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The Suitability of Pyrogen Test in vitro with Human Peripheral Blood Mononuclear Cell for Haemorrhagic Fever with Renal Syndrome Bivalent Vaccine

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ABSTRACT

To investigate the suitability of Pyrogen Test in vitro with Human Peripheral Blood Mononuclear Cell (PBMC) for Haemorrhagic Fever with Renal Syndrome Bivalent Vaccine, We collected 9 batches vaccines from different manufacturers. The recovery was determinated by human PBMC- IL-6 in vitro pyrogen test with spiking bacterial endotoxin. The recoveries of batches were between 50%-200%, as the test solutions at a dilution less than the maximum valid dilution (MVD). The results showed all of the 9 batches vaccines were qualified products in pyrogen test. the human PBMC-IL-6 in vitro pyrogen test is suitable for Haemorrhagic Fever with Renal Syndrome Bivalent Vaccine to control the pyrogen.

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Abbreviations: PBMC, Peripheral blood mononuclear cell; MVD, Maximum valid dilution; HFRS, Hemorrhagic fever with renal syndrome; VLP, Virus-like particles

Introduction

Human Peripheral Blood Mononuclear Cell in vitro Pyrogen Test is a novel method for the detection or quantization of pyrogen contaminants, which is developed based on the principle that exogenous pyrogens (e.g. bacterial endotoxin from Gram negative bacteria, toxins from Gram positive bacteria, fungi and virus) can stimulate the human peripheral blood

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monocytic cells (PBMC) to produce endogenous heat generating factor (IL-6) [1]. Compared with previous pyrogen testing methods (rabbit pyrogen test and bacterial endotoxin test), Human Peripheral Blood Mononuclear Cell in vitro Pyrogen Test (Referred to:PBMC-IL-6 pyrogen test) has many advantages including detection of all types of pyrogens quantitatively with high accuracy and no species difference [2]. After research for 30 years, this method has been formally validated and recommended by several authorities, such as European center for the validation of alternative methods (ECVAM) and National institute for biological standards and control (NIBSC). And this method has been adopted by European Pharmacopoeia and US Food and Drug Administration (FDA) [3-4].

Although for ethic reasons, such as the adoption of human blood, this method cannot be popularized and standardized to replace the current pyrogen testing method, further detection and research for its application for samples not suitable for current pyrogen testing methods can be performed as a beneficial alternative.

Hemorrhagic fever with renal syndrome (HFRS), caused by HFRS virus, is a natural focal infectious endemic disease disseminated by rodents, characterized by wide prevalence, acute and critical symptom, with high mortality rate. HFRS vaccine is one of the major prophylaxis methods for HFRS, two types of vaccines have been successfully developed so far, i.e. inactivated cell culture vaccine and inactivated mouse brain vaccine. Potential adverse effect of vaccination includes fever, local swelling and induration at the injection site, inappetence, emesis, stomachache, allergy and etc, among which fever is the most common effect [5].

The fever is caused by two reasons, first, it may be caused by the characte--ristics of the vaccine and this type of fever is usually not severe and no other harmful outcome will occur; second, it may be caused by quality problems of the vaccine, i.e. the peyrogenic effect resulted in the pyrogen in the vaccine, major clinical features include fever, shivering and emesis.

Currently, pyrogen in HFRS vaccine is controlled through bacterial endotoxin test in the quality specification. Since alumin--ium hydroxide is included in the vaccine as an adjuvant, which could adsorb bacterial endotoxin and lead to inaccurate detection of bacterial endotoxin in the vaccine, so a clinical risk exists[6]. Concerning problems exist in the pyrogen detection in inactivated bivalent HFRS vaccine, the method suitability of PBMC-IL-6 for pyrogen testing of HFRS vaccine was performed to develop a novel alternative pyrogen testing method.

Material and method

1.Materials

1.1Reagents:

National Standard bacterial endotoxin was provided by National Institute for Food and Drug Control, 10000 EU/vial; RPMI1640 culture medium and lymphocyte separation medium were purchased from Sigma Co. Ltd.; Human IL-6 ELISA kit was purchased from Xinbosheng bioscience Co. Ltd.; Water for bacterial endotoxin test was provided by National Institute for Food and Drug Control, 5mL/vial.

1.2 Testing Samples

Four manufacturers have the manufactur--ing authorization of inactivated bivalent HFRS vaccine in our country totally, 3 batches of samples were obtained from 3 manufacturers respectively as testing samples (9 batches in total), and the capacity of which was 1.0mL/dose, clinical dosage of this vaccine was one dose a time (1.0mL, Table 1).

1.3 Instruments

Vortex mixer is manufactured by US Scientific Industries Inc.(model:GENIE-2); CO_2 incubator was manufactured by US Thermo (model:3141); Microplate reader was manufactured by US BioTek (model: synergyHT); Microplate washer was manufactured by SYM-BIO (Shanghai) Co. Ltd.(model: EGEAE2310).

1.4 Human peripheral blood monocytic cells

The experiments were conducted accord-ing to the experiment method recommended by The Interagency Coordinating Comm--ittee on the Validation of Alternative Methods (ICCVM) [7]. It is required that PBMC from 4 different donors should be applied respectively or mixed in PBMC-IL-6 pyrogen test. Mixed PBMC was adopted in this method. Fresh peripheral blood was phlebotomized from 4 healthy volunteers and was anti--coagulated by 15U/mL heparin sodium aqua. In 4 hours, PBMC was extracted from the blood with lymphocyte separation medium by density gradient centrifugation, then PBMC from all donors was mixed and diluted to about 2.0 $\times$ 10^6cells/mL with RPMI1640 culture medium. Finally, mixed serum from the donors was added to a concentration of about 1%-2%.

Table 1:Manufactures and batches of HFRS Bivalent Vaccine

Manufacturers	Lots	Number
A	201411073 201409059 201410067	3
B	20141022 20141021 20141020	3
C	20141123 20141022 20141021	3

2. Methods

Standard of endotoxin was taken as the criterion in PBMC-IL-6 pyrogen test to evaluate the overall pyrogen contamination of drugs and reflect the pyrognenicity of drugs. Endotoxin concentration obtained from the standard dose-effect curve of bacterial endotoxin is equivalent to the concentration of pyrogen contaminants. Therefore, standard curve and recovery test should be prepared with standard bacterial endotoxin.

2.1 Bacterial endotoxin standard curve

Reconstitute 1 vial of national standard of endotoxin with 1mL of water for bacterial endotoxin test, mix with vortex mixer for 15 minutes. Dilute the mixture with RPMI1640 culture medium to required concentrations, each dilution should be followed by 30 seconds of vortex mixing. Concentrations of standard of endotoxin for standard curve were 0, 0.0125, 0.025, 0.05, 0.1, 0.2 and 0.4EU/mL, respectively (from low to high, 4 replicate wells for each concentration).

2.2 Calculation of the pyrogen limit and the maximum valid dilution

The pyrogen limit of the samples (L) was calculated with the equation: L=K/M, where K is the threshold human pyrogenic dose of endotoxin per kg of body weight (EU/(kg • h), for general injections, K=5EU/(kg • h), for radiopharmaceutical injections, K=2.5EU/(kg • h), for intrathecal injections, K=0.2EU/(kg • h). M is equal to the maximum recommended human dose of product per kg of body weight in a single hour period (expressed by mL/(kg • h), mg/(kg • h) or U/(kg • h)), the average Chinese body weight was calculated as 60kg and body surface area as 1.62m^2. If the injection duration is less than 1 hour, then calculate as 1 hour. Calculated with the equation L=K/M, when K=5EU/(kg • h) and M=1.0mL/(60kg • h), L= 300EU/mL.

Then calculate the maximum valid dilution (MVD) with the equation MVD=C • L/ λ , where L is the pyrogen limit of the sample, C is the concentration of the sample solution. When L is expressed with EU/mL, C is 1.0mL/mL, when L is expressed with EU/mg or EU/U, the unit of C should be mg/mL or U/mL. λ is the lowest concentration point of the prepared bacterial endotoxin standard curve, 0.0125EU/mL. MVD=300EU/ml ÷ 0.0125 EU/mL =24000, i.e. MVD of inactivated bivalent HFRS vaccine was 24000, the dilution ratio of 500 was applied in actual experiment.

2.3 Preparation of test solution and solution for recovery test

Dilute the sample with RPMI1640 culture medium, and the dilution ratio should be less than the MVD. The spiked standard bacterial endotoxin concentration in the solution for recovery test was 0.05 EU/mL.

1.5.4 PBMC-IL-6 pyrogen test

Add PBMC to a U-bottom 96-well microplate, then add dilution standard bacterial endotoxin solution, test solution and solution for recovery test to the corresponding well (4 replicates for each solution) successively. Solution volume was 125 μL of PBMC solution/well +125 μL of standard solution or test (recovery) solution/well. Mix homogenously, then incubate at 37±1℃ in 5% CO_2 for 18–24h. At the end of incubation, test as directed in the user instruction of IL-6 ELISA kit.

Results

According to the bacterial endotoxin test in China Pharmacopoeia, when the dilution ratio is less than the MVD, the recovery rate should be within 50%-200% to confirm that the test solution has no interference to the test under this experiment condition. Under this experiment condition, the recovery rates of 500-time diluted inactivated bivalent HFRS vaccines from 3 manufacturers were all within 50%-200%, indicating the test solution had no interference to the test. See Table 2 for detailed results.

Based on the qualified interference test, concentrations of pyrogen in 9 batches of samples were all not more than 300 EU/mL, which were compliant with the requirement. Since the detection limit of this experiment was 0.0125EU/mL, and the test solution was diluted 500 times, when the testing results were lower than the detection limit, the corresponding pyrogen concentration in the sample should be less than the product of the dilution ratio multiply by the detection limit, i.e. <6.25 EU/mL. Results are listed in Table 3.

Table 2: The recovery of HFRS Bivalent Vaccine in the PBMC- IL-6 pyrogen test

Manufacture	Lots	The recovery（%）
A	201411073	179
	201409059	123
	201410067	115
B	20141022	78
	20141021	106
	20141020	88
C	20141123	56
	20141022	78
	20141021	81

Table 3: The pyrogen concentration of Haemorrhagic Fever with Renal Syndrome Bivalent Vaccine in the PBMC- IL-6 pyrogen test

Manufacture	Lot	The pyrogen concentration (EU/ml)
A	201411073	< 6.25
	201409059	8.57
	201410067	10.34
B	20141022	< 6.25
	20141021	< 6.25
	20141020	< 6.25
C	20141123	12.21
	20141022	24.50
	20141021	7.83

Discussion & Conclusion

PBMC-IL-6 was applied to systematically investigate the method suitability for pyrogen detection in 9 batches of inactivated bivalent HFRS vaccines from 3 manufacturers, when the sample was diluted 50 times, the recovery rates of PBMC-IL-6 were all within 50%-200%, indicating that the sample has no interference to this method. The sample origins covered 75% of all the manufacturers of inactivated bivalent HFRS vaccines in this country, which could fairly reflect the overall features of inactivated bivalent HFRS vaccines, so the pyrogen in this vaccine can be detected using PBMC-IL-6.

Adjuvant in vaccines could strongly adsorb proteantigen in the solution and precipitate, when the vaccine is vaccinated, an “antigen repository” can be formed in the body to slowly release antigen and elongate the action time of antigen sufficiently, meanwhile, macrophage response at the injection site can be promoted [8]. Adjuvant is added in many types of vaccines to enhance the antigen specific immunogenicity, and aluminium hydroxide is the one of the most extensively used adjuvant. But the adjuvant, as an adsorbent, could adsorb bacterial endotoxin and other pyrogen contaminants in the vaccine besides proteantigen, and adsorbed bacterial endotoxin cannot be detected by bacterial endotoxin test, so certain risks and problems exist in the pyrogen detection of this type of vaccine.

Human cells and vaccine are co-incubated in PBMC-IL-6 test to simulate the slow release of antigen and

the pyrogen in the body, which is closest to the clinical situation and could detect the pyrogen in samples accurately. So it is recommended that manufacturers of this type of vaccines could take PBMC-IL-6 as a supplement of pyrogen test and the actual pyrogen contamination of the vaccines can be detected to ensure clinical drug safety.

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