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INCQS

APPLICABILITY OF MONOCYTE ACTIVATION TEST (MAT) IN THE ROUTINE OF THE QUALITY CONTROL LABORATORY

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INTRODUCTION

Contaminations of parenterals with pyrogens can induce inflammatory reactions, including a rise in body temperature, but also more severe adverse reactions, such as septic shock, and even death. Therefore, the testing of parenterals prior to batch release is obligatory for manufacturers. Pyrogens usually are detected in the Rabbit Pyrogen Test (RPT) or the *Limulus* Amoebocyte Lysate (LAL), both with specific advantages and disadvantages. RPT covers a broader range of substances and it is closer to the human immune system but less sensitive and animal consuming. The LAL test has not been able to replace RPT fully, since it is defined not as a pyrogen test, but as an endotoxin test. Since 2010, five in vitro tests called as Monocyte Activation Test (MAT) are included in the European Pharmacopoeia as a third method for detection of pyrogen. Despite the potential to detect all types of pyrogens, one identified limitation of the MAT is the lack of data to detect endotoxin in a sufficient number of injectables and their responses to pyrogens other than endotoxins that are currently detected by RPT. However, a potential advantage of MAT is that they are derived from human tissues, which avoids potential uncertainty associated with cross-species extrapolation [1,2]

OBJECTIVE

The aim of this study was to use MAT in hyperimmune sera previously analyzed in the routine of quality control by RPT and presented results considered negative and positive. Moreover, Zymozan spiked sample were also studied as non-endotoxin pyrogen.

METODOLOGY

Samples - MAT was used with the same hyperimmune sera samples used in RPT and that were repeated in a second test (suspicion of contamination). Non-pyrogenic hyperimmune sera were spiked with 0.5 UE/mL Lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 (Sigma–Aldrich, Steinheim, Germany) as positive control. Zymosan A (Sigma–Aldrich, Steinheim, Germany) from Saccharomyces cerevisae (Sigma, Deisenhofen, Germany) were also used as non endotoxin pyrogen (50 to 1000 pg/ml). The spikes were diluted in pyrogen free saline solution 0,9%.

Whole blood incubation: cryo-preserved whole blood (from 4 healthy donors) was diluted with 900 µL to 0.9% saline and stimulated with 100 mL of immune stimulis in polypropylene vials (Eppendorf, Hamburg, Germany). After incubation for 20 h at 37°C/5% CO_2, vials were closed and stored at -80°C until cytokine measurement [3,4].

Cytokine determination : Sandwich ELISA for human whole blood was used (IL-1β, R&D Systems, Wiesbaden, Germany). Cytokine release was induced in pooled blood and data are Mean ± SEM of triplicates. The dilutions and the intra-assay results were accepted if the coefficient of variation (CV) was below 25%.

Rabbit Pyrogen Test: RPT was conducted in adult New Zealand rabbits and in accordance with the recommendations outlined in the Brazillian Pharmacopeia. We have been injected each selected samples and concentrations (1 mL/kg of body weight) into a marginal ear vein of each of 3 rabbits. After the injection, the rectal temperatures of the animals were recorded with a PyroMon® System (Ellab, Hillrod, Denmark) over a 3-h period. For each rabbit, the response was defined as the difference between the basal temperature (obtained prior to injection) and the maximum temperature recorded after injection. When none of the rabbits showed a temperature increase of ≥0.5 °C over its basal temperature, the concentration tested was classified as pyrogen-free. When only one rabbit showed a temperature increase of ≥0.5 °C, the test was repeated using five different rabbits. If no more than three of the eight rabbits tested showed an individual temperature increase of ≥0.5 °C or more and if the sum of the temperature increases in the eight rabbits did not exceed 3.3 °C, the concentration tested was also classified as pyrogen-free [5].

RESULTS AND DISCUSSION

Table 1 shows results of different hyperimmune sera, comparing RPT and MAT. When RPT was clearly non-pyrogenic or pyrogenic, MAT presented the same result. When RPT needed to be repeated using new rabbits and the final result was non-pyrogenic, MAT presented both results. Nevertheless, it seems to be a limit range that lead MAT to be positive or negative, depending on the intensity of rabbit response. In the cases where RPT final result was positive, MAT has the advantage of detecting earlier, which means that the use of more animals can be avoided.

Table 1: Comparison between RPT and MAT for hyperimmune sera previously analyzed in the routine of quality control. ABS – Anti-*Bothrops* venom sera; ACS – Anti- *Crotalus* venom Sera; ASS – Anti-Scorpion Sera; ABLS – Anti-*Bothrops* and *Lachesis* venoms Sera; ARS – Anti Rabies Sera; NP – Non Pyrogenic; P – Pyrogenic; R – Repeat.

Sample	Individual Variation rabbit ºC.	Animals >=0.5°C	S	RPT	MAT
ABS 01	0,10; 0,00; 0,00	0	0,1	NP	NP
ABS 02	0,20; 0,20; 0,00	0	0,4	NP	NP
ABS 03	0,20;0,20 ;0,00	0	0,4	NP	NP
ABS 04	0,10,0,10,0,20	0	0,4	NP	NP
ABS 05	0,20;0; 0,00	0	0,2	NP	NP
ABS 06	0,00; 0,00; 0,10	0	0,1	NP	NP
ABS 07	0,40; 0,40; 0,50; 0; 0,10; 0; 0,10; 0	1	1,5	R/NP	NP
ABS 08	0,10; 0,50; 0,10; 0,10; 0,10; 0; 0; 0,40	1	1,3	R/NP	NP
ABS 09	0,10; 0,50; 0,10; 0,10, 0,00; 0,00; 0,40	1	1	R/NP	NP
ACS 10	0,50; 0,10; 0,00; 0,20; 0,00; 0,20; 0,00; 0,10	1	1,1	R/NP	NP
ASS 11	0,35; 0,00; 0,50; 0,20; 0,45; 0,05; 0,10; 0,2	1	1,85	R/NP	NP
ASS 12	0,35; 0,00; 0,50; 0,20; 0,45; 0,05; 0,10; 0,3	1	1,85	R/NP	NP
ASS 13	0,50; 0,30; 0,40; 0,10; 0,10; 0,20; 0,20; 0,30	1	2,1	R/NP	NP
ABLS14	0,20; 0,10; 0,60; 0,10; 0,10; 0,20; 0,30; 0,40	1	2	R/NP	NP
ABLS 15	0,20; 0,10; 0,60; 0,10; 0,10; 0,20; 0,30; 0,40	1	2	R/NP	NP
ABS 16	0,60; 0,30; 0,30; 0,10; 0,40; 0,60; 0,20; 0,00	2	2,50	R/NP	P
ABS 17	0,30; 0,50; 0,10; 0,20; 0,50; 0,30; 0,30; 0,50	3	2,7	R/NP	P
ABS18	0,60; 0,20; 0,40; 0,50; 0,50; 0,30; 0,20; 0,30	3	3	R/NP	P
ABS19	0,40; 0,40; 0,50; 0,00; 0,10; 0,00; 0,10; 0,00	1	1,5	R/NP	P
ARS 20	0,30 ;0,10; 0,60; 0,10; 0,15; 0,00; 0,10; 0,00	1	1,35	R/NP	P
ABS 21	0,30; 0,40; 0,70; 0,50; 0,50; 0,40; 0,50;0,60	6	3,9	P	P
ABS 22	0,30; 0,70; 0,50; 0,30; 0,50; 0,60; 0,40; 0,40	4	3,7	P	P
ARS 23	0,75; 0,30; 1,05; 0,30; 0,40; 0,30; 0,40; 0,65	3	4,15	P	P

Analysing results using a 2x2 table, data presentes 100% sensitivity and 75% specificity. It means that there was no false negative. The positive findings for some negative RPT assays shows MAT is capable to detect some limit endotoxin concentration that rabbit cannot detect due to biological variation.

Table 2: RPT assay for Zymosan. Pyriogenic response was seen at 5,000 ng/kg (1 mL/kg). NP – Non Pyrogenic; P – Pyrogenic; R – Repeat

Zymosan A	Individual Variation rabbit ºC.	Animals >=0.5°C	S	RPT	MAT
Saline 0,9%	0,0; 0,0 ;0,1	0	0,1	NP	NP
1000 ng/kg	0,40; 0,30; 0,30	0	1	R/NP	NP
2500 ng/kg	0,30; 0,40; 0,50; 0,30; 0,40; 0,40; 0,00; 0,30	1	2,6	R/NP	P
5000 ng/kg	0,60; 0,30; 0,70; 0,30; 0,70; 0,30; 0,40; 0,50	4	3,8	R/P	P

Zymosan was pyrogenic to animals at the dose of 5,000 ng/kg/mL (Table 2). Zymosan was positive in MAT at the concentration of 2,500 ng/mL when compared to LPS 1ng/mL (5EU/mL) limit dose (Figure 1). It indicates that MAT is more sensitive in detecting Zymosan pyrogenicity. Expressing results in Equivalent Endotoxin Units we find 3,9 EEU/mL (1000 ng/mL), 5,6 EEU/mL (2500 ng/mL) and 9,5 EEU/mL (5000 ng/mL). Therefore, to limit dose of LPS 5 EU/mL match to limit dose of Zymosan 5,6 EUU/mL.

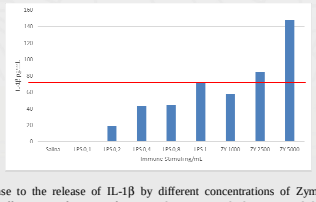

Figure 1: Response to the release of IL-1β by different concentrations of Zymosan in cryopreserved blood. The red line represents the cut off 5 EU/mL from LPS from *E. coli* O55: B5, which correspond the positive control.

CONCLUSION

Results showed that MAT was more sensitive than RPT. Biological variation of rabbits may difficult the correspondence of MAT results in the limit range. Nevertheless, these findings demonstrated that MAT can be used for biologicals products and have a good response to pyrogens other than endotoxins like Zymosan. This study suggests that MAT may replace RPT for hyperimmune sera as well as for detecting non-endotoxin pyrogens in the quality control of biologicals.

REFERENCES

[1] SCHINDLER S, VON AULOCK S, DANESHIAN M, HARTUNG T,. Development, validation and applications of the Monocyte Activation Test for pyrogens based on human whole blood. **ALTEX**; 26: 265-77, 2009.

[2] ICCVAM. Interagency Coordinating Committee on the Validation of Alternative Methods. Validation status of five *in vitro* test methodsd proposed for assessing potential pyrogenicity of pharmaceuticals and other products,. **ICCVAM test method evaluation report,** NIH publicatio, n. 08, p.6392, 2008.

[3] SCHINDLER, S.; SPREITZER, I.; LOESCHNER, B. International validation of pyrogen tests based on cryopreserved human primary blood cells. J.Immunol. Methods, v. 316, p. 42-51, 2006.

[4] EUROPEAN PHARMACOPOEIA. Monocyte-Activation Test. In: EUROPEAN Pharmacopoeia. 7 th Edition. Strasbourg: Concil of Europe, 2010. v.1, p.192-197.

[5] BRASILEIRA. Pirogênios. In: FARMACOPEIA Brasileira. 5 ed. Brasília: Anvisa, 2010. v.1, p. 229-230.