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TOXOID FORMATION OF PNEUMOLYSIN AND STREPTOLYSIN O BY GLUCURONAMIDE

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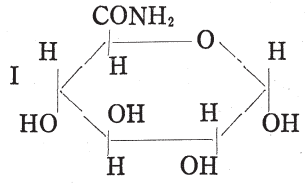
Pneumolysin and streptolysin O were changed respectively to toxoid by treatment with glucuronamide *in vitro*. The mechanism of the toxoid formation was discussed.

INTRODUCTION

Pneumolysin, an oxygen-labile or O hemolysin, is liberated from pneumococcus especially on autolysis. Streptolysin O is produced by almost all strains of Group A streptococci and by many strains of group C and G. These two toxins have not been obtained in pure form, but since the toxins are readily destroyed by proteolytic enzymes, they would appear to be protein in nature. The toxins are related serologically to each other as shown by Todd[1].

This paper reports on the detoxication of the toxins by a glucuronic acid derivative, α-D-glucuronamide. The structure of α-D-glucuronamide is shown in I.



MATERIALS AND METHODS

Pneumolysin

Concentrated cell suspensions in 0.01 M phosphate-buffered saline (PBS) (viable count: $1.2\text{–}3.5 \times 10^8$ per ml) were obtained by centrifuging 16 to 18 hour cultures of *Diplococcus pneumoniae* type I (the Neufeld strain) in brain heart infusion or trypticase soy broth (BBL). The cell-free preparation containing high titer of pneumolysin was prepared by centrifuging the degraded cell suspensions which had been treated for 10 minutes with sonication (10 kc), or by an autolysis method (10 cycles of freezing and thawing). However, it was found that the pneumolysin was readily liberated into PBS by incubating the cell suspensions at 37°C for 3 hours. At the end of incubation period the cell suspensions were centrifuged at 40,000 g for 30 minutes and the supernatant

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fluid was obtained. This PBS extract was also used as pneumolysin preparations for the experiments. There were no living cells in the pneumolysin preparations obtained by the above mentioned three methods.

The hemolytic titers of these preparations obtained by the three method were the same when the preparations were derived from a lot of culture. Thus, pneumolysin could be obtained without any destruction of the living cells by the last method.

Measurement of hemolytic titer of pneumolysin

The measurement was made as follows; to test tubes containing 0.5 ml of serial two-fold dilutions of pneumolysin were added 0.5 ml of 0.5 per cent suspension of rabbit red cells or of sheep red cells, and the mixtures were incubated at 37°C for 2 hours. Another serial dilution system made with PBS containing 0.05 M $Na_2S_2O_4$ was prepared. There was no difference in the hemolytic titer between both systems when the preparation was fresh. Therefore, the highest dilution which gave a complete hemolysis was defined as the hemolytic titer.

Measurement of antipneumolysin titer of serum

A 0.5 ml of two-fold dilutions of rabbit serum was mixed with equal volume of pneumolysin which contains 64 units. The mixtures were incubated at 37°C for 30 minutes. A 1.0 ml of 0.5 per cent suspension of rabbit red cells or of sheep red cells was added to the mixture, and the mixtures were incubated at 37°C for 2 hours. The highest dilution of the serum which gave complete inhibition of hemolysis was defined as the antipneumolysin titer.

Streptolysin O (SLO)

SLO used in the present experiments was the liophylized sample of the purified SLO supplied from Nihon Eiyo Kagaku Co. (Eiken), Japan.

Measurement of hemolytic titer of SLO

The measurement was made as follows; to test tubes containing 0.5 ml of serial two-fold dilutions of SLO were added 0.5 ml of 0.5 per cent suspension of rabbit red cells, and the mixtures were incubated at 37°C for 45 minutes. The highest dilution which gave complete hemolysis was defined as the hemolytic titer. For the dilution of SLO and the preparation of the suspension, PBS containing 0.05 M $Na_2S_2O_4$ was employed to prevent the inactivation by oxygen.

Measurement of ASLO

The serum antistreptolysin O (ASLO) titers of the rabbits were determined according to the method of Rantz and Randall[2].

Glucuronic acid and its derivatives

Sodium glucuronate, glucuronolactone and glucuronamide were used. These materials were the products of The Central Laboratory of Chugai Pharmaceutical Co., Japan.

RESULTS

Toxic action of pneumolysin

The viable cell counts did not diminished even after the extraction of the pneumolysin with PBS at 37°C for 3 hours by the last method described above. This indicates that pneumolysin is not a product liberated only upon autolysis of pneumococci.

CFW strain or CF 1 strain of mice (body weight: 10±1 g) were inoculated intranasally or intravenously with pneumolysin. The mice were killed by intranasal inoculation with 0.1 ml or by intravenous inoculation with 0.25 ml of the pneumolysin of which titer was higher than 256 resulting in pulmonary hemorrhage within 12 hours. Pulmonary hemorrhage caused by the pneumolysin preparations seemed to depend upon the activity of pneumolysin itself according to the following four evidences. (1) When the pneumolysin was adsorbed off from the preparation with rabbit red cells, the supernatant failed to produce pulmonary hemorrrhage. (2) When the preparation was inactivated by an addtion of a minute amount of cholesterol or by heating, the effect to produce pulmonary hemorrhage was lost. (3) When the preparation was mixed with homologous antiserum, the effect was abolished. (4) When the preparation was stored, the hemolytic activity and the effect to produce pulmonary hemorrhage were concurrently diminished. Addition of sodium hydrosulfite to the stored pneumolysin preparations resulted in recovery of both activities.

Detoxication of pneumolysin by glucuronic acid derivatives

Hemolytic activity of the pneumolysin preparation was inactivated gradually when it was mixed with an equal volume of 10 per cent solution of sodium glucuronate, glucuronolactone, or glucuronamide in PBS (pH 7.2) and incubated at 37°C as shown in Fig. 1. The inactivation could not be recovered by an addition of sodium hydrosulfite. Inactivation by glucuronolactone occurred most rapidly and was completed within about 24 hours. However, this may be due to the rapid decline of pH of the mixture to 3.6. Complete inactivation of pneumolysin by glucuronamide or sodium glucuronate occurred respectively within 48 hours or 96 hours. In these cases, the final pH of the mixtures was 6.4 to 6.8, and was almost similar to that of the mixture of pneumolysin preparation and PBS as control. The inactivation of pneumolysin in control was occurred slower than that of the mixtures of pneumolysin and glucuronic acid derivatives, and about 25 per cent of the activity was retained even after 96 hours. The results are shown in Fig. 1 and Fig. 2. Glucuronolactone should be neutral, but the solution indicates actually lower pH than neutral because of the presence of contaminated glucuronic acid[3]. Similar data were obtained in another experiment where the final concentration of the glucuronic acid derivatives was 2.5 per cent.

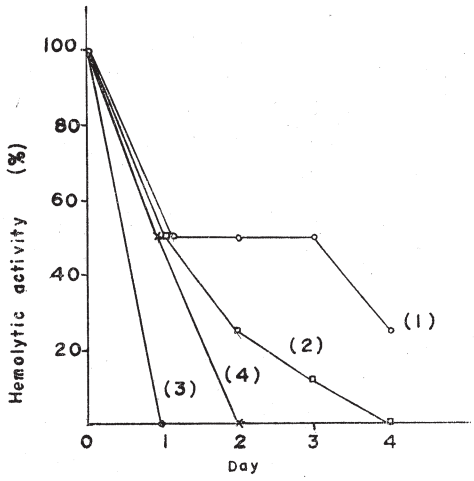

FIG. 1

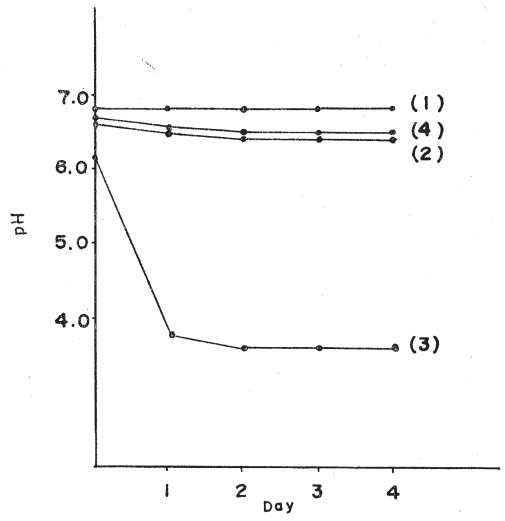

FIG. 2

FIG. 1. Inactivation of pneumolysin with sodium glucuronate, glucuronolactone and glucuronamide. Pneumolysin preparation was mixed with an equal volume of (1) phosphate buffer solution (pH 7.2, 0.1 M), (2) 10% sodium glucuronate, (3) 10% glucuronolactone, and (4) 10% glucuronamide in phosphate buffer, respectively. They were incubated at 37°C.

FIG. 2. Change in pH in mixtures of pneumolysin and glucuronic acid derivatives. Materials were the same as shown in Fig. 1.

Production of antibody by glucuronamide-inactivated pneumolysin

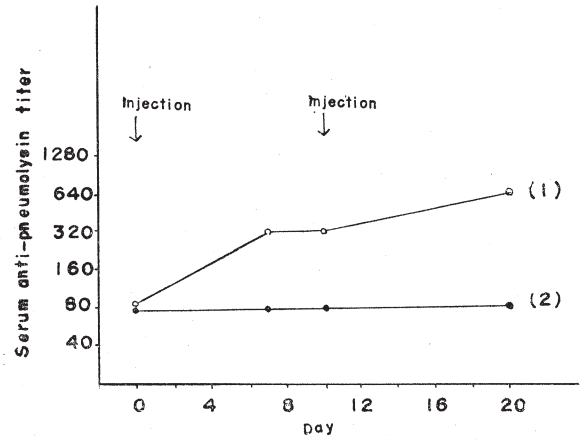

FIG. 3. Production of anti-pneumolysin in rabbits injected intravenously with pneumolysin inactivated with glucuronic acid derivatives. (1) Injected twice with 8 ml of glucuronamide inactivated-pneumolysin. (2) Injected twice with 8 ml of glucuronolactone-inactivated pneumolysin. Pneumolysin titer before inactivation was 64 units.

When a rabbit was injected intravenously twice with 8 ml of the glucuronamide-inactivated pneumolysin preparation of which titer before inactivation was 64, the antipneumolysin level in the serum was significantly elevated. On the other hand, two intravenous injections of 8 ml of the glucuronolactone-inactivated pneumolysin preparation could not elicit the production of antipneumolysin. These results are shown in Fig. 3.

Detoxicatiou of streptolysin O by glucuronic acid derivatives

Each of 20 per cent solution of sodium glucuronate, glucuronolatone, and glucuronamide in PBS was added to the equal volumes of SLO solution of which titer was 64. The mixtures were incubated at 37°C. Since it has been observed that the SLO solution in PBS lost about 90 per cent of its activity within 24 hours when incubated at 37°C, the measurement was made in this experiment at 2 to 3 hours intervals for the first 12 hours, and at 4 hours intervals for the following 12 hours. SLO was completely inactivated in 2 hours by the addition of glucuronolactone, in 8 hours by the addition of glucuronamide, whereas sodium glucuronate did not give any detectable effect on the inactivation of SLO, *i.e.*, the inactivation curve of SLO treated with sodium glucuronate (final concentration was 10 per cent) was similar to that of SLO alone as control. These results are shown in Fig. 4. When the mixtures were kept at 0°C, none of the glucuronic acid derivatives had any inactivating effect as shown in Fig. 5.

The effect of the concentration of glucuronic acid derivatives to the activity of SLO was studied. Glucuronamide in final concentration of 5 per cent did not influence so much as in the case of 10 per cent on the inactivation of SLO

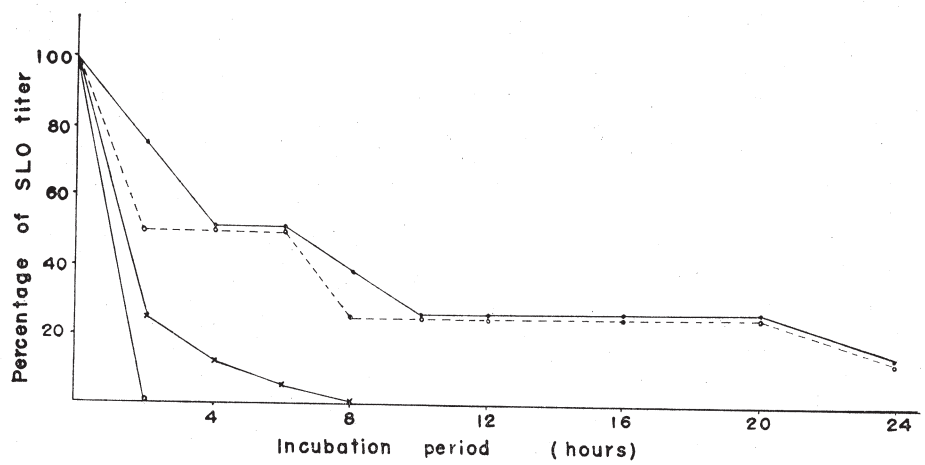

FIG. 4. Inactivation of streptolysin O (SLO) with glucuronic acid derivatives at 37°C.

×——×: 10% glucuronamide, ○——○: 10% glucuronolactone,
○ - - - ○: 10% sodium glucuronate, ●——●: control.

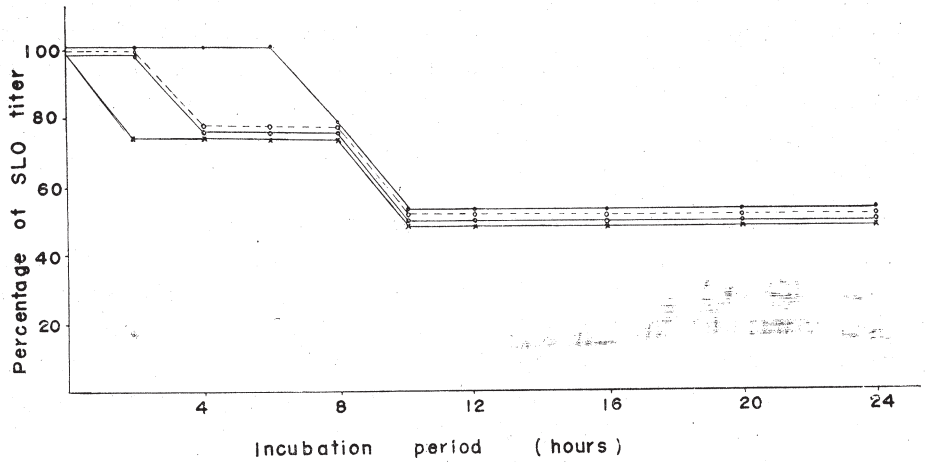

FIG. 5. Inactivation of SLO with glucuronic acid derivatives at 0°C.
Glucuronic acid derivatines were the same as shown in Fig. 4.

at 37°C, and glucuronamide in concentration of 2.5 per cent did not show any effect. Complete inactivation of SLO was observed in 4 hours by 5 per cent glucuronolactone, in 6 hours by 2.5 per cent glucuronolactone at 37°C, whereas it was observed, as mentioned above, in 2 hours by 10 per cent glucuronolactone.

Observations were made whether pH of the mixtures dealt with the inactivating effect. As can be seen in Fig. 6 and Fig. 7, pH of the mixture of SLO and sodium glucuronate (10 per cent) and of the mixture of SLO and glucuronamide (10 per cent) did not differ so much from that of the control,

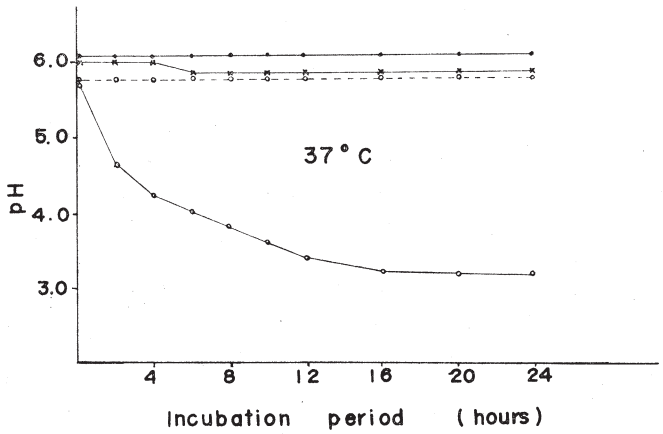

FIG. 6. Changes in pH of a mixture of SLO solution and glucuronic acid derivative at 37°C.
×——×: 10% glucuronamide, ○——○: 10% glucuronolactone,
○- - -○: 10% sodium glucuronate, ○——○: control

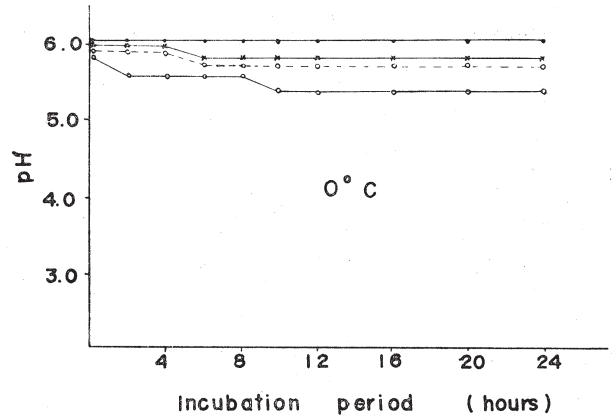

FIG. 7. Changes in pH of a mixture of SLO solution and glucuronic acid derivative at 0°C.
×——×: 10% glucuronamide, ○——○: 10% glucuronolactone, ○- - -○: 10% sodium glucuronate, ●——●: control.

SLO alone, throughout the 24 hour incubation at 37°C, whereas pH of the mixture of SLO and glucuronolactone (10 per cent) declined rapidly to 3.2 after 16 hours incubation. The decrease of pH of the mixture of SLO and glucuronolactone (10 per cent) was, however, not so distinct when kept at 0°C as shown in Fig. 6 and Fig. 7. The pH of the mixture of SLO and glucuronolactone (5 per cent) and that of SLO and glucuronolactone (2.5 per cent) declined respectively to 3.6 after incubation at 37°C.

Immunogenicity of SLO treated with glucuronic acid derivatives

An attempt was made to know whether the inactivated SLO with glucuronolactone or with glucuronamide still retained the immunogenicity. Inactivated SLO was injected intravenously to rabbits and then anti-SLO (ASLO) titer of the serum was estimated by determing the Todd units according to the Rantz-Randall method. To 10 ml of SLO solution (hemolytic titer: 64) were added 10 ml of 20 per cent glucuronamide or glucuronolactone, and incubated for 8 hours for the mixture of SLO and glucuronamide, for 2 hours for the mixture of SLO and glucuronolactone. The treated SLO solutions were used as immunogenic materials for injection. A couple of injections was done a week apart, and the ASLO titer was followed for two weeks. The results are shown in Fig. 8. When injected with glucuronamide inactivated SLO, the serum ASLO titer was elevated after the second injection, whereas when injucted with glucuronolactone inactivated SLO, the elevation of ASLO titer of the serum did not occur.

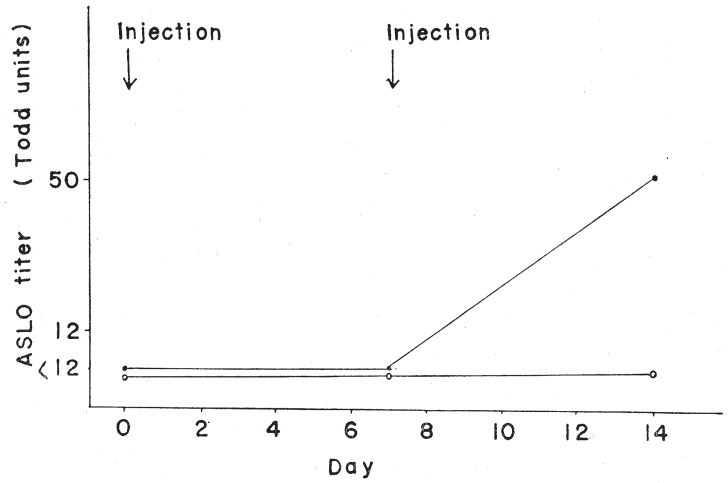

FIG. 8. Serum anti-streptolysin O titers in rabbits injected intravenously with SLO inactivated with glucuronamide or glucuronolactone.
●——●: SLO inactivated with 10% glucuronamide
○——○: SLO inactivated with 10% glucuronolactone
Rabbits were injected with 10 ml of inactivated SLO.

DISCUSSION

Viral toxin which is found in viruses of MNI (Myxovirus) group is inseparably associated with virus particles. Though its chemical nature is still obscure, it is easily inactivated by heat, ultraviolet ray, or formol, and is neutralised by the respective antiviral serum. Pyrogenic, skin lesion-producing and pulmonary consolidation-producing activities of the viruses are belived to be due to the toxic action of the virus. These activities were not inactivated by treatment with sodium glucuronate, but were destroyed by treatment with glucuronolactone *in vitro*[4)5)]. It seemed to be that the viral toxin was destroyed by lowered pH which brought by glucuronolactone. The result suggests that the nature of the viral toxin is not alike to the exotoxin derived from some bacteria. Yamagata *et al.*[6)7)] has proved that diphtheria toxin incubated with sodium glucuronate for 4 to 6 weeks became non-toxic but keeping antigenicity. They have concluded that the diphtheria toxin could became toxoid by *N*-glucuronide conjugation with sodium glucuronate as Ishidate *et al.*[8)] found that *N*-glucuronide conjugation of glucuronic acid with amines was revealed without enzyme system *in vitro*. Akatsuka[9)] confirmed the toxoid formation of diphtheria toxin by glucuronic acid. Ogasawara *et al.*[10)] found that when sodium glucuronate or glucuronamide was added to the α-toxin of staphylococci and incubated at 37°C, the hemolytic activity of the toxin decreased gradually until it loses the activity completely in a week. When rabbits were inoculated with the toxin which was detoxified completely by treatment with sodium glucuronate

or glucuronamide, high antitoxin production was obtained in the rabbits. Akatsuka[11] confirmed that the toxicity of α-toxin which was supplied by us was inactivated by treatment with glucuronic acid or glucuronamide. He observed that the toxicity of α-toxin disappeared rapidly by treatment with 2, 4-dinitrofluorobenzene, suggesting that the free amino group in the toxin protein may be responsible for the toxicity.

Two toxins, pneumolysin and streptolysin O, would appear to be protein nature. They may be detoxified and changed to toxoid forming *N*-glucuronide with glucuronamide or glucuronic acid. It may be supposed that amino group of lysine of the toxins combinds with glucuronamide or glucuronic acid resulting in disappearance of the toxicity of the toxins. The toxicity of glucuronamide is very low. LD_{50} of glucuronamide in mice is 20.0 g per kg. Hence, glucuronamide is more suitable for preparing toxoid vaccines than formol.

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