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Review Article

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A Review on Whole Blood Pyrogen Assay

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ABSTRACT

The whole blood pyrogen test was first described in journal exactly twenty years ago.it employs the cytokine response of blood monocytes for the detection of microbiological contaminates with the potential to finally replace the still broadly used rabbit test. Its measure the fever reaction following injection of the sample to the animals. LAL which is measure the coagulation in a lysate prepared from blood of the horseshoe crab specifically initiated by endotoxins. Pyrogen detection is important in pharmaceutical industry, laboratories and health care institution. As an alternative to the animal consuming rabbit pyrogen test or limulus amoebocytes lysate test, the monocyte activation test was introduced as a gold standard method in European pharmacopoeia. The monocyte activation test has not gained wide acceptance in practice. We stimulate bovine whole blood with different endotoxin preparation (lipopolysaccharide E. coli) as well as the non-endotoxin pyrogen peptidoglycan and lipoteichoic and Prostaglandin E_2 (PGE_2) served as read out. The test is the first solution enabling adequate pyrogen testing of cell therapies, including blood transfusion, and medical device. As the test can quantitatively assess human relevant airborne pyrogens, the contribution of pyrogens to chronic obstructive lung diseases and childhood asthma can for the first time be defined and home and work place safety improved in the future.

Key-words: pyrogen, 3R, Limulus Amebocyte Lysate test, human whole blood test, Interlukin-1β, endotoxin, cytokine, prostaglandin E_2, monocyte activation test, lipoteichoic acid.

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INTRODUCTION:-

The term "pyrogen" was coined by Burdon-Sanderson in 1875 for a hypothetical substance in bacteria-free extracts of putrid meat, which caused fever upon injection into animals (for review see clough, 1951). [1]When Wechselman in 1911 showed that the febrile reaction in patients after injection of Salvarsan were due to contamination of the distilled water, presumably by bacteria, this sparked much interest to address this problem. Salvarsan was the first effective treatment for syphilis, the modern chemotherapeutic agent, but has to be injected as it not orally bioavailable. This drug spurred the wide-spread use of injectable drugs and the need for pyrogen control. Hort and Penfeld developed the first rabbit pyrogen test in 1912 and demonstrated that in rabbits Gram-negative bacteria were pyrogenic but Gram-positive very much less so(reviewed in Bennett and Beeson,1950)[2].We observed the same when we developed a rabbit blood pyrogen test to compare the responsiveness of rabbit and human blood to pyrogen. I.e. rabbit blood, human blood . Its show a high responsiveness to LPS but a lower responsiveness to Gram-positive pyrogens than human blood. This difference in rabbit responsiveness between Gram-negative and Gram-positive bacteria, which does not translate to human. Pyrogen research almost entirely on pyrogen from Gram-negative bacteria, i.e. endotoxin (known as Lipopolysaccharide (LPS) [3]).

The most potent stimuli are endotoxin from Gram-negative bacteria. These LPS of the outer cell membrane induce a cascade of defence mechanisms known as inflammation and fever. When cells of immune system, blood monocytes and macrophages come into contact with pyrogenic contamination, they release mediators which transmit the fever reaction within the organism. The most prominent representative of these endogenous pyrogens is the pro-inflammatory cytokine Interleukin-1β (IL-1β).

The new pyrogen test (Hartung and Wendel, 1995, 1996) is based on this principle: The sample to be tested is incubated with a small amount of blood taken from a healthy donor. Any pyrogenic activity, independent of its chemical nature, induces the formation of IL-1β which can be determined by ELISA [4].

COURSE OF EVOLUTION:-

As an academic researcher in the mid-nineties, I was pretty about the acceptance process for an alternative method. Some validation seemed necessary, but otherwise promising animal welfare legislation in Europe requesting the use of an alternative method if reasonably available seemed to be an easy way to reach implementation.The immediate interest by the European Pharmacopoeia was very promising. The chair of the pyrogen working group, Dieter Kruger, initiated an independent evaluation and financed this via Roche / Boehringer-Mannheim. Markus Weigandt [5](with help from Peter Lexa and Michael Jahnke) carried out a thesis at the University of Heidelberg supervised by Hans- Gunter Sonntag. Some highlights of his results Many false- negative results for LPS-spiked samples in the rabbit assay were detected correctly in the human whole blood assay of 84 blood samples from healthy donors none displayed spontaneous cytokine release but all reacted to LPS in a homogenous manner with inter-individual absolute amounts of interleukin-1 (IL-1) release; the test was positive for Mycobacterium terrae, a Gram-positive bacterium; the limit of detection (final concentration) was in the low pg range for LPS, i.e., similar to LAL."These study results prove that the HVP [human whole blood pyrogen test] represents a sensitive, reliable pyrogen test for drugs suitable for routine use, which is clearly superior to both pharmacopoeia methods."

Whole blood assays	Cryo-blood assays
Blood donor dependent, primary cells, no isolation, cell suspension (Daneshian , 2009)	Standardized and pretested primary cell pool, no isolation after thawing, cell suspension (Schindler et al., 2004)
Internationally validated (Hoffmann.,2005)	Internationally validated (Schindler , 2006)
Saline, thermoblock incubation possible	Culture medium, thermoblock incubation
extensive supportive data (90 different,50 fungal spores, 5 exotoxins, etc.), incriminated samples and > 80 products, direct comparisons to rabbit testing; not disturbed by adjuvants	Supportive data for pyrogens and routine product testing

Variants: commercial kit available, medical devices/solid materials (Hasiwa,2007), airborne pyrogens (Kindinger,2005), adsorb/wash-extraction/enrichment (Daneshian et al., 2006, 2008), cell therapies	Variants:whole blood assays, storage at -80°C or liquid nitrogen

[6] "These test methods are based on an endotoxin-induced pyrogenic mechanism that is scientifically valid in principle. The validation studies present results that exhibit a high correlation with the rabbit pyrogen test, but it re- mains necessary to increase the number of compounds and implement validation at multiple laboratories before these test methods can be adopted as an alternative test method. At present, these test methods are not suitable as a replacement for endotoxin tests.

PYROGEN DETECTION METHOD

- Bovine whole blood assay
- Monocyte activation test (MAT)

BACKGROUND:-

The detection of pyrogenic contamination is an essential part of drug safety testing in the pharmaceutical industry, reference laboratories as well as health care institutions. Patient safety, pyrogenic contamination have been determined and must not be exceeded. Monocyte activation test (MAT) as most suitable test for pyrogen testing. (1) Rabbit pyrogen test (2) Limulus amoebocyte lysate (LAL) test.

Disadvantages of Rabbite pyrogen test is low sensitivity for pyrogens and The LAL is unable to detect non-endotoxin pyrogen. Both test are animal consuming tests which, according 3R concept –Replacement, reduction, refinement should be avoided.

The MAT utilize human blood is characterized by a high sensitivity for detecting endotoxin and non-endotoxin pyrogens. Human whole blood or producing larger amount of cryoblood for use in the MAT is certainly logistic challenge. cryoblood is tested for sterility and HIV, HAV, HCV and HBV [7].

IN PRINCIPLE,

A designated breed animal blood would be used for pyrogen detection because the animal can be under standardized specific pathogen free condition. [8]

Method's

Blood collection

Blood is obtaining via venepuncture from healthy cattle into 7.5 ml heparinized tubes (Li-heparin 19 IU/ml). All blood donors are female, non –lactating cow and are fed with hay ad libtium. The age range from 2.5 to 13 years.

In vitro assay using bovine peripheral blood

225 µl lithium heparin blood from different donors are pipette into 96-well culture plates and stimulate for 24 hours with 25 µl pyrogen solution or vehicle. After incubation at 37◦c and 5% CO_2, the 96-well plates are centrifuged at 2272×g for 10 minutes, the supernatants are collected and frozen at -80◦c until analysis.

Comparison with the Pyro detect system

The Pyro Detect System is use according to the manufacture's instruction as quantitative test with the exception that sample are not test at different dilutions. The Quantitative test is described by the procedures as method A. By using method A, a quantitative comparison of the samples with the standard endotoxin is possible. The stimulating agents are pipette out as 20 µl portion into 96-well plate under a horizontal flow bench. The spike and blank wells –the former is fill with stimulant and spike RPMI medium, with a total of 40µl RPMI-20µl RPMI are add to each well. The cryoblood vials (included in the kit) are thaw in a water bath for 1 minute and dilute immediately with 8 ml RPMI 1640 cell culture medium each(included in the kit). Cryopreserved blood mixture is pipette into the plate at a volume of 200 µl per well. After incubation for 16 hours at 37◦c with 5% CO_2 the mixture in the wall is resuspend five times and transfer to ELISA plate (included in the kit). The IL-1β ELISA is perform following the manufacture's instructions. To compare this test system with the bovine whole blood assay the same stimulants (dilute in RPMI) are test simultaneously using the blood of 6 animals (separately).

Statistical analysis

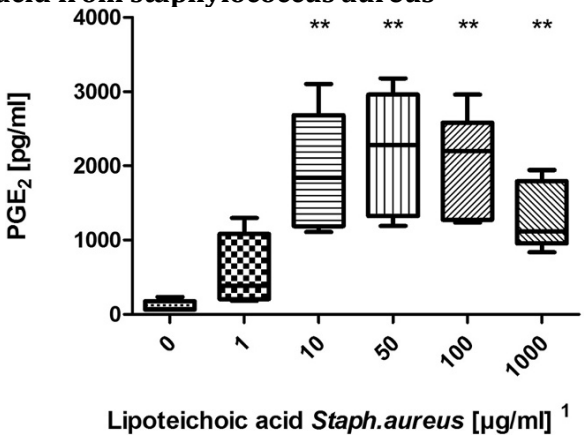
Measurable examination is completed utilizing the product SAS 9.3 (SAS, Cary, NC, USA). Information is check for ordinary circulation by visual investigation and the Kolmogorov-Smirnov test. Some information sets demonstrate a left-skewed appropriation and fizzled the typicality test.

In this way a stage test (10000 changes) is use for computing a randomize complete piece outline (equal to correct Friedmann Test) and P values littler 0.05 is consider noteworthy. Figurings finished with the SAS full scale. Information are spoken to as box-plot with middle and min to max hairs.

Pyrogen stimulation

LPS from E. coli 0127:B8 is use as a stimulating agent and we found a dose-dependent increase of PGE_2 starting at a dose of 0.08 EU/ml and reaching a plateau at 0.16 EU/ml. Using the WHO standard endotoxin (LPS E. coli 0113:H10) we discovered a dose-dependent increase of PGE_2 release starting at 0.25 EU/ml. Peptidoglycan (PGN) from Bacillus subtilis induce a dose-dependent increase of PGE_2 in concentrations of more than 10 µg/ml. As like, lipoteichoic acid (LTA) from Staphylococcus aureus provoke a significant increase of PGE_2 at concentrations of 10 µg/ml and above (1 µg/ml provoke an increase as well, but fell just short of the level of significance, $p = 0.056$). The maximum PGE_2 production seen at 50 µg/ml, but although release elicited by higher LTA concentrations decline, it remained significantly higher compare to un- stimulate blood.

Stimulation with lipoteichoic acid from staphylococcus aureus



Bovine whole blood assay compared to Pyrodetect system

After 16 hours of stimulation –analogously to the manufacturer's lab procedure-the pyrodetect system ELISA detected the presence of 0.25 EU/ml standard endotoxin, the pyrodetect system also detected the presence of all other pyrogens at all concentration use. The color reaction of the ELISA was so intense that we were unable to quantify it precluding the specification of EU equivalents.

The simultaneous stimulation of bovine blood from 6 animals with the same pyrogen resulted in detection limits comparable to those obtained from the previous experiments. Results of one animal were excluded from analysis because of pre-existing PGE_2 release from the unstimulated blood.

HUMAN FEVER REACTION:

pyrogen → leucocytes → cytoxines (1L-1β) → FEVER (rabbit pyrogen test)

HUMAN PYROGEN TEST:

Test sample → whole blood → cytoxines (1L-1β) → ELISA (Human whole blood)

In this diagram principle on which the human whole blood assay base.

The properties of the human whole blood assay is compared with the ones if rabbit test and the LAL.it is evident that among the two in vitro tests, gram positive and fungal contamination escape detection by the LAL while the blood assay detects them.one of the characteristics of the whole blood assay is that the direct contact between the relevant cells (monocytes/macrophage) and with the surface of any material of interest is the key event that initiates the signal that is taken as final dout.

Rabbits and the WBT, the rabbit whole blood test was thus developed. This test uses the same species as the established in vivo test, but the same material and endpoint as the new in vitro test with human blood. Several steps were necessary to establish the rabbit whole blood test:

i) Production of recombinant rabbit IL-1β in E. coli (positive control for the ELISA, substance to immunise the animals in step two),
ii) Immunisation of a sheep with the IL-1β antigen,
iii) Immunisation of mice with IL-1β and production of monoclonal antibodies,
iv) Establishment of a sandwich
ELISA (Enzyme Linked Immunosorbent Assay) with the antibodies and the antigen. These steps were successfully completed recombinant IL-1β was produced in a reliable quality and sufficient quantity.
Monoclonal (mice) and polyclonal (sheep) antibodies against rabbit IL-1β were isolated. The ELISA assay allows the quantitative determination of the endogenous rabbit fever signal IL-1β.

Comparison of three pyrogen tests

		Test:	Rabbit	LAL	WBT
Pyrogens	Bacteria gram-negative		+	+	+
	Bacteria gram-positive		+	-	+
	Fungi		+	-	+
Applications	Biologicals	+		-	+
	Pharmaceuticals	+		+	+
	Medical Devices	-		+	+
	Air quality	-		(+)	+
	Blood components	-		-	+

IMPORTANCE:-
The importance of commercialization
It Is quit incredible that, after the acceptance of the MAT, rabbit use for pyrogen testing even increase by about 10000 to more than 170000 rabbits use.
Pyrogen test requirement also for injectable below 25 ml many of these are lipophilic and can not be test in LAL and are test in the RPT [9].

ADVANTAGES AND DISADVANTAGES OF DIFFERENT MAT VARIENTS

Source of monocytes	Advantages	Disadvantages
Cell line	- No donor	-high variability,laborious
PBMC	-Most sensitive	-handling artifacts, infection risk
Fresh whole blood	-cell suspension -physiological, strongly buffered	-infection risk
Cryopreserved whole blood	-physiological, standerization -commercial kit available	-DMSO present

The importance of international harmonization
Medications are sold universally, which implies that just cluster discharge tests, e.g. pyrogen tests, that are acknowledged in all business sectors will be utilized. This is an executioner for all modify local strategies.
LAL is viewed as a trade for the rabbit test that obviously is confined to endotoxins, while there is Sample confirm that the entire blood pyrogen test covers all known human-important pyrogens. In 2012, the FDA issued "Direction for Industry: Pyrogens and Endotoxin Testing: According to the ICCVAM website[10], "This report is tended to organic item, medication, and gadget makers and is mean to clear up FDA's present position on pyrogen testing and acknowledgment criteria. The direction talks about methodologies, for example, pooling tests for testing that could lessen creature utilize and expresses that in vitro monocyte enactment pyrogen tests like tests assessed by ICCVAM might be utilized as a part of lieu of the rabbit pyrogen test or the bacterial endotoxin test given fitting item particular acceptance."

CONCLUSION
In conclusion, with regard to the aim of reduction, refinement and replacement (3R) of animal experiments the introduction of MAT seem promising for the reduction of the use of RPT and LAL. This objective does not

achieving because the MAT is not widely used . Here we show the potential of detecting endotoxin and non-endotoxin pyrogen using a bovine whole blood assay, further efforts are indispensable to improve the method's functionality, detection limits and robustness as well as to verify whether it can detect further pyrogens including lipopeptides. If the BWBA meets these requirements it should be possible to produce large standardized batches of bovine blood in reference laboratories which could then offer pyrogen testing services using the BWBA as an alternative to the RPT.

Human whole blood test (WBT) utilizing an ELISA, was universally approved by ECVAM. It quantifies cytokine generation, for this situation IL-1ß, by human monocytes taking after a test with pyrogens. It is not so much costly but rather more delicate than the rabbit test and has the extra point of interest of having the capacity to look at the response quality straightforwardly in human material.

Most as of late, extra tests utilizing interleukin-6 discharge as endpoint and fringe blood mononuclear cells (PBMC) or the monocytoid cell line MONO MAC 6 (MM6) tests were deductively accepted and the legitimacy affirmed by the ECVAM Scientific Advisory Committee.

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