PYROGEN TESTING OF HUMAN VACCINE
BY MONOCYTE ACTIVATION TEST

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1st DCVMN 3Rs Experts Working Group
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MISSION: Promotion and protection of national and international public health through research, surveillance, regulation, control, prevention, communication, counseling and training activities.
IMMUNOLOGY UNIT
COCCIA’S TEAM INVESTIGATES:

- Immune-pathogenic mechanisms of infectious diseases and escaping strategies evolved by pathogens;
- Gene expression in response to infectious agents;
- Immunotherapy of infectious diseases;
- Alternative experimental model to test in vitro vaccine pyrogenicity and potency.

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02/12/2019

1ST DCVMN 3RS EXPERTS WORKING GROUP, DECEMBER 2ND, 2019 – BANGKOK
“VACCINE BATCH TO VACCINE BATCH COMPARISON BY CONSISTENCY TESTING”
PROJECT (VAC2VAC)

OBJECTIVES AND AMBITION

- Report on pyrogenicity assessment of human Tick-borne encephalitis virus (TBEV) vaccine (ENCEPUR®) using monocyte activation test (MAT) in human PBMC.

- To replace the existing pyrogenicity test in rabbit by performing the monocyte activation test MAT assay described in the European Pharmacopoeia by using human peripheral blood mononuclear cells (h-PBMC).

- Develop, optimise & evaluate non-animal methods that cover key parameters for demonstrating batch consistency, safety and efficacy.

- (Pre-) validate methods and define with regulators guidance for regulatory approval and routine use.
Flavivirus
Small enveloped virus
Positive-sense, single-stranded RNA
3 structural proteins

NO INTRINSIC PYROGENICITY
**TICK-BORNE ENCEPHALITIS VACCINE (INACTIVATED)**

**Vaccinum encephalitidis ixodibus advectae inactivatum**

**DEFINITION**

Tick-borne encephalitis vaccine (inactivated) is a liquid preparation of a suitable strain of tick-borne encephalitis virus grown in cultures of chick-embryo cells or other suitable cell cultures and inactivated by a suitable, validated method.

**FINAL LOT**

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde, bovine serum albumin (where applicable) and pyrogens and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

**IDENTIFICATION**

The vaccine is shown to contain tick-borne encephalitis virus antigen by a suitable immunochemical method (2.7.1) using specific antibodies or by the mouse immunogenicity test described under Assay.

**TESTS**

- **Aluminium (2.5.13):** maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.
- **Free formaldehyde (2.4.18):** maximum of 0.1 g/l.
- **Bovine serum albumin:** If bovine serum albumin has been used during production, the vaccine contains not more than 50 ng per single human dose, determined by a suitable immunochemical method (2.7.1).
- **Sterility (2.6.1):** The vaccine complies with the test for sterility.
- **Pyrogens (2.6.8):** The vaccine complies with the test for pyrogens. Inject into each rabbit, per kilogram of body mass, one dose of vaccine.

**PRODUCTION PROCESS**

Barrett et al., 2008 (2)

Risk of cellular, viral, bacterial and fungal contaminations

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(1) Background Document on Vaccines and Vaccination against Tick-borne Encephalitis [Vaccine, 2011;29(48):8769-70]

(2) Tick borne encephalitis virus vaccines.[Vaccines pp. 841-856]
Exogenous Pyrogens
bacteria, virus, fungi

Innate Immune System
monocytes, macrophages
neutrophils, dendritic cells

Endogenous Pyrogens
TNF-α, IL-1β, IL-6

Fever

PGE₂

Hypothalamous

Testing of pyrogens in chemicals or biologicals, including vaccines, prior to batch release is relevant
**RPT- Rabbit pyrogen test**
(Qualitative measurement of endotoxin and non-endotoxin pyrogens)

“The test consists of measuring the rise in body temperature evoked in rabbits by the intravenous injection of a sterile solution of the substance to be examined” (Chapter 2.6.8 Ph. Eur.).

**BET-Bacterial endotoxin test / LAL – Limulus amebocyte lysate test**
(Limit /quantitative test of endotoxin; does not detect not-endotoxin pyrogens)

“The test is used to detect or quantify endotoxin from gram-negative bacteria using amebocyte lysate from the horseshoe crab (Limulus polyphemus or Tachyleus tridentatus)” (Chapter 2.6.14 Ph. Eur.).
PYROGEN/ENDOTOXIN TESTS (II)

→ rFC- Recombinant factor C test
(Quantitative measurement of endotoxin)

The test is used to quantify endotoxin from gram-negative bacteria by mean of a non-animal-derived reagent namely Recombinant Factor C. (Coming soon as chapter 2.6.32 Ph. Eur.).

→ MAT- Monocyte activation test
(Semi-quantitative/quantitative measurement of endotoxin and non-endotoxin pyrogens)

“The MAT is used to detect or quantify substances that activate human monocytes or monocytic cells to release endogenous mediators such as pro-inflammatory cytokines, for example TNF-α, IL-1β and IL-6” (Chapter 2.6.30 Ph. Eur.).
MONOCYTE ACTIVATION TEST
- workflow -

1. BLOOD DONATION
   - Plasma
   - PBMCs
   - Density gradient medium
   - Erythrocytes and granulocytes

2. PBMCs

3. MONOCYTIC CELL LINES

4. VACCINE

Stimulation with vaccine/reference standard endotoxin (RSE) or reference vaccine serial dilution

22 ± 2 h

TNF-α, IL-1β or IL-6 quantification (commonly by ELISA)
**CELL SOURCE FEATURES**

<table>
<thead>
<tr>
<th>Whole Blood</th>
<th>PBMCs</th>
<th>Monocytic Cell Lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Polimorphonuclear and Mononuclear Cells]</td>
<td>[Mononuclear Cells]</td>
<td>[Mono-MAC-6 and THP1]</td>
</tr>
<tr>
<td>Donor variability</td>
<td>Donor variability</td>
<td>Very low variability</td>
</tr>
<tr>
<td>For unspecified pyrogens</td>
<td>For unspecified pyrogens</td>
<td>For known pyrogens</td>
</tr>
<tr>
<td>Presence of cytokines and antibodies in plasma</td>
<td>Basal activation due to PBMC isolation procedures</td>
<td></td>
</tr>
</tbody>
</table>

**Donor variability**

- For unspecified pyrogens
- Very low variability

**For known pyrogens**

- For unspecified pyrogens
- Basal activation due to PBMC isolation procedures
RABBIT PYROGEN TEST (RPT) VS MONOCYTE ACTIVATION TEST (MAT) (I)

STATE OF ART FOR VACCINE TESTING

✓ **RPT**: multivalent DTwP-HepB vaccine, vaccines against HepB, rabies, TBEV, pneumococcal and meningococcal polysaccharide vaccine;

✓ **MAT**: *Neisseria meningitidis* group B vaccine (BEXSERO®); Salmonella vaccine (Typhim Vi® - ANSM communications to OMCL annual meeting – Sarajevo 2018);

↓

**MAT** is not applied so far for the batch release of other vaccines
MAT is a non-animal alternative to the RPT (in agreement with the 3Rs principle);

Since RPT was originally developed to test pyrogens in parenterals (administered intravenously in large volume), the method is not appropriated for testing pyrogens in intramuscularly or subcutaneously administered vaccines (dilution is needed);

MAT execution (from purchase of material/animals to data report) is quicker with respect to the RPT;

MAT allows the testing of human vaccine in human setting;

MAT incubation time (22 ± 2 hours) is longer than RPT one (3 hours), thus allowing the detection of delayed inflammatory response.
2.6.30. MONOCYTE-ACTIVATION TEST

1. INTRODUCTION

The monocyte-activation test (MAT) is used to detect or quantify substances that activate human monocytes or monocytic cells to release endogenous mediators such as pro-inflammatory cytokines, for example tumour necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6). These cytokines have a role in fever pathogenesis. Consequently, the MAT will detect the presence of pyrogens in the test sample. The MAT is suitable, after a product-specific validation, as a replacement for the rabbit pyrogen test.

Pharmaceutical products that contain non-endotoxin pyrogenic or pro-inflammatory contaminants often show very steep or non-linear dose-response curves in comparison with endotoxin dose-response curves. Preparations that contain or may contain non-endotoxin contaminants have to be tested at a range of dilutions that includes minimum dilution.
onset of encephalitis in children. Pertussis vaccination coverage dropped precipitously in some countries (such as the United Kingdom), and vaccination against pertussis was halted in others (Sweden), resulting in national pertussis outbreaks of a magnitude not seen for decades [7,8]. The response of the scientific community was to search for purified antigens (or sub-units) capable of inducing a protective immune response and with improved reactogenicity profiles [9]. The resulting acellular pertussis vaccines containing between one and five purified antigens demonstrated lower rates of local and systemic reactions after vaccination compared with whole-cell vaccines [10]. Nevertheless, the duration of immunity induced by acellular pertussis vaccines appears to be shorter than expected [11–13], underlining the need for regular booster doses in older children as well as in adolescents, adults, and the elderly. At the same time, the search continues for improved pertussis vaccines that induce more durable protection [14].

Other new vaccine approaches were developed to address a range of technical and implementation-related challenges. For example, recombinant technologies allowed the production of vaccines for pathogens unable to be grown in vitro. Pathogens with multiple disease-causing strains/serogroups required methods to combine multiple antigens into a single vial. Efforts were also made to improve vaccine acceptance and coverage using complex multi-valent vaccines targeting multiple different diseases in the same injection.

While vaccines containing a limited number of purified antigens generally have improved safety profiles compared with live-attenuated and whole-pathogen vaccines, they are also often less immunogenic due to the removal of pathogenic features of the organism (Figure 1) [15].

**Characteristics of Antigens and Adjuvants Used in Licensed Vaccines**

Table 1. Characteristics of adjuvants used in licensed vaccines.

<table>
<thead>
<tr>
<th>Adjuvant</th>
<th>Composition</th>
<th>Origin</th>
<th>Other Uses</th>
<th>Major Immune Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aluminum</strong></td>
<td>Aluminum as salts mixed with antigen (adsorption)</td>
<td>Naturally occurring present in soil, water, air</td>
<td>Medicines, cosmetics, food industry</td>
<td>Increases local inflammation, improves antigen update by APCs. Acts to increase antibody production</td>
</tr>
<tr>
<td><strong>Virosomes</strong></td>
<td>Vesicles where influenza antigens in aqueous volume are enclosed within a standard phospholipid membrane bilayer</td>
<td>Natural phospholipids, Seasonal influenza glycoproteins</td>
<td>None</td>
<td>Increases uptake by APCs. May interact with B cells leading to T-cell activation.</td>
</tr>
<tr>
<td><strong>AS04</strong></td>
<td>(3-deacyl-monophosphoryl lipid A) derived from LPS from Salmonella Minnesota, Aluminum salts</td>
<td>Natural exposure to LPS from Gram-negative bacteria occurs frequently</td>
<td>None</td>
<td>Directly stimulates TLR-4 increasing APC maturation and Th1 responses.</td>
</tr>
<tr>
<td><strong>MF59</strong></td>
<td>Squaene</td>
<td>Animal source (shark liver oil). Found naturally in human tissues: adipose tissues, skin, arterial walls, skeleton, muscles, lymph nodes</td>
<td>Cosmetics, moisturizers</td>
<td>Increases APC recruitment and activation. Promotes antigen uptake and migration of cells to lymph nodes.</td>
</tr>
<tr>
<td><strong>AS03</strong></td>
<td>Vitamin E (α-Tocopherol)</td>
<td>Naturally occurring in humans.</td>
<td>Vitamin</td>
<td>Promotes local production of cytokines and recruitment of innate cells.</td>
</tr>
<tr>
<td></td>
<td>Surfactant polysorbate 80</td>
<td>Surfactant and emulsifier</td>
<td>Used in foods, eye drops &amp; intravenous injections</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Squalene</td>
<td>Animal source (shark liver oil). See above</td>
<td>Naturally occurring. See above</td>
<td></td>
</tr>
<tr>
<td><strong>Thermo-reversible oil-in-water</strong></td>
<td>Squalene</td>
<td>Animal source (shark liver oil). See above</td>
<td>Naturally occurring. See above</td>
<td>Not reported</td>
</tr>
<tr>
<td>(Influenza-pandemic)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ISA51</strong></td>
<td>Mineral oil DRAKEOL 6 VR</td>
<td>Refined mineral oil of vegetable origin</td>
<td>Food industry</td>
<td>Strongly immunogenic</td>
</tr>
<tr>
<td>(therapeutic vaccine NSCLC)</td>
<td>Surfactant mammide-mono-oleate</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D = diphtheria, T = tetanus, IPV = inactivated poliomyelitis vaccine, HPV = human papilloma virus, LPS = lipopolysaccharide, APC = antigen presenting cells, TLR = toll-like receptor, NSCLC = non-small cell lung cancer, MPL = monophosphoryl lipid A.
MAT METHODS

**METHOD A**
**QUANTITATIVE TEST**
Method A involves a *comparison of the product examined with standard endotoxin dose-response curve*. The contaminant limit concentration (CLC) of the preparation being examined is to be less than the contaminant limit concentration to pass the test.

**METHOD B**
**SEMI-QUANTITATIVE TEST**
Method B involves a *comparison of the product examined with standard endotoxin*. The contaminant concentration of the *product* is to be **less than the CLC** to pass the test. The highest product concentration must be chosen for the pass decision, unless otherwise justified and authorized.

**METHOD C**
**REFERENCE LOT COMPARISON**
Developed to address extreme donor variability in response to certain product containing high level of pyrogen contaminants. Method C involves a *comparison of the preparation being examined with a validated reference lot of that preparation*. 

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The MAT optimized for the TBEV vaccine was set-up by using as cell source cryopreserved peripheral blood mononuclear cells (PBMCs). According to Ph.Eur., human PBMCs have been qualified:

- PBMCs remain viable (≥ 95%) when stored at -196°C up to 18 months;
- Reproducibility of the response to scalar doses of reference standard endotoxin (RSE) at 12 and 18 months after PBMC freezing.

IL-6 was chosen as read-out providing the robust production as compared to TNF-α and IL-1β after PBMCs stimulation with RSE, and the two non-endotoxin TLR agonists R-848 and FSL-1.
SETTING OF MAT CONDITIONS FOR THE TBEV VACCINE (II)

ASSURANCE OF CRITERIA FOR ENDOTOXIN STANDARD CURVE

INTERFERENCE IN THE DETECTION SYSTEM

TEST FOR INTERFERING FACTORS

METHOD VALIDATION FOR NON-ENDOTOXIN MONOCYTE-ACTIVATING CONTAMINANTS
**ACTIVE SUBSTANCE:** TBEV inactivated by formaldehyde - ENCEPUR®;

**EXCIPIENTS:** Aluminum hydroxide, TRIS buffer, sucrose. Traces of tetracycline, gentamicine, neomycine and formaldehyde.

**Cell source:** human peripheral blood mononuclear cells (PBMCs);

**Read-out:** IL-6 release;

**V1, V2, V3:** Defined vaccine serial dilution;

**E1, E2, E3, E4, E5:** RSE chosen serial dilutions showing a linear correlation.
First application of MAT to a vaccine;

Recombinant fusion proteins NHBA and fHbp and recombinant protein NadA of MenB; MenB outer membrane vesicles (OMV);

OMV contain: endotoxin, porins, peptiglycan, muramylpeptides, lipoproteins (highly pyrogenic);

To test MenB vaccine by RPT, higher dilutions are needed then in MAT;

Successful application of Method C: data expressed as “Relative Pyrogen Units” with respect to a reference vaccine lot.
MenB vaccine is distributed in several countries, which do not perform local testing for batch release. In these countries (Australia, Argentine, Canada, Chile, Brazil, Turkey, Israel, New Zealand, Hong Kong), the OCABR released by European NCLs is sufficient to get market authorization for a MenB vaccine lot. In addition, the company provides the summary protocol, including all the results obtained on the distributed lot, through internal testing;

FDA and USP position remains vague, although recently, MAT and RPT comparison test on the same vaccine lots has been requested.
WHO REQUIREMENTS FOR RPT
- State of art -

<table>
<thead>
<tr>
<th>PRE-QUALIFIED VACCINES</th>
<th>TRS N°</th>
<th>Stage of RPT execution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>980/Annex 6/2014</td>
<td>RPT or LAL on intermediate production stage and final lot</td>
</tr>
<tr>
<td>D, T, aP, wP, HepB, IPV, Hib single or combined</td>
<td>978/Annex 4/2013</td>
<td></td>
</tr>
<tr>
<td></td>
<td>980/Annex 4/2012</td>
<td></td>
</tr>
<tr>
<td>HPV (bi-, nine- and quadri-valent)</td>
<td>999/Annex 4/2016</td>
<td>If there is interference with LAL, RPT on final lot</td>
</tr>
<tr>
<td>JE (inactivated)</td>
<td>963/Annex 1/2007</td>
<td>RPT on final lot</td>
</tr>
<tr>
<td>MenA</td>
<td>962/Annex 2/2011</td>
<td>RPT on final lot</td>
</tr>
<tr>
<td>MenAC</td>
<td>924/Annex 2/2004</td>
<td>If there is interference with LAL, RPT on final lot</td>
</tr>
<tr>
<td>MenACYW-135</td>
<td>594/Annex 2/1975</td>
<td>RPT on final lot</td>
</tr>
<tr>
<td>PCV</td>
<td>977/Annex 32013</td>
<td>RPT on intermediate production stage; RPT or LAL on final lot</td>
</tr>
<tr>
<td>Rabies</td>
<td>941/Annex 2/2007</td>
<td>RPT on final lot</td>
</tr>
<tr>
<td>VICPS</td>
<td>840/Annex 1/1992</td>
<td>RPT on final lot</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>OTHER VACCINES</th>
<th>TRS N°</th>
<th>Stage of RPT execution</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepE</td>
<td>WHO/BS/2018.2348</td>
<td>RPT on final lot</td>
</tr>
<tr>
<td>Ebola</td>
<td>1011/Annex 2/2018</td>
<td>RPT or LAL on final lot</td>
</tr>
<tr>
<td>HFRS (inactivated)</td>
<td>848/Annex 2/1993</td>
<td>RPT on final lot</td>
</tr>
<tr>
<td>RTS (Malaria)</td>
<td>980/Annex 3/2014</td>
<td>RPT or LAL on final lot</td>
</tr>
<tr>
<td>TBEV</td>
<td>889/Annex 2/1997</td>
<td>RPT on final lot</td>
</tr>
</tbody>
</table>
SUMMARY AND CONCLUSIONS

✓ MAT is intended as a replacement of the rabbit pyrogen test;
✓ The method has been already described in the general chapter of the Ph. Eur. and therefore does not require re-validation per se while tests for product (vaccine)-specific optimization are needed;
✓ MAT represents a human setting for testing human vaccines;
✓ MAT sensitivity could be adjusted to face the heterogenicity of vaccine formulation: ranging from the possibility to chose between primary cell or monocytic cell to three different methods of analysis;
✓ To rule out the presence of endotoxin and non-endotoxin pyrogens in vaccines, the MAT could be a useful tool during development of the production process (R&D), manufacturing process or for batch release;
✓ Pharmacopoeia harmonization is not too far since China has announced MAT implementation in the Pharmacopeia for 2020 while Health Canada and the National Institute of Health Science in Japan are on the way.
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