Comparative Study of Streptococcal DNase by Rapid and Quantitative Assay Systems

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Clinical isolates of group A, B, and G streptococci were compared in regard to their extracellular deoxyribonuclease (DNase) activities by the modified assay system for DNase using DNA-methyl green complex as a substrate. DNase activity in a culture supernatant of each strain was measured as a percent decrease in the substrate-specific absorbance at 640 nm. After 1 hr incubation, DNase activities in all the strains of group A streptococci were found to be markedly higher than those of any strain in the other groups, although the DNase activities in groups B and G were considerably heterogeneous. These findings were also seen when commercially available DNA-dye complex reagent for the determination of anti-DNase B antibody titer was used as a substrate in the assay system. Our results suggest that this newly established assay system could be applicable for the rapid measurement of DNase activity to distinguish group A streptococci from the other groups.

BETA haemolytic streptococci are well known as a causative agent of upper respiratory and skin infections. They are also thought to be important etiologic factor for acute glomerulonephritis or rheumatic fever. Several kinds of extracellular enzymes, e.g., streptolysin, streptokinase, and streptodornase (deoxyribonuclease), are produced by some beta haemolytic streptococci. Detection of antibodies for these enzymes is useful for the clinical diagnosis of streptococcal infection^{1,2}

Although the characterization of deoxyribonuclease (DNase) produced by group A streptococci is being investigated³, little information has been reported on the comparison of the

DNase activities between group A and the other groups of streptococci⁴. In a previous study, Yamada et al.⁵ established the DNA-agar method for qualitative DNase assay and showed that most strains of group A produced DNase more extensively than those of the other groups, B, C, and G.

In the present study, DNA-methyl green complex, which was used for determination of serum DNase⁷, was employed as a substrate for the quantitative assay of the extracellular DNase produced by group A, B, and G streptococci. To confirm the above assay system, commercially available DNA-dye reagent which is generally used for the determination of anti-DNase B antibody titer, was also tested as a substrate in the assay system. We showed that our assay system is applicable for distinguishing the isolates of group A from those of the other groups.

Key Words:

Beta haemolytic streptococci
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Fig.1. The time course of the decrease in DNA-dye complex by extracellular DNase action of group A, B and G streptococci. The number of strains tested was 65, 155 and 20 for group A, B and G streptococci, respectively. Each strain was cultured overnight in heart infusion broth at 37°C. After centrifugation, 0.5 ml of supernatant was mixed either with 2.5 ml of a DNA-methyl green complex solution or with Kyorin DNA-dye complex solution, and was incubated at 37°C. Absorbance at 640 nm of the incubation mixture was measured after 1, 2, 3 and 24 hrs. The percent decrease (D) in the absorbance was calculated from the equation described in the text. The mean D value within each serological group was plotted for the incubation time. A: DNA-methyl green complex, B: Kyorin DNA-dye complex. The symbols, (●), (○) and (▼) represent group A, B and G streptococci, respectively. The width of a vertical bar on each plot represents the standard error of the mean.

MATERIALS AND METHODS

Bacterial strains: All strains used were clinical isolates. The number of strains was 65, 155, and 20 for group A, B, and G streptococci, respectively. The cultures were stored by freezing at -20°C. Serological groupings were carried out according to the method using the coagglutination technique⁶

Culture medium: Heart infusion agar (Eiken Chemical Co., Ltd., Tokyo) supplemented with 5% sheep blood was used for the subcultures of the isolates. Heart infusion broth was used for overnight culture at 37°C for 18 hrs.

DNase assay: DNase activity was measured by using two kinds of DNA-dye complexes as substrates. DNA-methyl green complex was used as a substrate for the assay system with a partial modification of the method of Kurnick⁷ and the complex solution was prepared as follows: 1.25 ml of 0.1% calf thymus DNA (Sigma Chemical Co., St. Louis, Mo.) in distilled water with 0.02%

NaN₃ was diluted with an equal volume of 0.1 M tris-HCl buffer (pH 8.7) containing 4 mM CaCl₂, 2 mM MgSO₄, and 0.02% NaN₃. One percent methyl green (Chroma Gesellschaft, Stuttgart-Untertürkheim) in 0.02 M acetate buffer (pH 4.2) was prepared by repeated extraction with chloroform to remove crystal violet, a side product in the stain solution, and then stored at 4°C. Five μl of the stock methyl green solution was added, by stirring, to 2.5 ml of the DNA solution, mixed thoroughly, and kept at room temperature for about 10 min.

The reagent of the DNA-dye complex in the streptonase-B kit for determination of anti-DNase B antibody titer (Carter-Wallace, Inc., N.J., supplied by Kyorin Pharmaceutical Co., Kyorin DNA-dye complex) was also used as a substrate. In this study the complex was dissolved in 0.05 M imidazole buffer (pH 8.0) containing 2 mM CaCl₂, 1 mM MgSO₄ and 0.02% NaN₃ and then, 2.5 ml of this solution was used as a substrate solution.

Fig.2. Distribution of DNase activities of streptococci after 1 hr incubation with DNA-methyl green complex. The D value of the individual isolates tested in the experiment shown in Fig. 1A was plotted in each serogroup, A, B and G. A horizontal bar indicates the mean D within each serogroup.

Both substrates exhibited the maximal absorbance at 640 nm. Accompanied by depolymerization of the substrates, the color of the DNA-methyl green complex solution changes from green to colorless, and that of the Kyorin DNA-dye complex solution changes from blue to pink. In practice, the lyophilized Kyorin DNA-dye complex was dissolved just before use, as the color changes spontaneously.

For DNase assay either 2.5 ml of a DNA-methyl green complex solution or a Kyorin DNA-dye complex solution was mixed with 0.5 ml of the supernatant of an overnight culture of each strain in a glass tube (13 mm in diameter, Iwaki Glass Co., Ltd., Tokyo), and then incubated in a water bath at 37°C. As a control, 2.5 ml of the complex solution was mixed with 0.5 ml of fresh heart infusion broth and was incubated similarly. After 1, 2, 3, and 24 hrs of incubation, the decrease in the absorbance at 640 nm of these mixtures was monitored with Bausch & Lomb spectrometer (spectronic 20A). DNase activity was measured as a percent decrease (D) of the absorbance. The D value was calculated from the following equation and plotted against the time of incubation.

$$D = (1 - X_t/C_t) \times 100$$

where X_t is the absorbance at 640 nm of the experimental sample at incubation time t , and C_t is that of the control sample at the same time.

RESULTS

Extracellular DNase activities of streptococci assayed on DNA-methyl green complex. By using DNA-methyl green complex as a substrate, the extracellular DNase of 240 isolates of group A, B, or G streptococci were assayed. Figure 1A shows the time course of the mean percent decrease in the substrate-specific absorbance at 640 nm and the standard errors within each serological group. In the experiment with group A isolates a marked decrease of absorbance occurred immediately after the start of incubation, and reached a plateau by 2 to 3 hrs. Standard errors showed that the decreasing pattern among the isolates was quite similar. On the other hand, in the experiments with group B and G streptococci, only slight decreases were observed after 3 hrs.

These results indicate that a great difference in the mean DNase activity between group A streptococci, and group B and G streptococci can be seen after 1 hr of incubation. The difference in the DNase activity between group B and G isolates became evident after 24 hrs. These results suggest that DNase assay in our experimental system makes it possible to distinguish isolates of group A from those of the other groups, B and G.

The validity of this suggestion was clearly shown by plotting the percent decrease in the absorbance after 1 hr for all the individual strains tested in this experiment (Fig. 2). The distribu-

Fig.3. Distribution of DNase activities of streptococci after 1 hr incubation with Kyorin DNA-dye complex. The D value of the individual isolates tested in the experiment shown in Fig. 1B was plotted in each serogroup, A, B and G. A description of the figure follows Fig. 2.

tions of the plots indicated that the DNase activities of all the isolates of group A streptococci were markedly higher than those of any isolates of group B and G streptococci.

Extracellular DNase activities of streptococci assayed on Kyorin DNA-dye complex. A DNase assay was also performed using Kyorin DNA-dye complex as a substrate. The pattern of the mean percent decrease in the absorbance with the time course within each serological group was similar to that using DNA-methyl green complex, showing a great difference in the activities between group A, and group B and G streptococci (Fig. 1B). In addition, it was shown that the sensitivity of Kyorin substrate to DNase was higher than that of DNA-methyl green complex and that a difference between groups B and G was exhibited at 2 hrs.

By plotting the individual activities of the isolates after 1 hr of incubation, it was again confirmed that DNase assay makes it possible to distinguish group A streptococci from group B and G streptococci (Fig. 3). Figure 3 also showed that DNase activities in groups B and G streptococci were very heterogeneous. We could not, however, distinguish the isolates of group B from those of group G by plotting the individual activities obtained from the assay using either method.

DISCUSSION

In the present study, we used two kinds of DNA-dye complexes as substrates for the quantitative assay of the extracellular DNase produced by group A, B, and G streptococci. The results show that all the isolates of group A streptococci tested produce extracellular DNase more extensively than any isolates of group B, and G streptococci. These findings are in good agreement with the previous report of Yamada et al. on the DNA-agar method for qualitative DNase assay, although the DNase assay for group C streptococci was not tested in the present study. From the results shown in Figs. 2 and 3, it should be emphasized that our DNase assay system with an incubation period of only 1 hr makes it possible to distinguish group A streptococci from the other groups, B and G streptococci. As far as we know, this is the first study which compares the DNase activities between group A and other groups of streptococci by a quantitative assay system.

The assay system used here is advantageous for the rapid measurement of crude DNase activities, because most of the other methods are laborious and are limited to fairly pure enzymes. Furthermore, in the assay with Kyorin DNA-dye complex, we found that the critical point for a color change from blue to pink coincides with the mid-point of the percent decrease in the

absorbance at 640 nm. In the case of group A streptococci, therefore, the color change can be clearly observed after 30 min of incubation under the present conditions. It may be possible that by applying this finding, one can distinguish group A streptococci from the others by determining the difference of their DNase activities with the naked eye, without using a spectrometer. A study of the practical application of this method is in progress in our laboratories.

The sensitivity of the Kyorin DNA-dye complex to enzymatic action is higher than that of DNA-methyl green (Fig. 1). Although the procedure for the preparation of the Kyorin DNA-dye complex is not generally informed, the pattern of agarose gel electrophoresis of the complex showed a broad band (data not shown). It is probable that partially digested DNA has been used for preparing the complex to enhance the sensitivity to enzymes. It was apparent that the DNase activities of the isolates of groups B and G are of very heterogeneous (Figs. 2 and 3). It may be that the relationship between the DNase activities of streptococci and their virulence should be investigated. The DNase assay after 1 hr incubation showed that only a few strains of groups B and G have relatively higher activities. This suggests the possibility that some isolates may be picked up as false positives when our DNase assay system is used to distinguish group A from the others.

Physicochemical and immunological characterizations of the extracellular DNase of the strains of each group are in progress in our laboratory.

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