated adsorbed adult tetanus-diphtheria toxoids (Td) mixture containing approximately 7.5 Lf of tetanus toxoid and 1.5 Lf of diphtheria toxoid (Eli Lilly & Co., Indianapolis, Ind.). The Td mixture also contained 0.01% sodium ethylmercurithiosalicylate (trimerosal) and 1.275 mg aluminum potassium sulfate per dose (0.5 ml).

Venous blood samples were obtained before and 10 to 21 days after immunization in all subjects. In many individuals, particularly those with rises in certain experimental param-

Submitted for publication August 9, 1976.

<sup>1</sup>This work was supported by United States Public Health Service Grants HD02662 and HD07850.

<sup>2</sup>J. N. was supported by the United States Public Health Service Training Grant HD00062.

<sup>3</sup>Abbreviations used in this paper: DPT, diphtheria pertussis tetanus; Td, tetanus-diphtheria toxoid; RAST, radioallergosorbent; RIST, radioimmunosorbent test; AU, antitoxin units.

ters, additional blood samples were obtained at varying intervals for a period of up to 19 months post-immunization. All serum samples were divided into small aliquots and frozen at  $-70^{\circ}\text{C}$  until analyzed.

**Hemagglutination (IgG) antibody assay.** Tetanus and diphtheria antibody levels were estimated by passive hemagglutination by using the method of Levine *et al.* (20) with modification. Tanned sheep red blood cells (SRBC) were suspended in 0.075 M phosphate-buffered saline solution, pH 6.4, and sensitized with tetanus toxoid (37 Lf/ml) in a 1:4 ratio for 10 min. Diphtheria toxoid (40 Lf/ml) was used in a 1:10 ratio for 30 min. The sensitized SRBC were then washed and suspended in 0.9% saline containing 1% heat-inactivated normal rabbit sera. The sera tested were heat inactivated for 30 min at  $56^{\circ}\text{C}$  and after 2-fold serial dilutions were made, 0.1 ml of sensitized tanned SRBC was added. Hemagglutination reactions were read after 18 hr of incubation.

United States standard tetanus (5 AU/ml) or diphtheria (6 AU/ml) antitoxin (Division of Biological Standards, NIH, Bethesda, Md.) was diluted and served as a standard for the hemagglutination determinations. Tetanus and diphtheria antibody titers were converted to AU/ml of sera by comparison with the results of the standard antitoxin hemagglutination.

In order to establish that the tetanus and diphtheria hemagglutinating antibodies were of the IgG class, heat inactivation, and mercaptoethanol (ME) treatment with the method of Levine *et al.* (21) was performed on six representative sera. A 0.1-ml aliquot of sera was mixed with 0.30 ml of 0.133 M ME in phosphate-buffered saline, pH 8.2. This mixture was then heated to  $56^{\circ}\text{C}$  for 1 hr and then used in the hemagglutination reactions without further inactivation or dialysis. There was no significant change in tetanus or diphtheria hemagglutinating antibody after ME treatment. Therefore, these antibodies were primarily IgG.

**Radioallergosorbent (IgE) test.** Tetanus and diphtheria-specific IgE was measured by the radioallergosorbent (RAST) method. Paper discs, 6 mm in diameter were cut with a punch from Fisher qualitative filter paper. Activated discs were prepared by the method of Ceska *et al.* (22) with slight modification. Ten grams of filter paper discs were allowed to swell in distilled water. After 30 min, the water was decanted and 200 ml of 5% (w/v) BrCN at  $10^{\circ}\text{C}$  were added to the discs. The pH was maintained between 10.0 and 10.5 with dropwise addition of 1 M NaOH and constant agitation. After 3 min the suspension was immediately poured into 2 liter of 0.005 M  $\text{NaHCO}_3$  at  $4^{\circ}\text{C}$ . The activated discs were then washed four times with 500 ml of acetone (reagent grade) at  $4^{\circ}\text{C}$  and dried. The activated discs were then coupled with purified preservative-free fluid tetanus toxoid (graciously provided by Wyeth Labs, Philadelphia, Pa.) containing 223 Lf (purity 1123 mg/PN) of toxoid/ml in borate saline containing 0.2 M borate buffer, 0.04 M NaOH and 0.16 M NaCl, pH 11.0, at  $4^{\circ}\text{C}$  for 72 hr. Discs were then washed with constant agitation twice with 0.1 M  $\text{NaHCO}_3$ , and once with 0.05 M  $\beta$ -ethanolamine in 0.1 M  $\text{NaHCO}_3$ . The discs were then rewashed with three changes of 0.1 M acetate buffer, pH 4.0, and two changes of incubation buffer containing 0.9% NaCl, 0.05 M phosphate buffer, 0.3% BSA, and 0.05% sodium azide at pH 7.4. This same procedure was followed for coupling of diphtheria toxoid. Diphtheria toxoid (procured from the Massachusetts Department of Public Health) containing 930 Lf (purity 937 mg/PN) of purified fluid diphtheria toxoid and 0.005% trimersol in borate saline was coupled to the activated discs by incubation at  $4^{\circ}\text{C}$  for 72 hr. Discs were then stored at  $4^{\circ}\text{C}$  under commercial RAST

buffer containing TWEEN 20 (Pharmacia Diagnostics, Piscataway, N. J.). Discs prepared and stored in this fashion appeared to have no apparent decrease in activity after 6 months of storage.

In the first step of the RAST, antigen-coupled discs were incubated for 3 hr with 50  $\mu\text{l}$  of subject sera at  $20^{\circ}\text{C}$ . Discs were then washed three times with 2 ml of 0.9% saline and incubated overnight with 50  $\mu\text{l}$   $\text{I}^{125}$ -labeled rabbit antibody to human IgE (Pharmacia Diagnostics). Total radioactivity added was determined for each sample with a gamma counter (Nuclear-Chicago, Des Plaines, Ill.). The radiolabeled discs were washed three times with 2 ml 0.9% saline and specific radioactivity for each sample was again determined. All RAST results are expressed as the number of counts bound to the antigen-coupled disc divided by the total counts added  $\times 100$ . Tests were performed in duplicate and results were expressed as the mean. Casein and hydrolyzed casein-specific IgE antibodies were also determined by the RAST procedure. Casein (Fisher Scientific, Fairlawn, N. J.) and hydrolyzed casein media (NZ Case, Humko Sheffield Chemical, Oneonta, N. Y.) were coupled to the sensitized discs and used in the RAST procedure previously described.

**Total serum IgE.** Total serum IgE was measured in all subjects by the radioimmunosorbent test (RIST) by using a commercially available kit (Phadebas IgE Test, Pharmacia Diagnostics). The IgE standard in the test kit is calibrated against the British Research Standard for Human Serum Immunoglobulin E 68/341, from the WHO International Reference Centre for Immunoglobulins, Lausanne, Switzerland (23). One unit (U) of this standard is approximately 2 ng of IgE (24).

## RESULTS

Figure 1 displays the tetanus and diphtheria IgG antibody values before and after Td immunization. Mean tetanus IgG responses increased by a factor of 2 logs to a geometric mean of 1.49 AU/ml of sera. Mean diphtheria IgG responses rose 1 log to 0.025 AU/ml sera. Ten (25.6%) of the individuals had negligible rises in diphtheria IgG when studied at 10 to 21 days post-immunization. When diphtheria antibody values were redetermined 4 to 6 weeks post-immunization, five of these 10 low responders (triangles in Fig. 1) demonstrated a rise in diphtheria antibody.

Figure 2 shows the anti-tetanus and anti-diphtheria IgE values pre- and 10 to 21 days post-immunization. Tetanus IgE pre-immunization was  $2.0 \pm 0.2$  and post-immunization  $3.1 \pm 0.6\%$  counts bound. The difference between these mean tetanus IgE antibody values for this study population was not significant ( $p > 0.1$ ). Anti-diphtheria IgE pre-immunization was  $1.3 \pm 0.0\%$  counts bound. This rose to  $3.0 \pm 0.7\%$  counts bound for the entire population after immunization. The difference between these mean specific IgE antibody values was significant ( $p < 0.05$ ).

Figure 3 compares the anti-tetanus IgE responses with the presence or absence of atopy. In the atopic group pre-immunization anti-tetanus IgE was  $2.4 \pm 0.4\%$  counts bound. This rose to  $4.1 \pm 1.3\%$  counts bound after immunization. The difference between these values is not significant ( $p > 0.1$ ). Two atopic individuals rose greater than 2 standard deviations above the post-immunization mean to 15.4 and 22.0% counts bound. Neither of these individuals had elevated pre-existing tetanus antibodies of the IgG class and both had pre-immunization anti-tetanus IgE values within 1 standard deviation of the mean. The nonatopic group had a pre-immunization anti-

tetanus IgE value of  $1.7 \pm 0.1\%$  counts bound. This rose to  $2.2 \pm 0.2\%$  counts bound after immunization. This difference is also not significant ( $p > 0.05$ ). No nonatopic individual rose greater than 2 S.D. above the post-immunization mean.

Figure 4 displays the anti-diphtheria IgE responses in the atopic and nonatopic groups. In the atopic subjects anti-diphtheria IgE rose after immunization from a value of  $1.5 \pm 0.2\%$  counts bound to  $4.0 \pm 1.2\%$  counts bound. This difference was significant ( $p < 0.05$ ). The nonatopic group had a rise from  $1.2 \pm 0.0\%$  counts bound to  $2.2 \pm 0.5\%$  counts bound after immunization and was significant ( $p < 0.05$ ). There was one nonatopic and two atopic individuals who rose after immunization greater than 2 standard deviations to 11.3, 12.5, and 23.6% counts bound. These individuals did not have, before immunization, elevation of either specific tetanus or diphtheria IgG or IgE antibodies. One of these individuals responded with high specific IgE production to both tetanus (22.0% counts bound) and diphtheria (23.6% counts bound) antigens, whereas the other two individuals responded to only one of the two antigens.

Figure 5 compares total serum IgE in the atopic and nonatopic groups. Mean for the atopic group was  $737 \pm 312$  units/ml and for the nonatopic group  $40 \pm 11$  units/ml. This difference was significant ( $p < 0.05$ ). Mean for the population was  $362 \pm 153$  units/ml.

Figures 6 and 7 demonstrate the relationship between total serum IgE and specific tetanus and diphtheria IgE in the atopic and nonatopic groups. A positive correlation coefficient

was obtained between total serum IgE and post-immunization anti-tetanus and anti-diphtheria RAST values by using the Kendall tau test (25). Coefficients were 0.16 and 0.01 for tetanus and diphtheria in the atopic group and 0.03 and 0.40 in the nonatopic group. The magnitude of these coefficients was not sufficient to be predictive.

Longitudinal follow up of seven selected atopic individuals with rises in anti-tetanus IgE is shown in Figure 8. None of these individuals returned to pre-immunization specific IgE levels in 10 or more weeks after immunization. Two individuals with marked rises (22.0 and 27.0% counts bound) continue to demonstrate significant antibody elevation 9 and 18 months after immunization.

## DISCUSSION

In a previous study in our laboratory it was noted that 42 of 67 (63%), randomly selected individuals manifested immediate hypersensitivity reactions (wheal and flare) after skin testing with intracutaneously administered tetanus toxoid. Because specific IgE antibodies have been shown to be related to other immediate hypersensitivity phenomena, the question was raised whether antibodies of the IgE class might be found to tetanus and diphtheria toxoids after routine booster immunization with an adult Td toxoids mixture. Since adverse local hypersensitivity reactions have been considered to be related to elevated levels of specific IgG antibodies and "hyperimmunization" (26), the individuals in the present study were evaluated for elevated tetanus IgG before booster Td immunization. Only individuals who had pre-immunization tetanus antitoxin values within 1 log of the accepted protective level of 0.01 tetanus AU/ml sera were immunized (27). The interval since the last immunization ranged from less than 1 year to greater than 15 years. Many individuals had no booster immunization for diphtheria for 15 or more years which corresponds with the low pre-immunization diphtheria antitoxin values found in this and other studies (28, 29). After Td immunization with a commercial preparation containing 1.5 Lf of diphtheria toxoid, 10 of the 30 subjects did not achieve recommended antitoxin levels of  $>0.01$  diphtheria AU/ml (29), although only two were below the protective level of 0.001 diphtheria AU/ml sera. This finding has previously been reported by Edsall *et al.* (29) and Wishart and Johnson (30) and may be related to the small 1.5 Lf recall dosage administered and a long interval since prior immunization. Of the 39 individuals immunized only four had "adverse reactions." These reactions consisted of mild induration, erythema and in two individuals, slight fever and were not associated with a rise in either diphtheria or tetanus IgE antibodies. None of the individuals who did develop greatly elevated specific antibodies of the IgE class demonstrated any adverse local or systemic reaction; however, one of these individuals had a positive prick test with tetanus toxoid when tested several months after immunization. Only one of the four with an adverse local reaction demonstrated high post-immunization IgG antibodies ( $>10$  AU/ml) to either the tetanus or diphtheria component of the vaccine. Nine other individuals with post-immunization IgG titers ( $>10$  AU/ml) experienced no recognizable reactions.

Several factors may be important in the development of the specific IgE antibodies observed in this report. The alum and aluminum hydroxide adjuvants in the Td antigen have been shown to enhance reaginic antibody production in several mammalian species (31, 32). The alum adjuvant may have contributed to the magnitude of the diphtheria and tetanus

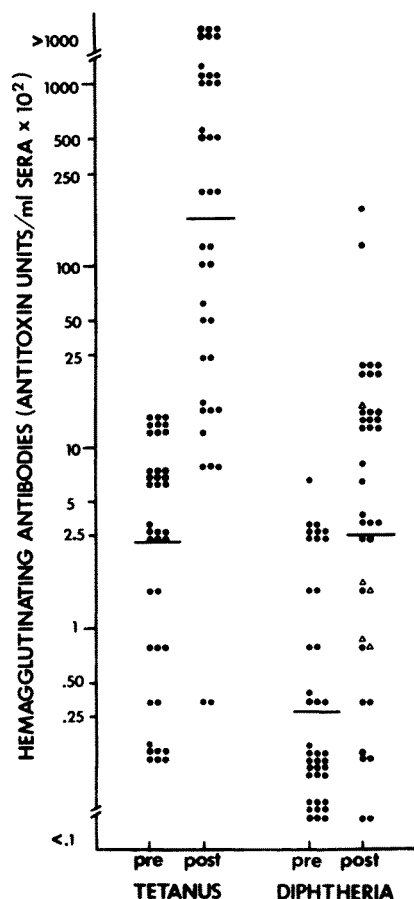


Figure 1. Tetanus and diphtheria hemagglutinating antibody levels (antitoxin units/ml serum) before (pre) and 10 to 21 days after (post) immunization with combined Td vaccine. Triangles indicate blood samples obtained 28 to 42 days after immunization. Geometric means are indicated by bars.

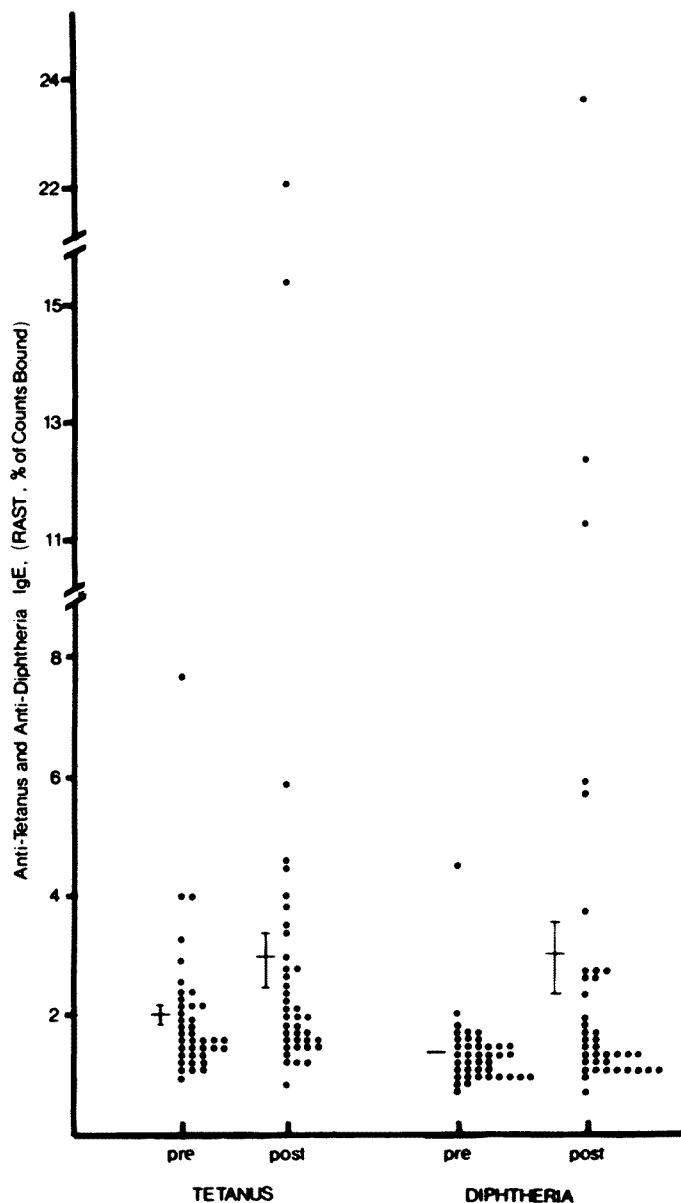


Figure 2. Anti-tetanus and diphtheria IgE antibody expressed as percentage of counts bound pre- and postimmunization. Geometric mean  $\pm$  S.E.M. are indicated by a bar and bracket, respectively.

IgE responses but there are no published reports documenting increased IgE antibody synthesis in man with alum adjuvants. This possibility is currently under investigation in our laboratories. The persistence of increased specific IgE as shown in Figure 8 was found to be long, especially in view of the short (2.8 day) half-life of serum IgE. Kuhns and Pappenheimer (33) have previously reported finding a nonprecipitating antitoxin with most of the properties of what is now termed IgE in the serum of several subjects after booster immunization with diphtheria toxoid. This antibody was also noted to persist for "weeks." This prolonged response may be a result of the use of an alum-precipitated toxoid; however, persistence of positive skin tests and specific serum IgE antibodies are regularly observed in atopic individuals after natural sensitization and may represent the unique pattern of IgE response in atopic individuals. Small amounts of antigen administered frequently are also known to enhance reagin production in both animal models and humans (23, 28). Since the exact previous immunization history of the subjects in this study cannot be established with certainty we are not able to exclude this possibility;

however, the possibility of hyperimmunization seems remote because the study population was screened before immunization for elevated tetanus antitoxin levels. The intramuscular route of antigen administration may also enhance reaginic antibody production through the presence of soluble antigen antibody complexes prolonging antigen stimulation. This phenomenon has been previously noted to occur after intramuscular immunization; however, it has primarily been associated with delayed reactions (34). Another question to be considered is whether the toxoid antigens might be contaminated with casein or casein hydrolysis products from the culture media. Since both toxoids were prepared in a hydrolyzed casein media, each of the subject's sera which demonstrated anti-tetanus or anti-diphtheria IgE activity were tested for IgE directed against casein and the hydrolyzed media by using the RAST method. No IgE antibodies were detected to either the casein or the hydrolyzed media. One may also wonder whether the IgE antibody synthesized after immunization might be directed against other potential impurities in the toxoids such

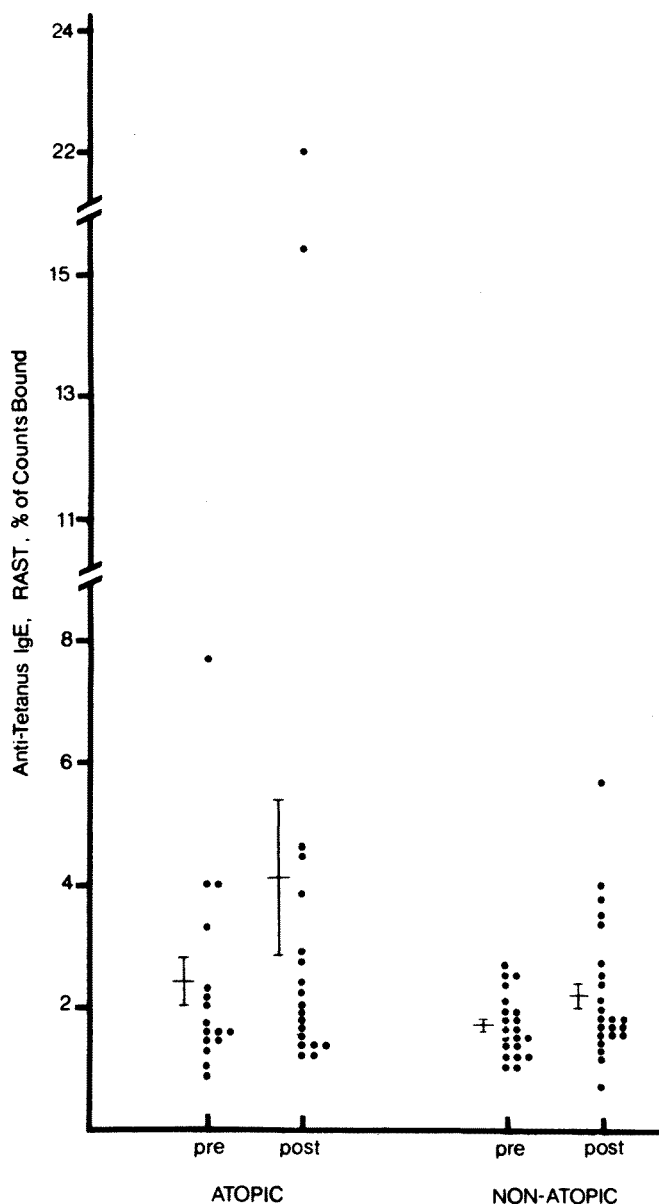


Figure 3. Anti-tetanus IgE antibody expressed as percentage of counts bound in atopic and non-atopic groups pre- and postimmunization. Geometric mean  $\pm$  S.E.M. are indicated by a bar and bracket, respectively.

as components of tetanus or diphtheria bacilli since the antigenic determinant for the IgE response may not necessarily be the same as that used to elicit antitoxin (IgG) response. Our present data do not enable us to exclude that possibility.

There are multiple reports of immediate wheal and flare reactions after cutaneous testing with bacterial antigens in patients with allergic disease (35-39). Facktor *et al.* (19) found that 63% of a group of normal volunteers demonstrated immediate cutaneous hypersensitivity to skin testing with intradermal tetanus toxoid and the frequency was similar in atopic and nonatopic individuals. Immediate wheal and flare reactions were found in 30.5% of a group of Schick-negative individuals when intradermally tested with diphtheria toxoid. Of these individuals, 60% were found to have a positive atopic history, whereas, only 7% of the nonatopic group had immediate reactions (12). Skin testing, with one exception, was not performed after booster Td immunization on the individuals in this study because we did not want to enhance, possibly, their immune responses with additional antigen.

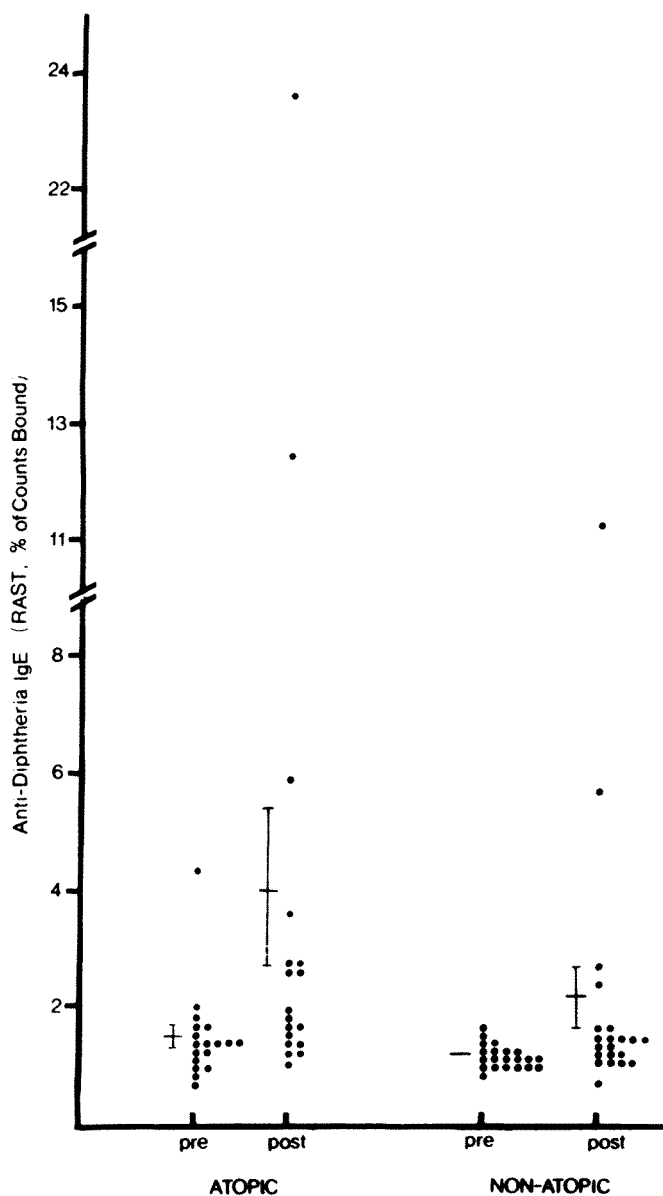


Figure 4. Anti-diphtheria IgE antibody expressed as percentage of counts bound in atopic and non-atopic groups pre- and postimmunization. Geometric mean  $\pm$  S.E.M. are indicated by a bar and bracket, respectively.

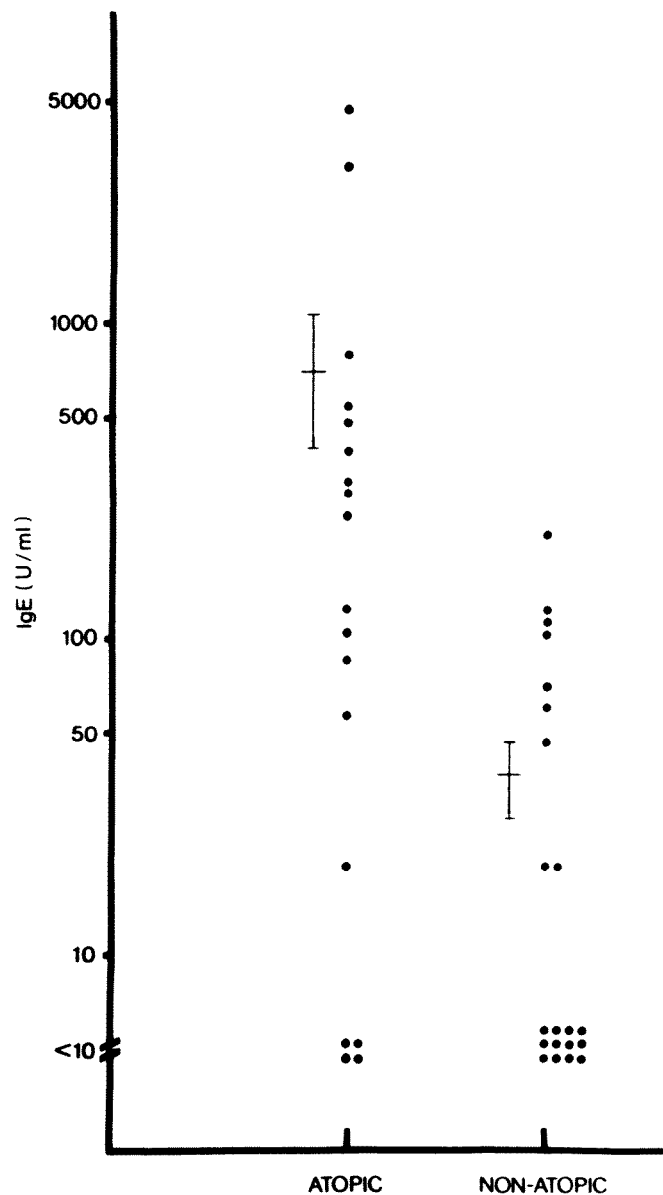


Figure 5. Total serum IgE levels (units/ml serum) are plotted logarithmically for the atopic and non-atopic groups. Geometric mean  $\pm$  S.E.M. are indicated by a bar and bracket, respectively.

This study also compared the synthesis of specific diphtheria and tetanus IgE antibodies in subjects with and without a personal history of atopy. Total serum IgE levels were measured in all individuals and increased IgE levels were found in 12 of 18 (67%) atopic subjects, an incidence similar to data reported by others (40). Even though the mean total serum IgE values were statistically greater in atopic as compared to nonatopic subjects, the pre-immunization mean-specific diphtheria and tetanus IgE antibodies were similar in both groups. The lack of elevation in the atopic subjects of pre-immunization-specific tetanus and diphtheria IgE antibody values may be explained by our screening for elevated IgG antibodies and excluding those individuals with elevated titers from immunization. Several studies have demonstrated immune responsiveness or nonresponsiveness in both animals and man to be under genetic control (41, 42) and by preselecting only individuals with tetanus antitoxin (IgG) values less than 0.125 AU/ml, irrespective of the interval since their previous immunization, a population of low or nonresponders may have been created. Several atopic individuals (IgE >2000 units/ml) who had

greatly elevated tetanus IgG ( $>10$  AU/ml) also had elevated ( $>2$  S.D.) pre-existing specific tetanus IgE values. Because of elevated pre-existing antibody values this group of individuals was not given booster immunizations.

In this report mean diphtheria IgE antibodies increased significantly after booster immunization in both atopic and nonatopic subjects. The increment of increase in specific diphtheria IgE antibody was greater in the atopic than the nonatopic subjects but the difference between the two groups was not statistically significant. Even though positive correlation coefficients were calculated when the post-immunization-specific tetanus and diphtheria antibody titers were compared to both total serum IgE and a history of atopy, the degree of correlation was not sufficient to be predictive.

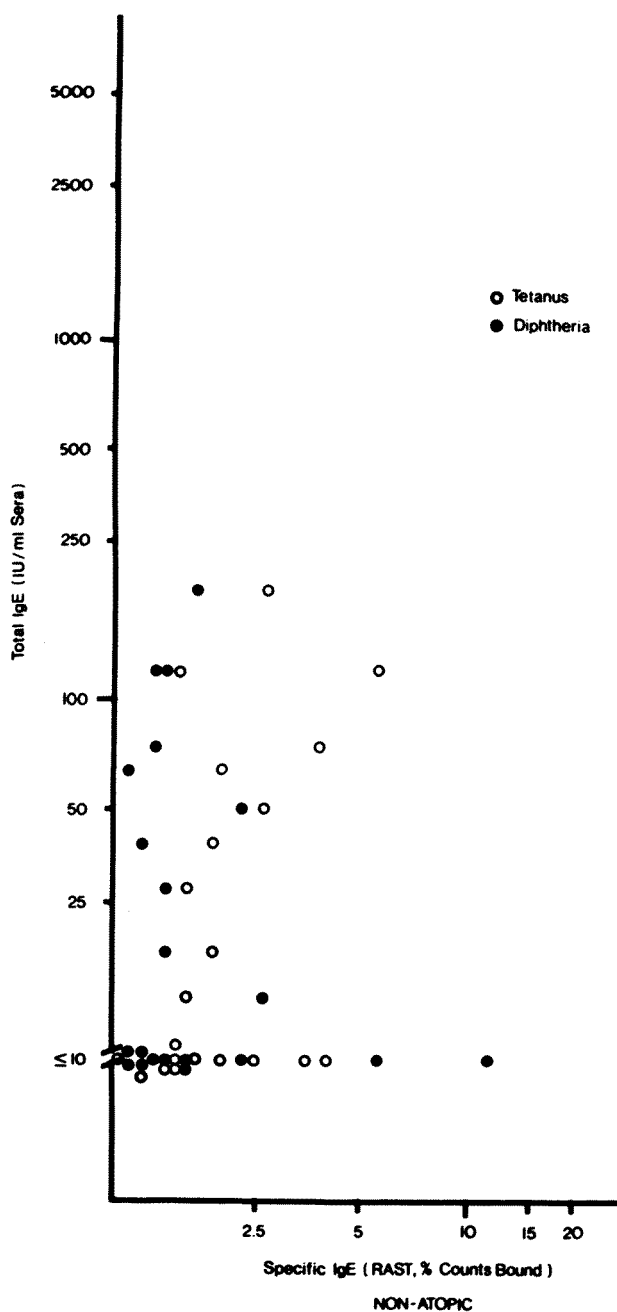


Figure 6. Total serum IgE level (units/ml serum) is plotted logarithmically on the ordinate and specific anti-tetanus and diphtheria IgE (percentage of counts bound) RAST values are plotted logarithmically on the abscissa for the non-atopic individuals.

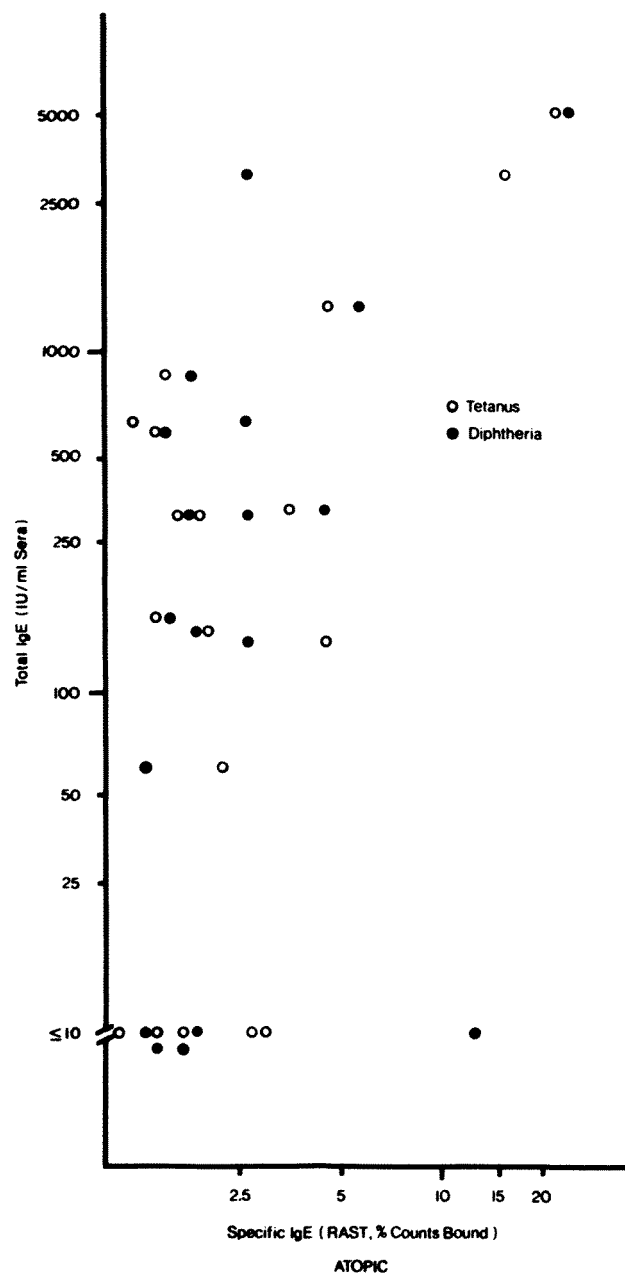


Figure 7. Total serum IgE level (units/ml serum) is plotted logarithmically on the ordinate and specific anti-tetanus and diphtheria IgE (percentage of counts bound) RAST values are plotted logarithmically on the abscissa for the atopic individuals.

This study has documented for the first time specific IgE antibody synthesis after booster immunization with tetanus and diphtheria toxoids. Since tetanus and diphtheria toxoids are capable of inducing immediate wheal and flare reactions, as well as specific IgE production, the possibility exists that other bacterial and perhaps viral products are also capable of sensitizing the human host via induction of specific IgE synthesis. Since a variety of cutaneous and respiratory phenomena resembling immediate hypersensitivity reactions may occur during infectious diseases, a role for the specific IgE antibodies in the host response to infectious agents must be considered. Interaction of IgE antibodies with appropriate antigens could directly initiate release of mediators or activate complement via the alternative pathway.

A strong correlation has been demonstrated between high specific antitoxin values and adverse local reactions noted after

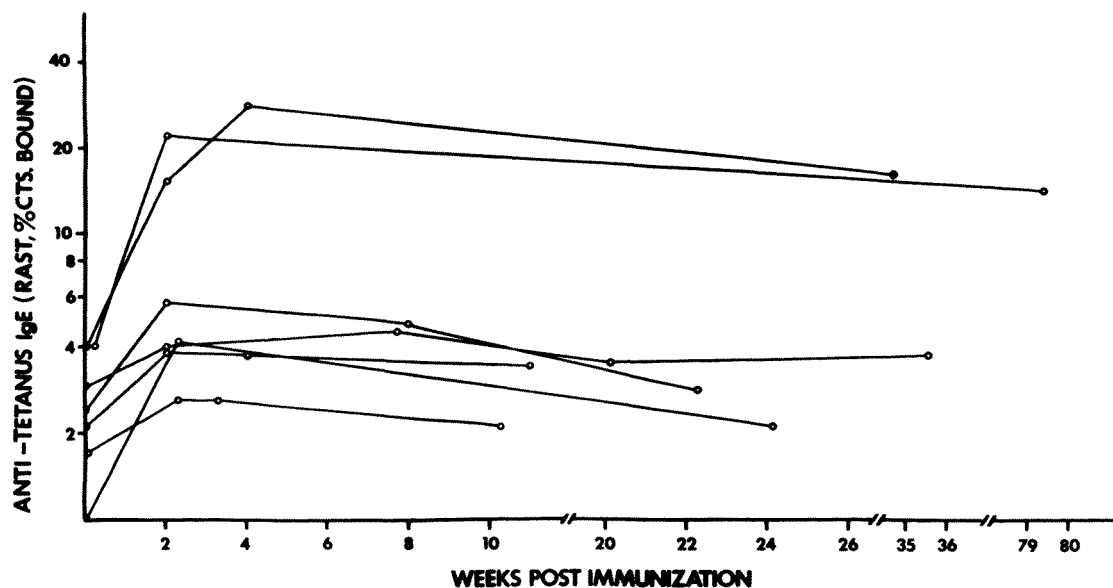


Figure 8. Anti-tetanus IgE response RAST (percentage of counts bound) is plotted logarithmically for seven individuals at various intervals (weeks) postimmunization.

booster immunization with tetanus or diphtheria toxoid (8, 43, 44). Patient surveys have indicated that many of the individuals with local reactions possessed antitoxin titers greatly in excess of protective levels (8, 45, 46) and this finding has led numerous authorities to recommend that booster immunizations be spaced at 5- to 10-year intervals or longer (45, 47, 48). Our finding that IgE class antibodies directed against tetanus or diphtheria may be synthesized by both atopic or nonatopic individuals after booster immunization lends additional support to these recommendations that the overuse of tetanus or Td toxoids should be avoided.

**Acknowledgment.** The authors are grateful to Dr. Floyd H. Taylor for statistical consultation, Dr. Gilbert Friday for allowing us to study his patients, and Mrs. Sylvia Ciota for assistance in preparation of the manuscript.

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