

Immunoglobulin E-Suppressing and Immunoglobulin G-Enhancing Tetanus Toxoid Prepared by Conjugation with Pullulan

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Immunoglobulin E (IgE) antibody response was found to be suppressed selectively and antigen specifically in mice given an antigen conjugated with pullulan, a linear copolymer of maltotriose, whereas IgM and IgG antibody responses were enhanced. On the basis of this finding, tetanus toxin was conjugated with pullulan by cyanuric chloride in the hope that the toxin would be detoxified by the conjugation procedure and could be used as an IgE-suppressing and IgG-enhancing toxoid without the aid of an aluminum adjuvant. This procedure of tetanus toxin-pullulan conjugation apparently detoxified the toxin. Administration of the resulting tetanus toxoid, tetanus toxin-pullulan conjugate, to mice induced strong suppression of IgE antibody response with fairly good IgG response, whereas the alum-precipitated toxoid or plain toxoid, customarily used for vaccination, elicited high IgE antibody formation. The IgE antibody response was minimal, but the IgG antibody response was maximal in the conjugate-primed mice even after a booster injection with an IgE antibody-inducing dose of the alum-precipitated toxoid.

In spite of recent advances in technology for purification of tetanus or diphtheria toxin, several investigators have reported that vaccination of the tetanus Formol toxoid causes occasionally acute systemic allergic reactions (1-3) and that administration of toxoid with an aluminum adjuvant induces an anaphylactic reaction (4, 13) and even death, in one case where the vaccinee's pathological examination strongly suggested that the anaphylactic shock was due to immunoglobulin E (IgE) antibodies (17). Aluminum hydroxide-precipitated tetanus toxoid (APTT) may stimulate the formation of IgE antibody, since it has been shown that an aluminum compound is a good adjuvant for IgE antibody formation in experimental animals (6, 10, 12). In fact, we demonstrated IgE antibody production to tetanus toxoid by enzyme immunoassay in individuals who had been injected with APTT. Additionally, one of us developed sneezing, rhinorrhea, and respiratory difficulties after inhaling tetanus toxin powder and had a high IgE antibody titer (H. Ikegami et al., Abstr. no. 87, 31st Congr. Jpn. Soc. Allergol., Utsunomiya, 21 October 1981).

Recently we have found that ovalbumin conjugated with pullulan, a linear copolymer of maltotriose, in small part maltotetraose, with α -1,6-glucoside linkage and bearing no branches

(20), enhances IgM and IgG anti-ovalbumin antibody production in mice while suppressing IgE antibody production (18). It was also found that conjugation of pullulan with toxins can abolish their toxicities (14). It appears, therefore, that a tetanus toxin-pullulan conjugate (TTPC) would be an ideal immunogen for vaccination against tetanus, diphtheria, and so on.

The purpose of this study was to investigate IgM, IgG, and IgE antibody responses in mice against TTPC. The results were compared with those obtained by immunization with APTT or plain tetanus toxoid (PTT).

MATERIALS AND METHODS

Animals. Female ddy mice (4 to 7 weeks of age) were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan. Female Sprague-Dawley rats (9 to 12 weeks of age) for the passive cutaneous anaphylaxis (PCA) assay were purchased from the Charles River Japan, Kanagawa.

Tetanus toxin and toxoid. Purified tetanus toxin with about 2,800 times the limit of flocculation (LF) per mg of protein nitrogen was prepared and detoxified by the method of Kameyama and Kondo (7). The Formalin-treated toxoid was used as PTT for immunization. This preparation was also used for sensitization of sheep erythrocytes in passive hemagglutination (HA) tests and as a challenging antigen in the PCA reaction.

APTT. APTT was prepared following the procedure

of Yamamoto et al. (21), using PTT. Aluminum content was adjusted to 0.45 mg for each APTT dose.

TTPC. Pullulan (molecular weight, 345,000) was kindly supplied by the Hayashibara Biochemical Laboratory, Okayama, Japan. Fifty milligrams of pullulan dissolved in 5 ml of 0.017 M phosphate-buffered saline (pH 7.0) was cooled in an ice bath. To the solution was added 20 mg of cyanuric chloride dissolved in 1 ml of cooled *N,N*-dimethylformamide, and the mixture was stirred in an ice bath for 4 to 5 h, keeping the pH of the mixture at 6.8 to 7.0 by carefully adding a few drops of a 5% Na_2CO_3 solution. Although the mixture was turbid at the beginning of the reaction, it became clear as the reaction progressed. Thereafter, tetanus toxin (ca. 20 mg of protein) was added to the mixture, which was stirred at 37°C for about 6 h. The mixture was frequently monitored by cellulose acetate membrane electrophoresis according to the method traditionally used in our laboratory until the band closer to the cathode was observed as shown in Fig. 1. To stop the cyanuric chloride, an adequate amount of glycine was added to the mixture, which was kept with additional stirring in a refrigerator overnight. The resulting mixture was chromatographed on DEAE-Sephacel equilibrated with 0.05 M Tris-hydrochloride buffer (pH 7.5). Fractions were eluted stepwise by 0.05 M Tris-hydrochloride buffer containing NaCl at increasing concentrations. Fraction 1 was eluted with 0.05 M Tris-hydrochloride buffer. Fraction 2 was eluted with the same buffer containing 0.05 M NaCl, and the remaining fractions were eluted with the same buffer and NaCl concentrations as follows: fraction 3, 0.1 M; fraction 4, 0.2 M; fraction 5, 0.3 M; and fraction 6, 0.5 M. Detoxification of each fraction was then tested as follows. Two mice were injected with 0.5 ml of each fraction and observed for 7 days. None of the mice showed any abnormal signs during the observation

period. The molar ratio of toxin to pullulan was calculated as 1:2.8 for fraction 1 and 1:1 for fraction 2 according to Usui and Matuhasi (18). Since TTPC is highly hydrophilic and the conjugate-antibody complex is too transparent to be read by the usual flocculation test, the antigenic potency of TTPC could not be determined accurately. Therefore, the combining power (CP) of TTPC along with cyanuric chloride-modified tetanus toxoid (CCTT) and PTT was measured by the method of Moloney and Hennessy (15).

Immunization. Mice were injected subcutaneously with TTPC, and with APTT, CCTT, and PTT as controls, on the schedules described in Results.

Antibody titrations. The passive HA test was performed, and the titer was expressed as HA units (HAU) per milliliter as described by Kameyama and Kondo (7). Treatment of antiserum with 2-mercaptoethanol (2-ME) was performed according to the procedure described by Kameyama et al. (8). The toxin neutralization test was carried out at a Limes-Tod/1,000 level in mice, and antitoxin titer was expressed in international units per milliliter (7). IgE antibody titer was determined by the PCA reaction in rats according to Mota and Wong (16) and Gerbrandy and Bienenstock (5) with slight modification.

RESULTS

Analysis of TTPC by cellulose acetate membrane electrophoresis. The fractions chromatographed on DEAE-Sephacel along with the original reaction mixture, toxin, plain toxoid, and human serum were subjected to cellulose acetate membrane electrophoresis. Two membrane strips were prepared. One was stained with nigrosin, and the other was stained with Schiff reagent. The original mixture consisted of two bands stained by nigrosin (Fig. 1). One was closer to the cathode; its electrophoretic mobility was much lower than that of the other fraction and that of toxin or plain toxoid. The slower-moving band was also stained with Schiff reagent, whereas bands of toxin and plain toxoid were not. The other band of the original mixture moved considerably faster than toxin or plain toxoid and was not stained with Schiff reagent. The slower-moving band was found in fraction 1, with lesser amounts found in fractions 2 and 3. In contrast, fractions 4 and 5 contained mostly the faster-moving component, and fraction 6 contained no significant amount of either component. These results indicated that TTPC was present in fractions 1, 2, and 3. Accordingly, fraction 1 or 2 was used as TTPC for immunization, and fraction 4 or 5, which we considered to be CCTT, was used as a control.

Kinetics study of antibody responses against TTPC and APTT. To avoid undesirable influences on antibody responses by frequent bleedings, four groups of five mice each were injected subcutaneously with 1 CP of TTPC or APTT. The four groups were bled 1, 2, 3, and 4 weeks, respectively, after injection. All mice were bled 1 and 2 weeks after the booster with 1 CP of

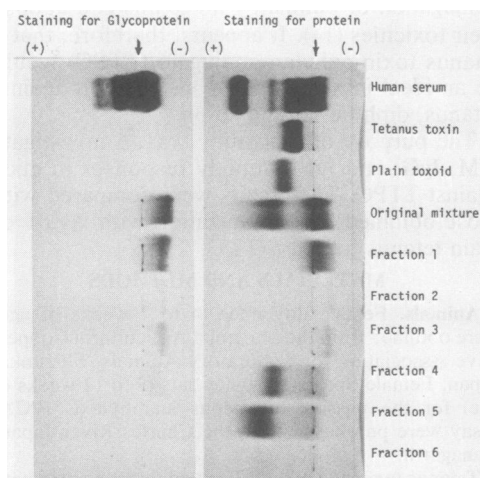


FIG. 1. Cellulose acetate membrane electrophoresis. Each fraction along with human serum, tetanus toxin, plain toxoid, and the original mixture after tetanus toxin-pullulan conjugation was subjected to electrophoresis. Protein was stained with nigrosin (right strip), and glycoprotein was stained with Schiff reagent (left strip). Arrows show the points where samples were applied.

homologous antigen at the 4th week. Serum specimens in each group were pooled. Figure 2 presents kinetics of IgE antibody responses to TTPC and APTT measured by the PCA reaction in rats. After the initial injection of 1 CP of TTPC, there was no IgE response up to the 4th week, whereas IgE response was observed as early as 1 week after injection of APTT. IgE response to TTPC was noted 1 week after the booster injection with TTPC, although the PCA titer was extremely low. In contrast, the PCA titer rose very sharply, reaching more than 100 1 week after the booster with APTT and declining 2 weeks later.

The same serum specimens were examined by the HA test for IgG and IgM antibodies. The HA titer of antisera to TTPC started to rise from the 3rd week, and that against APTT rose from the 2nd week (Fig. 3). Both titers were very similar at the 4th week. Upon booster with TTPC or APTT, titers reached their maxima 2 weeks later and tended to decline slightly thereafter. Treatment of antisera with 2-ME did not change their titers, indicating that these antibodies measured by the HA test belonged exclusively to the IgG class (data not shown).

Comparison of antibody responses against TTPC, APTT, CCTT, and PTT. Further experiments were performed by using PTT and CCTT in addition to another lot of TTPC and APTT. Four groups of mice, five animals each, were immunized with 1 CP of each of four different antigens and boosted with 1 CP of homologous antigens at the 4th week. Based on the above-described results of the kinetics study, they were bled for PCA and HA assays 4 weeks after the initial injection. They were also bled for the PCA assay 1 week and for the HA assay 2 weeks after the booster. In these experiments, titers of

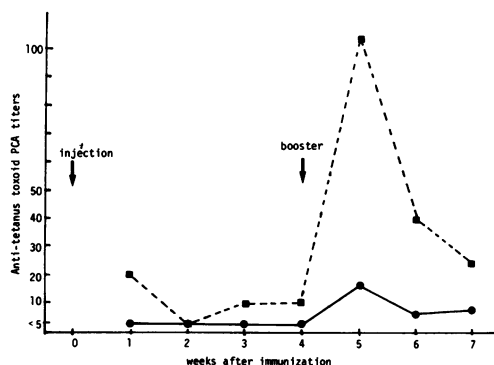


FIG. 2. Kinetics of IgE antibody responses to TTPC (●) and APTT (■). Mice were immunized with 1 CP of TTPC or APTT and boosted at the 4th week, as described in the text. Mice were bled at 1-week intervals, and the pooled sera were assayed.

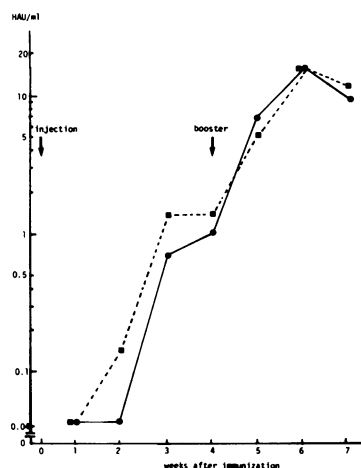


FIG. 3. Kinetics of HA antibody responses to TTPC (●) and APTT (■). The same sera examined in Fig. 2 were assayed for HA titers.

five individual antisera were determined by the HA test. Pooled sera of each group of animals were used for the PCA and toxin neutralization tests. In the HA test, the geometric means of antisera obtained 4 weeks after the initial injection were 1.08 HAU for TTPC, 0.34 HAU for CCTT, 0.14 HAU for PTT, and 1.08 HAU for APTT (Table 1). These values did not change significantly after the treatment of antisera with 2-ME, again indicating that antibodies detectable by the HA test belonged predominantly to the IgG class. IU values of these antisera paralleled their HA titers.

As expected, the PCA titer of APTT immune serum was maximal (titer of 20) and that of TTPC immune serum was minimal (titer of less than 5). CCTT and PTT immune sera gave a PCA titer of 5.

After the booster HAU values of TTPC immune sera and APTT immune sera increased almost 10-fold, as did those of the other antisera. Differences in these HAU values were not significant. Similar results were obtained with antisera treated with 2-ME. IU values also increased after the booster injection in all of these groups, with no significant differences among values. In contrast, the PCA titer of APTT immune serum was 320, and other immune sera gave significantly lower titers: 10 for TTPC, 20 for CCTT, and 57 for PTT.

Priming effect on IgE antibody response of TTPC and other antigens. Since the IgE-selective suppression was not strongly developed in mice given one injection of 2 CP of TTPC when challenged with an IgE antibody-inducing dose of APTT, mice were injected with TTPC three times at 1-week intervals, including control groups. Six groups of five mice each were inject-

TABLE 1. Antibody responses to various preparations of tetanus toxoid

Immunized group	Antitoxin titer							
	4 wk after initial injection ^a				2 wk after booster injection ^b		1 wk	
	HAU/ml ^c	HAU/ml (2-ME+) ^d	IU/ml ^e	PCA ^f	HAU/ml	HAU/ml (2-ME+)	IU/ml	PCA
TTPC	1.08 (0.44–2.64)	0.95 (0.27–3.29)	1.24	<5	9.34 (8.31–10.47)	6.61 (4.25–10.3)	6.84	10
CCTT	0.34 (0.14–0.84)	0.69 (0.26–1.84)	0.58	5	6.53 (3.00–14.2)	3.08 (1.17–8.12)	7.24	20
PTT	0.14 (0.05–0.46)	0.14 (0.04–0.54)	0.18	5	4.36 (2.73–6.96)	3.54 (1.87–6.71)	6.47	57
APTT	1.08 (0.74–1.59)	1.71 (1.00–2.86)	1.84	20	10.0 (6.70–15.0)	7.09 (4.80–10.5)	10.8	320

^a Four groups of mice, five animals each, were primed with 1 CP of the indicated antigenic preparation.

^b At the 4th week after the initial injection, each group was boosted with 1 CP of a homologous antigen.

^c Geometric means of HA titers in each group are presented. Numbers in parentheses show the fiducial limits at $P = 0.95$.

^d Each antiserum was treated with 2-ME as described in Materials and Methods.

^e Toxin neutralization test titer of pooled antiserum of each group.

^f Geometric means of PCA titers in two rats, using antisera pooled from each group.

ed three times at 1-week intervals with 2 CP of each of the four antigens. Two weeks later, the primed animals were boosted with 2 CP, with an IgE antibody-inducing dose of APTT, or with 2 CP of homologous antigen as controls. The PCA titer of pooled sera of mice primed with TTPC and boosted with APTT (I) was considerably lower than the titers of mice primed with other antigens (II, III, and IV) (Fig. 4). On the contrary, the HAU value of the pooled sera was the highest. The PCA titer of mice primed with TTPC and boosted with TTPC (V) was 10, which was the lowest among the groups, where the HAU value of these mice was reasonably high. On the other hand, the PCA titer of mice primed

with APTT and boosted with APTT (IV) was much higher than those of group I, III, V, and VI, but the HAU value of group IV was similar to that of group V. In mice primed with PTT and boosted with APTT, the IgE antibody titer was higher than the titers of mice primed with TTPC, suggesting that the priming effect for IgE response is greater for PTT than for TTPC. The IgE antibody titer of mice primed with CCTT and boosted with APTT was the highest among the groups, even though their HAU value was high. In mice primed and boosted with CCTT, the PCA titer was low, but the HAU value was lower than that of group V. These findings indicate that CCTT contamination of a TTPC preparation is undesirable.

It should be stressed that the PCA titers of mice primed with TTPC, regardless of booster antigens (i.e., TTPC or APTT), were very low, whereas their HAU values were higher than for the PTT- or APTT-primed group.

DISCUSSION

To search for an ideal tetanus vaccine, we used the TTPC procedure and found that it apparently detoxified the toxin. Although the exact mechanism(s) of detoxification by pullulan conjugation was not analyzed, it is possible that pullulan conjugated with toxin may block binding of the toxin to receptors of target cells by its steric hindrance. Alternatively, bridging of pullulan and toxin by cyanuric chloride via ϵ -NH₂ radicals of lysine, which is involved in the toxicity, may result in direct inactivation of the toxin. These possibilities are supported by the fact that toxicity was not found in the TTPC preparation whose toxin was conjugated to pullulan after free cyanuric chloride was dialyzed out in the conjugation procedure. However, in

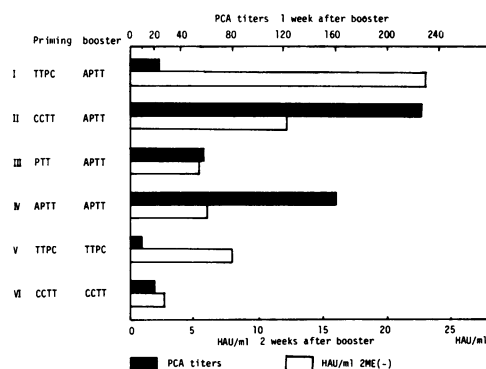


FIG. 4. Priming effect on IgE antibody response to TTPC and other antigens. Six groups of five mice each were primed three times at 1-week intervals subcutaneously with 2 CP of each antigen and boosted with 2 CP of APTT or homologous antigen at the 4th week. Mice were bled for the PCA test 1 week and for the HA test 2 weeks after the booster. Sera of each group were pooled and assayed.

other experiments we have found detoxification of tetanus toxin by treatment with cyanuric chloride alone. Therefore, we consider that tetanus toxin was detoxified by both binding to pullulan and modification with cyanuric chloride in the TTPC preparation used here.

TTPC was shown to be able to suppress IgE antibody response to tetanus toxin. Simultaneously, TTPC was shown to be an excellent immunogen for IgG antibody production. This was clearly shown in the kinetics studies (Fig. 2 and 3; Table 1). The IgE antibody response was minimal, but the IgG response was very strong in the TTPC-primed mice even after the booster injection with an IgE antibody-inducing dose of APTT (Fig. 4). This was in sharp contrast to the IgE and IgG antibody responses to APTT or PTT, which has been customarily used for human vaccination. These findings indicate that the priming effect of TTPC is strong for the IgG antibody response but very weak for the IgE response. On the other hand, it has been clearly established that protective antibodies against tetanus toxin belong to the IgG or IgM class, or both. In this respect, the above-mentioned observation would be of some importance in vaccination against tetanus.

Our previous study clearly demonstrated that the similar selective suppression of IgE response by ovalbumin-pullulan conjugate was antigen specific (18). In that study, the selective suppression of IgE response by TTPC was probably antigen specific and was considered to be caused by the same mechanism(s) as the suppression by ovalbumin-pullulan. Further studies on the selective suppression to ovalbumin have clarified several points (19): (i) the cells responsible for the suppression are transferable in our system; (ii) the cells are resistant to anti-Thy 1.2 or anti-brain-associated theta antigen serum plus complement; (iii) the cells are found in the populations of both those passed through and those adhered to a nylon wool column; (iv) macrophages do not participate in the suppression; (v) the cells are sensitive to puromycin, mitomycin C, cyclophosphamide, and X irradiation and (vi) the cells transferred into mice previously treated with cyclophosphamide do not induce the suppression. These findings suggest that the cells involved in suppression are different from those reported by Kishimoto et al. and others, who have shown the presence of IgE-specific T-suppressor cells in their serial experiments on inducing tolerance for the IgE antibody response (9, 11). In our experimental system, it is possible that several lymphocyte populations interact to manifest the suppression. The exact mechanism(s) responsible for the selective suppression of IgE antibody response, however, remains to be clarified.

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