EFFECTS OF pH AND LYSINE DURING DETOXIFICATION OF A HEMORRHAGIC PRINCIPLE OF HABU SNAKE (*TRIMERESURUS FLAVOVIDIDIS*) VENOM WITH FORMALIN ON THE IMMUNOGENICITY OF THE TOXOID

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SUMMARY: A hemorrhagic principle (HR1B) isolated from the venom of Habu snake (*Trimeresurus flavoviridis*) was detoxified with formalin at pH 5, 7 and 9. Higher pH shortened the period needed for detoxification and lowered the final formalin concentration required for complete detoxification. The extent of polymerization and the immunogenicity of the toxoid formed were dependent upon pH during detoxification; the lower the pH, the higher the extent of polymerization and the immunogenicity of the toxoid. The higher immunogenicity possessed by the toxoid with higher extent of polymerization was confirmed by fractionation of the toxoid prepared at pH 7 on Sephadex G-200 and by comparing the immunogenicity of the toxoid fractions. The presence of lysine during detoxification brought about a partially polymerized toxoid having new immunogenic characteristics and a higher immunogenicity.

INTRODUCTION

Attempts have been made by many workers at transforming venom to toxoids by treatment with formalin (1,2,3,4, 5,6). We have attempted to prepare formalin toxoids from the venom of Habu snake (*Trimeresurus flavoviridis*) (7,8,9, 10, 11, 12) and reported toxoids for human use (13,14).

Recently we found that lower pH of aqueous formalin in detoxification medium rendered the toxoid highly polymerized and immunogenic. We found also that the addition of lysine to the detoxification medium induced a partially polymerized toxoid having new immunogenic characteristics.

This report deals with the effects of pH and lysine during detoxification of a purified hemorrhagic principle of the venom with formalin on the immunogenicity of the toxoid formed.

MATERIALS AND METHODS

A highly purified hemorrhagic principle (HR1B) was obtained as described previously (15).

HR1B at 2 mg/ml was detoxified at 37°C at three different pH values. The buffers used were 0.2 M acetate, pH 5.0, 0.07 M phosphate, pH 7.0 and 0.03 M Tris, pH 9.0. Formalin was added initially to 0.1% and its concentration was increased by 0.2% increments at several days' intervals. At appropriate time intervals, aliquots of the reaction mixtures were withdrawn and dialyzed against physiological saline. When 0.1 ml of the dialysis residue induced no hemorrhagic spot in the rabbit skin, detoxification was regarded as complete. The toxoids were dialyzed against 0.025 M acetate-buffered saline (pH 6.0). The dialysis residue was diluted with the buffer to 1 mg/ml, and to this was added a half volume each of 0.077 M Na_3PO_4 and 0.077 M AlCl_3 in this order to obtain an aluminum phosphate gel suspension.

Immunization experiments were performed with four male and four female guinea pigs per dose. To examine for the effects of pH on the immunogenicity, three doses (0.002, 0.02 and 0.2 mg) of the toxoids were each injected subcutaneously twice at a 6-week interval. To examine for the immunogenicities of fractions of the toxoids and for the effects of lysine, three doses (0.0016, 0.008 and 0.04 mg) of the toxoids were each injected twice at a 4-week interval. All guinea pigs were bled 14 days after the 2nd injection. The anti-hemorrhagic activities of the sera were determined by the rabbit skin method (16) using purified HR1 as test toxin, and expressed in anti-HR1 units per ml (17). For statistical analysis, the validity tests were performed by the parallel-line-assay method (18). All tests for significance were made at a probability of $p=0.05$ and all confidence intervals were estimated at this probability.

The molecular weight was determined by SDS-polyacrylamide gel electrophoresis in 5% gel containing 1% SDS as described by Dunker and Rueckert (19). SDS-PAGE marker I (a mixture of heat-stable RNA-polymerases, Seikagaku Kogyo Ltd., Tokyo) was used to obtain the standard curve.

All chemicals used were of analytical reagent grade. Protein contents of toxoid preparations were estimated from the absorbancy at 280 nm of sample solutions with a conversion factor of 1.2 for purified HR1B.

RESULTS

HR1B, one of the major hemorrhagic principles of Habu venom, was detoxified with formalin at pH 5, 7 and 9.

Fig. 1. SDS-polyacrylamide gel electrophoresis of HR1B detoxified with formalin at various pH values.

Ten-microliter aliquots (about 20 μ g) of the toxoid preparations were subjected to SDS-polyacrylamide gel electrophoresis. The samples were treated with SDS and β -mercaptoethanol at 55 C for 1 hr, and then applied to 5% polyacrylamide gel containing 1% SDS (19).

The gels contained HR1B detoxified at pH 5 (1); at pH 7 (2); at pH 9 (3); native HR1B (4); marker protein of molecular weights of 180 000, 140 000, 100 000, 42 000 and 39 000 (from the top to bottom) (5). The faint band seen in the gel with native HR1B might have been produced by autodigestion of the principle during storage.

The final concentrations of formalin and the durations required for complete detoxification at those three pH values were 1.7, 1.3 and 1.1%, and 60, 34 and 28 days, respectively. As Fig. 1 demonstrates, marked polymerization occurred during detoxification of the hemorrhagic principle and the extent of polymerization was dependent upon pH. The lower the pH, the higher the extent of polymerization and the higher the immunogenicity of the toxoid (Fig. 2). The difference in the immunogenicity between the toxoids obtained at pH 5 and those at the other pH values was significant.

To correlate the polymerization with the immunogenicity, HR1B detoxified at pH 7 was molecular-sieved on Sephadex G-200, super fine, and the immunogenicities of the fractions were compared. A portion of each fraction was subjected to SDS-polyacrylamide gel electrophoresis; the fractions corresponding to monomer, dimer and highly polymerized (>trimer) toxoids were each pooled as indicated in Fig. 3. The difference in the immunogenicity between the monomer and the dimer fractions was statistically insignificant, whereas that between the monomer and the highly polymerized fractions was significant (Fig. 4).

Linggood et al. (20) reported that lysine aided to obtain a stable diphtheria toxoid. Since we have found that Habu toxoid regained occasionally its hemorrhagic activity in toxicity-reversion experiments, the effects of lysine on detoxification with formalin were investigated.

When L-lysine·HCl was added to the reaction mixture at pH 7 to a final concentration of 0.05 M, HR1B was detoxified in 21 days, whereas 35 days were required in the absence of the amino acid. Figure five shows the pattern of gel filtration on Sephadex G-200, super fine, of HR1B toxoid prepared in the presence of lysine. The presence of lysine

Fig. 2. Immunogenicities of HR1B-toxoids prepared at various pH values.

Anti-HR1 titers were determined 2 weeks after the 2nd injection. Geometrical means of titers in each immunizing group were plotted. The vertical bars indicate the confidence intervals ($p=0.05$). ●; pH 5, ○; pH 7, △; pH 9.

Fig. 3. Gel filtration on Sephadex G-200, super fine, of HR1B detoxified at pH 7. HR1B (20 mg) was detoxified at pH 7, and the toxoid solution was concentrated to about 2 ml under vacuum in a collodion bag. The concentrated solution was applied to a column (1.6 x 95 cm) of Sephadex G-200, super fine, equilibrated with 0.1 M NaCl-0.05 M Tris-HCl buffer (pH 8.5). The column was eluted with the same buffer, and 2-ml fractions were collected at 4 C.

Fig. 4. Immunogenicities of the fractions of HR1B-toxoid prepared in the absence of lysine.

The fractions of HR1B-toxoid used were the same as those described in the legend to Fig. 3. Geometrical means of anti-HR1 titers were plotted with the confidence intervals ($p=0.05$). Δ ; monomer fraction, $\circ$; dimer fraction, $\bullet$; highly polymerized (>trimer) fraction.

Fig. 5. Gel filtration on Sephadex G-200, super fine, of the toxoid prepared with lysine.

HR1B (45 mg) detoxified at pH 7 in the presence of lysine was subjected to gel filtration on Sephadex G-200, super fine. All the conditions used were the same as described in the legend to Fig. 3. Fractions from 33 to 46 and from 47 to 55 were pooled separately and designated as "polymerized" and "nonpolymerized", respectively.

Fig. 6. Immunogenicities of the fractionated HR1B-toxoids prepared with or without lysine.

The polymerized and nonpolymerized fractions prepared in the presence of lysine and the toxoids prepared without lysine were the same as those described in the legends to Figs. 5 and 3, respectively. Geometrical means of anti-HR1 titers were plotted with the confidence intervals ($p=0.05$).

- (a); with lysine, ○ : whole toxoid, △ : polymerized fraction,
 □ : nonpolymerized fraction.
 (b); without lysine, ● : whole toxoid, ▲ : highly polymerized fraction.

resulted in a partially polymerized toxoid (cf. Fig. 3). The toxoid was divided into polymerized and nonpolymerized fractions. These fractions as well as the whole toxoid (unfractionated) were injected into guinea pigs to determine the immunogenicities. For comparison, the whole toxoid prepared without lysine and its highly polymerized fraction, which were the same materials to those described in the legend to Fig. 3, were also injected into guinea pigs. As seen in Fig. 6(a), the polymerized and nonpolymerized toxoids obtained in the presence of lysine had immunogenicities comparable to each other. It is noteworthy, however, that the slopes of the dose-response curves are markedly different between the toxoids prepared in the presence (Fig. 6(a), $b=0.954$) and in the absence (Fig. 6(b), $b=0.411$) of lysine.

DISCUSSION

Many investigators demonstrated that polymerization of toxic protein(s) during detoxification with formalin (21,22,23,24,25), is dependent upon pH of the reaction mixture (23,26). Warren et al. (26) reported that staphylococcal enterotoxin B polymerized extensively in neutral (pH 7.5) and acidic (pH 5.0) conditions (maximum at pH 7.5) stimulated production of antibodies, while nonpolymerized toxin failed to do so. Morotz-Perlmutter et al. (6) demonstrated that the hemorrhagic principle isolated from the venom of *Vipera palestinae* polymerized over a wide pH range (pH 5.5 – 9.2) but the antigenicity was preserved only under acidic conditions.

As reported herein, the hemorrhagic principle (HR1B) from Habu snake (*Trimeresurus flavoviridis*) venom was detoxified easily with formalin under alkaline conditions (pH 9) with preservation of the immunogenicity. The hemorrhagic principle, when treated with formalin under acidic conditions (pH 5.0), was detoxified retaining higher immunogenicity and undergoing higher polymerization. Higher concentration of formalin and longer duration of the treatment were required for complete detoxification. The extent of polymerization paralleled the immunogenicity. Higher immunogenicity and polymerization of the toxoid were demonstrated by fractionating the toxoid by molecular sieving and by correlating the immunogenicity of individual fractions with the extent of polymerization. The immunogenicity of the highly polymerized toxoid was definitely higher than that of the monomer toxoid (Fig. 4), being consistent with earlier results (27, 28, 29, 30).

The reason why acidic conditions induce more extensive polymerization is not known. The amino groups in protein may show cationic repulsion against formalin in acidic milieu, while other functional groups, for example phenyl-, are modified forming intermolecular cross linkages as demonstrated for tetanus and diphtheria toxins (24, 31). The difference in pH value at which the maximum polymerization occurred for HR1B and staphylococcus enterotoxin B suggests different mechanisms of detoxification of these toxins.

Addition of lysine to the reaction mixture at a final concentration of 0.05 M shortened the detoxification period and lowered the final formalin concentration required for complete detoxification of the hemorrhagic principle. Moreover, as pointed out by Linggood et al. (20), the toxoid prepared with lysine did not restore its toxicity, whereas the toxoids prepared without added lysine sometimes restored the toxicity (data not shown).

It is noteworthy that the presence of lysine caused a remarkable change in the slope of the dose-response relationship of immunization. These phenomena may be due to incorporation of lysine into the toxoid as demonstrated with diphtheria toxoid (20, 32), suggesting that the toxoid shows different behavior in the immunocompetent system.

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