Bacterial Endotoxins Test and alternative methods using recombinant protein-reagents for endotoxin assay

(エンドトキシン試験法と測定試薬に遺伝子組換えタンパク質を用いる代替法)

Endotoxins, also called lipopolysaccharides, are present in the outer cell membrane of Gram-negative bacteria and exhibit various biological activities. Endotoxins, when entering the blood stream, can cause fever even in a very small quantity, and a large quantity of endotoxins is very toxic and can cause death due to endotoxin shock. In addition, endotoxins may contaminate pharmaceutical preparations during the production process because these are derived from Gram-negative bacteria widely present in the environment and because these are hard to be inactivated due to their heat-resistance. Endotoxins are designated as substances which should be controlled to ensure the safety of pharmaceutical preparations, etc., because these exhibit higher pyrogenicity than other well-known pyrogens which may contaminate them. Bacterial Endotoxins Test <*4.01*> is an *in vitro* test method that can detect endotoxins with high sensitivity using amoebocyte lysate prepared from blood corpuscle extracts of horseshoe crabs, and is applicable to injections, etc. On the other hand, recombinant protein-reagents for endotoxin assay have been developed as alternatives to lysate reagents for the purpose of protecting horseshoe crabs, ensuring a stable supply of reagents, reducing differences between reagent lots, and improving the continuity of the tests.

This General Information describes procedures and consideration in measurement when using recombinant protein-reagents for endotoxin assay as alternative methods, in addition to lysate reagents and test methods in Bacterial Endotoxins Test <*4.01*>.

1. Measurement principle of the Bacterial Endotoxins Test

Bacterial Endotoxins Test <*4.01*> is a test to detect or quantify bacterial endotoxins using amoebocyte lysate prepared from blood corpuscle extracts of horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). This test utilizes the reaction in which the hemocyte extract of horseshoe crab is coagulated by endotoxins, and the coagulation reaction is based on a chain reaction by multiple serine proteases triggered by endotoxins (Fig. 1). Endotoxins activate factor C contained in the hemocyte extract of horseshoe crab to convert to an active serine protease, which in turn successively activates factor B, and then proclotting enzyme. Finally, coagulogen, which is a coagulant protein, is hydrolyzed to result in coagulin, and insoluble gel is formed and solidified. In addition, the hemocyte extract of horseshoe crab reacts not only to endotoxins but also to β-glucans and coagulates by a chain reaction starting from factor G.

2. Measurement method in the Bacterial Endotoxins Tests

Bacterial Endotoxins Test <*4.01*> includes the gel-clot techniques, which are based on the gel formation of the lysate TS, and the photometric quantitative techniques, which are based on endotoxin-induced optical changes (Fig. 1).

The gel-clot techniques visually confirm the presence or absence of gel formation and require no special device for the determination. The gel-clot techniques include a limit test and a quantitative test. The former is a method for judging whether a sample contains endotoxins exceeding the endotoxin limit specified in each monograph, using the labeled sensitivity of a lysate reagent as an index. The latter is a method for quantifying the amount of endotoxins in a sample by determining an endpoint, which is defined as the maximum dilution of a sample solution showing the gel formation.

The photometric quantitative techniques include the turbidimetric technique and the chromogenic technique (Fig. 1). In both techniques, the lysate TS and a sample solution are mixed, and the reaction solution is measured after a given time or over time using a spectrophotometer. The turbidimetric technique measures turbidity changes accompanying gelation of the lysate TS using absorbance or transmittance, and the chromogenic technique measures the amount of chromophore released from a synthetic chromogenic substrate by the reaction of endotoxins with the lysate TS using absorbance or transmittance.

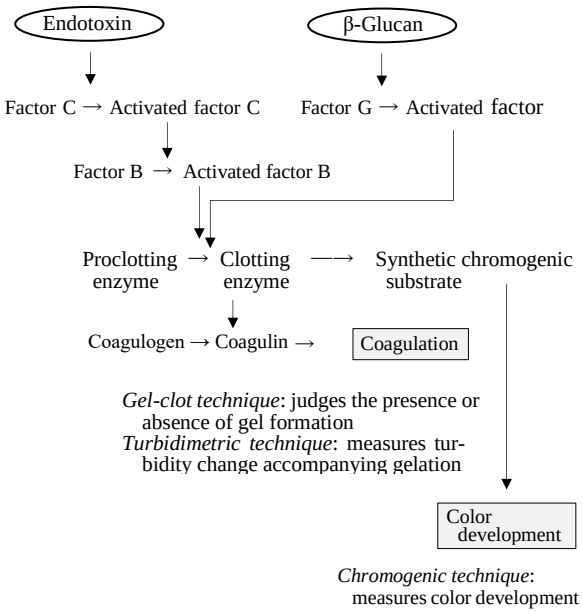

Fig. 1 Measurement principle and method in endotoxin assay

3. Reagents used for the Bacterial Endotoxins Test

There are several lysate reagents used for the Bacterial Endotoxins Test<*4.01*> corresponding to each test method. The reagents are classified into two types based on their reactivity to endotoxins and β-glucans. One is a reagent which contains both the reaction system starting from factor C and the reaction system starting from factor G. The other is a reagent which detects only endotoxins by the reaction system starting from factor because it does not contain factor G or suppresses the reaction of factor G system. Appropriate reagents should be selected depending on the sample to be examined and the purpose of the test.

On lysate reagents used for the gel-clot techniques, the lowest concentration of endotoxins that cause coagulation (gel formation) is set and labeled as the labeled lysate reagent sensitivity (endotoxin unit (EU)/mL) by the reagent manufacturers. The acceptance of a sample is judged using the labeled sensitivity as an index. In order to obtain accurate test results, confirm that the labeled sensitivity is appropriate according to 4.1.1. Test for confirmation of labeled lysate reagent sensitivity in Bacterial Endotoxins Test <*4.01*> 4.1. Preparatory testing. If the geometric mean endpoint concentration does not fall within the specified range, repeat the test after adjusting test conditions. If the geometric mean endpoint concentration does not fall within the specified range by the retest, the lysate reagent cannot be used.

When using lysate reagents for the photometric quantitative techniques, for both turbidimetric and chromogenic techniques, a standard curve is prepared using the standard solutions of three or more concentrations within the quantitative concentration range. Confirm that the test procedures of an operator and test conditions are appropriate according to 5.3.1. Test for assurance of criteria for the standard curve in the Bacterial Endotoxins Test <*4.01*> 5.3. Preparatory testing. Although the labeled sensitivity is not shown on the lysate reagents used for the photometric quantitative techniques, the lowest concentration of the standard solution used for the generation of the standard curve corresponds to the labeled sensitivity.

Most pharmaceuticals are found to interfere with the Bacterial Endotoxins Tests <*4.01*> performance, although to greater or lesser degrees. In general, the influence of interfering factors present in a sample solution can often be overcomed by dilution. In this case, samples should be diluted with water for bacterial endotoxins test within the range that does not exceed a maximum valid dilution for the measurement. The Maximum Valid Dilution is the maximum allowable dilution of a sample solution. As shown in Bacterial Endotoxins Test <*4.01*> 3. Determination of Maximum Valid Dilution, λ is a labeled sensitivity for a lysate reagent in the gel-clot techniques and is the lowest concentration of a standard curve for a lysate reagent in the photometric quantitative techniques; the smaller the λ, the larger the maximum valid dilution. λ for many lysate reagents used for the photometric quantitative techniques is smaller than those for lysate reagents used for the gel-clot techniques. If interfering factors contained in a sample are definite, perform procedures to reduce them. If interfering factors cannot be reduced or interference cannot be avoided because of indefinite interfering factors, consider using other lysate reagents or changing the test method.

4. Measurement by alternative methods using recombinant protein-reagents for endotoxin assay and points to consider in the measurement

Recombinant protein-reagents for endotoxin assay are classified into two types, namely both reagents are composed of the recombinant protein(s) derived from blood corpuscle extracts of horseshoe crabs. The first are reagents mainly composed of the recombinant protein of factor C (horseshoe crab: *Carcinoscorpius rotundicauda* or *T. tridentatus*), and the second are reagents composed of the recombinant proteins of factor C, factor B and proclotting enzyme (horseshoe crab: *T. tridentatus*). The former reagents measure the amount of fluorescence generated by cleavage of a fluorescent synthetic substrate by activated factor C (genetical recombination) which has been activated by endotoxins (Fig. 2a). In the case of the latter reagents composed of the three kinds of recombinant proteins, endotoxins activate factor C (genetical recombinant), and it further activates factor B (genetical recombinant) and proclotting enzyme successively in the same way as the measurement method of the chromogenic technique. Then, the amount of chromophore released from the synthetic chromogenic substrate is measured (Fig. 2b). In the both cases, a reagent solution and a sample solution are mixed, keeping at 37 ± 1℃, and the fluorescence intensity or absorbance of the reaction solution is measured optically after a given time or over time.

The recombinant protein-reagents for endotoxin assay do not correspond to "an amoebocyte lysate prepared from blood corpuscle extracts of horseshoe crab" specified in Bacterial Endotoxins Test <*4.01*>. If these reagents for endotoxin assay are used as an alternative method, confirm that accuracy, precision, sensitivity, specificity, etc. are equal or better compared to Bacterial Endotoxins Test <*4.01*> using the lysate TS. Among the recombinant protein-reagents for endotoxin assay, some have been reported to have sensitivity and specificity equal to or better than the method using the lysate TS. When bacterial endotoxins tests <*4.01*> for pharmaceuticals, etc. are performed using

recombinant protein-reagents, it is necessary to conduct 5.3.1.Test for assurance of criteria for the standard curve as with the Photometric quantitative techniques in the Bacterial Endotoxins Test <*4.01*>, and in that case the lowest concentration of the standard curve corresponds to λ (EU/mL). In addition, it is necessary to pay attention to interference, and Test for interfering factors should be performed. Particularly, in the case of methods that measure the amount of fluorescence, it is necessary to be careful of interference because even substances that do not disturb the measurement when using lysate reagents may exhibit interference such as the inhibition of the generation of fluorescence. In addition, regarding reagents prepared using the gene sequence of factor C of different kinds of horseshoe crab from horseshoe crab (*L. polyphemus* or *T. tridentatus*) specified in the Bacterial Endotoxins Test, it should be noted that the difference of factor C may affect the reactivity to endotoxins.

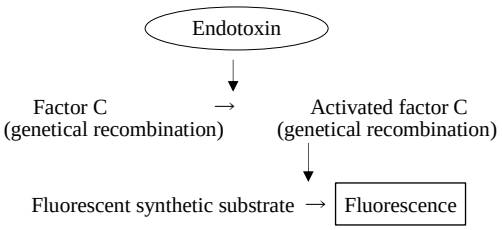

Fig. 2a Reagents for endotoxin assay mainly composed of recombinant factor C

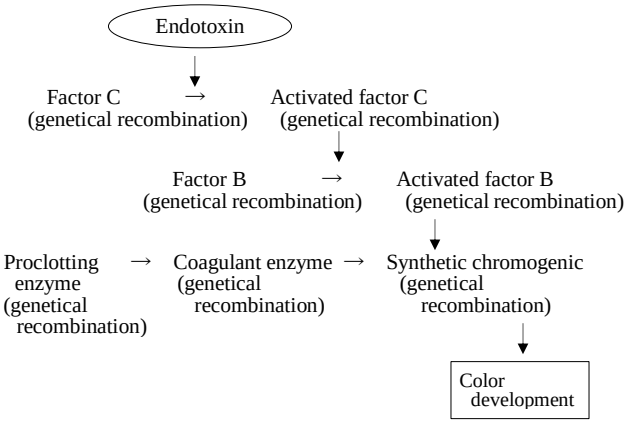

Fig. 2b Reagents for endotoxin assay composed of the three kinds of recombinant proteins