Performance of Monocyte Activation Test Supplemented with Human Serum Compared to Fetal Bovine Serum

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Abstract
The monocyte activation test (MAT) is used to detect pyrogens in pharmaceutical products and serves as replacement of the rabbit pyrogen test. The peripheral blood mononuclear cell-based MAT assay requires the addition of serum to the medium and is either performed with fetal bovine serum (FBS) or human serum (HS). Since the capacity to detect non-endotoxin pyrogens (NEPs) in a sensitive manner is an important strength of MAT compared to bacterial endotoxin test, the performance of the MAT using FBS and HS was investigated using endotoxin and several NEPs. The MAT was more sensitive for endotoxin when FBS was used, however for most NEPs the MAT was more sensitive when performed in HS. Furthermore, heat-inactivation of FBS affected the performance of the MAT for endotoxin to some extent and not for the NEPs. Interestingly, heat-inactivation of HS led to an almost complete loss of reactivity towards endotoxin, it reduced the response towards HKSA and peptidoglycan, while having minor or no effects on the responses towards R848, flagellin and Pam3CSK4. Therefore, to guarantee optimal performance of MAT heat-inactivated serum should be avoided. Moreover, product testing of a human blood-derived product in MAT using HS was beneficial since endotoxin spike recoveries were improved. This product is therefore currently batch released with the HS-based MAT assay. Overall, The HS-based MAT appears to be the first choice to replace the rabbit pyrogen test while in some cases the FBS-based MAT may be favored.

1 Introduction
Pharmaceutical products intended for parenteral use must be below a specified level of pyrogenicity to protect humans from “injection fever”, a marked rise in body temperature caused by injection/infusion of a drug product contaminated with pyrogens. Pyrogens are subdivided in endotoxins (lipopolysaccharides; LPS) derived from gram-negative bacteria and non-endotoxin pyrogens (NEPs) such as peptidoglycans (PGN), flagellin and β-glucans derived from gram-positive bacteria and fungi. Historically, the rabbit pyrogen test (RPT) was used to determine pyrogenicity of injectable drugs for humans, including plasma-derived products and implantable medical devices. However, the RPT assay requires the use of animals. In addition, the responsiveness between humans and rabbits to pyrogens differs, both species show a high reactivity to endotoxin, but humans usually show a higher reactivity to gram-positive pyrogens than rabbits (Schindler et al., 2003). The Monocyte Activation Test (MAT) is an in vitro-based alternative for the RPT and based on the capacity of human immune cells such as monocytes to respond to endotoxins and NEPs by production of pro-inflammatory cytokines like interleukin (IL)-6, IL-1β or Tumor Necrosis Factor (TNF) (Dinarello, 2004; Kikkert et al., 2008). The MAT is considered as a replacement for the RPT. The current European Pharmacopeia (Ph. Eur.) chapter 2.6.30 (07/2017) states that “MAT is suitable, after product specific validation, as a replacement for the rabbit pyrogen test” (Council of Europe, 2020). According to Ph. Eur. chapter 2.6.30 multiple cell sources can be used for the MAT assay, namely whole blood, peripheral blood mononuclear cells (PBMCs) or a monocyctic cell line as also reviewed by Poole and colleagues (Poole et al., 2003). These cells can either be used freshly isolated or cryopreserved and as single donor cells or pools with a minimum of 4 individual donors (Solati et al., 2015; Koryakina et al., 2014). The requirement of 4 donors is to include genetic variation in humans to the responsiveness to NEPs (Jaeger et al., 2015). The responsiveness to endotoxins is quite similar between humans (Copeland et al., 2005). The monocyctic cell line can be used according to Ph. Eur. chapter 2.6.30, but a cell line has the disadvantage that there is just one genetic background and it is derived from a tumor cell line and functional stability over passaging has to be ensured. In this study, the MAT is performed with cryopreserved pooled PBMCs from 4 donors.
combined with an IL-6 ELISA as cytokine readout. Previous results from our and other laboratories has shown that a PBMC-based MAT assay has high sensitivity and high specificity (Hoffmann et al., 2005; Solati et al., 2015; Vipond et al., 2019; Etna et al., 2020).

According to the Ph. Eur. (Council of Europe, 2020) each new product has to go through a product-specific validation to determine that the solution does not interfere with the MAT test consisting of 1) tests showing that the MAT detects endotoxins and NEPs when spiked in the product and 2) a test showing that the solution does not interfere with the detection system (ELISA). Subsequently, release testing can be performed on the production process qualification (PPQ) batches (for registration) and/or on each production batch. During release testing according to method A (quantitative test) or method B (semi-quantitative test) as described in Ph. Eur. chapter 2.6.30, three dilutions of the product are tested with and without an endotoxin spike, of which the 3 dilutions were determined during the product-specific validation. The lowest dilution with a valid endotoxin spike recovery, between 50 and 200%, is used to calculate the amount of endotoxin equivalent units per milliliter (EEU/mL) for method A. If the responses to dilutions of a preparation are not parallel to the responses of standard endotoxin dilutions than method B should be used. Method B is a pass/fail test, e.g. the contaminant concentration of the preparation should be below the Contaminant Limit Concentration (CLC) to pass.

A PBMC-based MAT assay requires the addition of serum to the cell culture medium in which proteins are present that aid in the immune reaction triggered by pyrogens. The MAT can either be performed with fetal bovine serum (FBS) or human serum (HS) as serum source. Since the capacity to detect NEPs in a sensitive manner is an important asset of the MAT, we compared the performance of the MAT in FBS and HS to gain more insights in the detection of endotoxins and NEPs by the PBMC-based MAT using these different serum sources. This is especially relevant since results might support a MAT format without use of animal-derived materials. In addition, we studied the effect of heat-inactivation of the serum on the response to endotoxins and NEPs and the effect of serum source on recoveries of the endotoxin spikes in a specific human blood-derived product.

2 Materials and Methods

2.1 Pyrogens

Endotoxin Standard Biological Reference Preparation (BRP) batch 5 (EDQM, Council of Europe, Strasbourg, FRANCE) was used to prepare endotoxin and NEP stock solutions. A stock solution of 2000 EEU/mL was prepared in endotoxin-free water, aliquoted (50 μL) and stored at -80°C. The endotoxin stock solution was diluted further in complete medium to the required endotoxin concentrations. Heat-killed S. aureus (HKSA), flagellin (S. typhimurium), Pam3CSK4, R848 and PGN (S. aureus) were all obtained from Invivogen (San Diego, CA, USA) and aliquoted and stored according to manufacturer’s instructions. The absence of endotoxins in these NEP preparations has been confirmed by the manufacturer and for HKSA, flagellin and PGN reconfirmed by us using the Limulus Amebocyte Lysate (LAL) test (Pam3CSK4 and R848 were not suitable for testing in the LAL). Of note, the endotoxin and NEP concentrations stated throughout this study are final concentrations in the well (after addition of cells to the well). Concentration in the sample can be calculated by multiplying the stated concentrations with 2 (since samples and cells are mixed 1 to 1).

2.2 MAT execution: Cell culture

The MAT was performed using the MAT Cell Set (Sanquin Reagents, Amsterdam, The Netherlands), following manufacturer’s instructions with adaptations as described. In short, a vial containing 5x10^6 (±1x10^6) cryopreserved PBMCs (1 mL) was thawed in a water bath of 37°C until a little clump of ice remained. The cell suspension was subsequently transferred to a new tube and diluted with 10 mL Iscove's Modified Dulbecco's Medium (IMDM; Lonza, Verviers, Belgium) supplemented with either 5% FBS or HS (both from Biowest, Nuaillé, France) as serum source. In the MAT cell culture plate, 100 μL of cells was added to 100 μL sample (tested in triplicate or quadruplicate where indicated). In case of spiking experiments, 5 mL of supplemented IMDM was added to the thawed cell suspension, and 50 μL of cells was added to 100 μL sample plus 50 μL endotoxin spike solution or 50 μL supplemented IMDM (for unspiked samples). Subsequently, the 96-well cell culture plate was incubated in a humidified incubator at 37°C in the presence of 5% CO2 for a duration of 18-24 h, with approximately 45,000 cells/well. Where indicated FBS or HS was heat-inactivated at 56°C for 45 minutes before use.

2.3 MAT execution: IL-6 ELISA

Cell culture supernatants were harvested and analyzed in 1:5 dilution, except if indicated otherwise, for the presence of IL-6 using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (PeliKine Compact™ human ELISA kit, Sanquin, Amsterdam, The Netherlands) following manufacturer’s instructions. Depicted are the ODs at 450nm with subtraction of the background OD at 540 nm.

2.4 Drug product testing with the MAT

To exclude interference between the test and the product being tested, the MAT has to be validated for that product according to the pharmacopoeia guidelines. Therefore endotoxin is spiked into uncontaminated batches of the product and tested in the MAT. The product is considered free from interfering factors if the mean recovery of the added endotoxin is in the range of 50-200%. During these experiments all samples were tested in quadruplicates and 50 μL of cells were added to 100 μL of sample and 50 μL of endotoxin spike or 50 μL complete medium (for unspiked samples). The endotoxin spike was 0.02 EEU/mL (final concentration in the well) for the FBS-based MAT or 0.16 EEU/mL for HS-based MAT. In addition, an endotoxin standard curve was included on the same plate in quadruplicate, ranging from 0.005 to 0.08 EEU/mL for FBS-based MAT and 0.02 to 0.32 EEU/mL in the well for the HS-based MAT. Of note, the endotoxin concentrations stated throughout

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this study are final concentrations in the well (after addition of cells to the well). Concentration in the sample can be calculated by multiplying the stated concentrations with 2 (since samples and cells are mixed 1 to 1).

2.5 Statistical analysis
GraphPad Prism 8.0.2. was used for the analysis. Area under the curve (AUC) of nonlinear fitted regression lines ([agonist] vs. response- variable slope (four parameters) were calculated. Paired t-test was used to compare the AUC of 2 different serum sources. Unpaired t-test with correction for multiple comparison with Holm-Sidak method was performed to analyze the effect of additional HS in FBS-based MAT for detection of endotoxin.

3 Results

3.1 Comparison of MAT responses to endotoxin and NEPs using FBS and HS as serum source
To investigate if the reactivity of the MAT is different for endotoxin and a selection of NEPs using FBS or HS as serum source, we either performed the MAT with medium containing a final concentration of 5% FBS or 5% HS respectively. Sensitivity towards endotoxin was lower in the HS-based MAT compared to the FBS-based MAT. The limit of detection (LOD) for endotoxin using FBS was ≤ 0.01, and for HS ≤0.03 (Fig. 1A) EU/mL per well. The HS-based MAT based was more responsive towards HKSA (amongst others a ligand for Toll-like Receptor-2; TLR-2 from gram-positive Staphylococcus aureus), Pam3CSK4 (a synthetic mimic of bacterial lipopeptides (TLR2/1 ligand)) and peptidoglycan (PGN; NOD1/2 ligand from S. aureus) in comparison to the FCS-based MAT. In case of flagellin (TLR5 ligand from gram-negative Salmonella typhimurium) and Imidazoquinoline compound R848 (TLR7/8 ligand), we did not observe a significant difference in reactivity of the MAT performed in FBS or HS. The observed differences between performing MAT in FBS or HS were not depending on the PBMC pool, batch of HS or batch of FBS (data not shown). Overall, we observed that for endotoxin the MAT is more sensitive when executed with FBS instead of HS, while for the NEPs the sensitivity was either lower or the same when executed with FBS compared to HS.

Fig. 1: Comparison of MAT performed in FBS and HS
MAT cells were o/n stimulated with endotoxin (LPS), HKSA, Pam3CSK4, PGN, flagellin and R848 in 2-fold concentration range using either FBS or HS as serum source. An IL-6 ELISA was performed on the culture supernatants. Supernatants of R848 were diluted 1:25 for all other conditions’ supernatants were tested in 1:5 dilution. A. Representative graphs are depicted, results are shown as mean OD± SD of triplicate measurements. B. Area under the curve (AUC) of fitted pyrogen curves of all experiments is calculated and depicted as mean ± SD. Endotoxin (LPS) (n=4), HKSA (n= 4), Pam3CSK4 (n=4), PGN (n=3), flagellin (n=5) and R848 (n=4). Paired t-test was performed. *p<0.05, ** p<0.001.

3.2 Effect of serum heat inactivation on reactivity of the MAT for endotoxin and NEPs
Serum heat-inactivation is usually performed to inactivate complement (Soltis et al., 1979). Heating of serum however also reduces or modifies serum growth factors and is often performed without any evidence of beneficial effect (Rahman et al., 2011). To study if the reactivity of the MAT is affected by heat-inactivation of the serum, we compared the responses in MAT using FBS or HS and their respective heat-inactivated forms. In case of FBS heat-inactivation resulted in a reduced reactivity of the MAT towards endotoxin (Fig. 2A,B). For the NEPs we did not observe an effect of heat-inactivation on the reactivity towards the NEPs in the FBS-based MAT.

The heat-inactivation of HS had a more pronounced effect compared to heat-inactivation of FBS (Figure 2C,D). It resulted in almost complete loss of reactivity of MAT cells towards endotoxin. Heat inactivation of HS had varying effects on reactivity of MAT cells towards NEPs. For Pam3CSK4 the reactivity of the MAT was not affected by heat-inactivation of HS. For R848 and flagellin the reactivity was significantly higher when using heat-inactivated HS, while for HKSA and PGN the response was significantly lower using heat-inactivated HS. Overall, heat-inactivation of HS had differential effects on the responses of the MAT cells on the pyrogens.

3.3 Interference of HS in an FBS-based MAT
Since the reactivity towards endotoxin was lower in the HS-based MAT compared to the FBS-based MAT, we wondered if HS contains either lower amounts of stimulatory constituents or that it contains (higher amounts of) inhibitory constituents
Fig. 2: Effect of serum heat inactivation on results MAT
MAT cells were o/n stimulated with endotoxin (LPS), HKSA, Pam3CSK4, PGN, flagellin and R848 in 2-fold concentration range. The effect of heat-inactivation of FBS (A, B) and HS (C, D) on the MAT response was tested. A, C. An IL-6 ELISA was performed on the culture supernatants. Supernatants of R848 were diluted 1:25 for all other conditions’ supernatants were tested in 1:5 dilution. Representative graphs are depicted, results are shown as mean OD± SD of triplicate measurements. B, D. Area under the curve (AUC) of fitted pyrogen curves of all experiments is calculated and depicted as mean ± SD. Endotoxin (LPS) (n=4), HKSA (n=4), Pam3CSK4 (n=4), PGN (n=3), flagellin (n=5) and R848 (n=4). Paired t-test was performed. *p<0.05, ** p<0.01, ***p<0.001.

Fig. 3: Interference of HS on endotoxin-induced response in an FBS-based MAT
MAT cells were stimulated o/n with 0.08 EU/mL endotoxin in MAT medium containing 5% FBS. Additional HS or FBS was added in graded dose as indicated. An IL-6 ELISA was performed on the culture supernatants, the OD value without additional serum addition was set to 1 and all other OD values were normalized to it. Data of 5 independent experiments are combined and represented as mean ± SD. Unpaired t-test with correction for multiple comparison with Holm-Sidak method was performed. ** p<0.01, *** p<0.001.

affecting the endotoxin response. Therefore, we performed an FBS-based MAT (5% FBS) using a fixed amount of endotoxin (0.08 EU/mL) and added increasing percentages of HS to investigate if this affected the response towards endotoxin. To correct for additional addition of serum we also added the same percentage of additional FBS to the FBS-based MAT. The experiments were performed with serum sources that were not heat-inactivated. The addition of HS caused a significant inhibition of the response towards endotoxin, while this was not the case for addition of extra FBS (Fig. 3). This inhibition was already observed at 0.16 % of HS, but was more pronounced at higher HS concentrations. Altogether, indicating that HS contains constituents that can inhibit the response to endotoxin in an FBS-based MAT.

3.4 Effect of serum source during drug product testing
In previous studies it was observed that certain plasma-derived products yielded invalid recoveries of the endotoxin spike which was added to the products, making it complex to test these products in the MAT (method A or B of Ph. Eur. 2.6.30) (Perdomo-Morales et al., 2011). Here a blood-derived product was tested in the MAT using either FBS or HS as serum source, to assess the effects on endotoxin recovery. The maximum valid dilution of a product is determined by dividing the contaminant limit by the LOD. With FBS based MAT the LOD was 0.008 EU/mL and this resulted in a maximum valid dilution (MVD) for the product of 625-fold. The product was tested at 150-, 300- and 600-fold dilutions, however the 150-fold dilution never yielded a valid endotoxin recovery (<50%) (Fig. 4). The 300-fold product dilution yielded recoveries
around 50% and the 600-fold around 60%. This resulted in many repeats of routine MAT assay due to invalid endotoxin spike recoveries. The same blood-derived product was also tested with the HS-based MAT. Since the LOD of this MAT was determined to be 0.03 EU/mL, the maximum valid dilution of the product was only 166-fold. Therefore, the product was tested with lower dilutions, namely 31.2-, 62.5- and 125-fold dilutions. All 3 product dilutions consistently yielded valid endotoxin recovery values between 50-200% (Fig. 4A). After product specific validation the routine measurements were performed with HS-based MAT and the data from 20 batches showed the same result: although the MAT assay based on HS serum requires higher product concentrations, the endotoxin recoveries of an endotoxin spike are better (Fig. 4B).

### 4 Discussion

In this study we compared the performance of the MAT either using FBS or HS as serum source. Except for a slightly lower sensitivity for endotoxin, the MAT was more reactive towards most NEPs when performed with HS. Furthermore, heat-inactivation of FBS affected the performance of the MAT for endotoxin to some extent and not for the NEPs. Interestingly, heat-inactivation of HS led to an almost complete loss of reactivity towards endotoxin, it reduced reactivity in the response towards HKSA and PGN, while having minor or no effects on the responses towards R848, flagellin and Pam3CSK4.

Moreover batch release testing of a certain blood-derived product in MAT using HS was beneficial since endotoxin spike recoveries were valid at lower product dilutions.

For the stimulation of TLR4 by endotoxin several co-factors are important such as LPS-binding protein (LBP), CD14 and MD-2 (as reviewed in (Raetz and Whitfield, 2002; Park and Lee, 2013)). MD-2 forms a receptor complex with TLR4 at the membrane and is required for the recognition of LPS molecules by the TLR4/MD-2 complex and subsequent signaling events. LBP is a soluble protein present in serum and is necessary for the extraction of LPS from the bacterial surface or from LPS aggregates and thereafter will transfer the LPS molecule to CD14. CD14 will successively present the LPS molecule to the TLR4/MD-2 complex. CD14 is present as a GPI-anchored membrane protein on monocytes and also present in soluble form in serum. Endotoxin was the only pyrogen tested that gave a higher reactivity in FBS compared to HS. Differences in LBP concentration between FBS and HS might explain the difference in reactivity of the MAT cells.

However since additional HS in an FBS-based MAT inhibited the response to endotoxin, this indicates that (in addition) inhibitory components are present in HS which might (partly) cause the lower response to endotoxin in HS-based MAT. It has been described that serum lipoproteins in HS, e.g. LDL and VLDL can inhibit the response to endotoxin (as reviewed in (Berbee et al., 2005; Wendel et al., 2007), which are absent or present in lower extent in FBS (Haylett and Moore, 2002; Forte et al., 1981). Furthermore, the presence of anti-LPS antibodies in human serum possibly blocking the binding to the TLR4/MD-2 complex can also not be ruled out (Fomsgaard et al., 1987; Nys et al., 1996).

Heat-inactivation of FBS led to a reduced response towards endotoxin, while heat-inactivation of HS almost completely blocked the response towards endotoxin, demonstrating that heat-inactivation of serum is not beneficial nor advisable for detection of endotoxin in the MAT. The effect of heat-inactivation might be explained by heat-sensitivity of LBP, Meszaros and colleagues have shown that heat treatment affected the LPS-potentiating effect of LBP (Meszaros et al., 1995).

The reactivity to the different NEPs in the MAT was much higher or the same when performed with HS compared to FBS. Especially the response towards HKSA and PGN increased substantially. The response towards Pam3CSK4, a synthetic lipopeptide that mimics the acylated amino terminus of bacterial lipoproteins (a constituent of the cell wall of gram-positive and gram-negative bacteria), was also enhanced in the HS-based MAT.
Heat-inactivation of HS had differential effects for the different NEPs tested, the most profound effect was the lowered response towards HKSA and PGN. Altogether the negative effects of heat-inactivation of HS on responses towards endotoxin, HKSA and PGN impacts the overall performance of the MAT in HS much more than the slight positive effect of heat-inactivation of HS observed for R848 and flagellin.

Drug product testing in the MAT requires that endotoxin spike recoveries in the product are between 50% and 200%. Some plasma-derived products show high interference in the MAT, rendering the assay unsuitable as a pyrogen test due to invalid spike recoveries (Perdomo-Morales et al., 2011). In some cases, especially with a highly sensitive MAT (low LOD), interference can be overcome by dilution of the product close to the MVD. Although our HS-based MAT has a lower MVD due to a higher LOD for endotoxin, testing of a blood-derived product in this assay resulted in valid spike recoveries at 10-20 fold lower product dilutions compared to the FBS-based MAT (31.2-fold vs 300-600 fold diluted). A possible explanation for obtaining valid spike recoveries at lower product dilutions using the HS-based MAT compared to the FBS-based MAT could be that an inhibitory component of HS provides a form of basal inhibition towards endotoxin in the assay (as indicated by a higher LOD). This may limit further inhibition of any possible inhibiting component in the drug product resulting in improved spike recoveries. This is supported by our finding that adding additional HS in the FBS-based MAT results in interference. Since 2018 the HS-based MAT is routinely used for releasing batches of this specific blood-derived medicinal product.

In conclusion, the MAT is more sensitive for endotoxin when FBS is used as serum source, however for most NEPs the MAT is more sensitive when performed in HS. Further, we show that HS-based MAT is preferred for certain blood-derived products. The HS-based MAT is therefore considered to be the first choice to replace the rabbit pyrogen test since the detection of certain NEPs is superior compared to FBS. HS also better reflects the human immune system and, importantly, the use of FBS as cell culture supplement is under debate due to animal welfare concerns and compliance to the 3Rs principle (van der Valk et al., 2018).

Recently several papers described the usefulness of MAT for testing of pyrogen levels in vaccines (Vipond et al., 2019; Etna et al., 2020; Rossi et al., 2020). Vaccines are complex products since they differ in formulations (e.g. type of antigens, adjuvant, excipient and stabilizer), resulting in vaccines that have inherent pyrogenic activities and others that lack pyrogenic activity. For vaccines with pyrogenic activity there should be a balanced amount of pyrogenicity, too much immune activation will lead to more severe side-effects (e.g. fever), too little will not provide protection. Especially for vaccines with pyrogenic activity the MAT can serve as a release test to determine the consistency between batches. The FBS-based MAT however could be more suitable for testing certain vaccines, especially those against diseases for which the donors of the HS may already have antibodies, which could interfere in the MAT assay e.g. by binding to the vaccine.

Finally, to guarantee optimal performance of the MAT heat-inactivation of serum should be avoided.

References
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Conflict of interest
Sanquin Diagnostics performs MAT testing for customers. Sanquin Reagents produces and distributes MAT cell kits.

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