9.0 Other Scientific Reports and Reviews

The individual BRDs submitted by ECVAM (i.e., one for each of the *in vitro* pyrogen test methods) are provided in **Appendix A** and were used in the performance analyses described in **Section 6.0** and **Section 7.0**. A *FR* notice (Vol. 70, No. 241, pp. 74833-74834, December 16, 2005) was published requesting the submission of data from the RPT, the BET, or an *in vitro* pyrogen test method. No data were received in response to this request.

NICEATM conducted a prescreen evaluation of the ECVAM BRDs to verify that the information contained within the documents fulfilled the requirements outlined in the ICCVAM submission guidelines (ICCVAM 2003). Based on this evaluation, the ICCVAM PWG requested a direct comparison on the accuracy analysis of the *in vitro* test methods with the reference test methods (i.e., the RPT and the BET) and data to support the claim that the *in vitro* test methods can detect non-endotoxin pyrogens. In response to these requests, ECVAM provided supplemental data from published (e.g., Francois et al. 2006) and unpublished studies in an attempt to address these issues (see questions #1 and #2 in **Appendix B**).

Hartung et al. (2001) provided a summary report of an ECVAM-sponsored workshop to review the current status of pyrogenicity testing, to review the capabilities of new pyrogen tests, and to provide recommendations for their continued development. The need for alternatives to the RPT and the BET was discussed, and their respective limitations were highlighted. The workshop compared the utility of the various methods (i.e., *in vitro* pyrogen test methods, BET, RPT) for testing a variety of pyrogenic materials. Workshop conclusions indicated a need for alterative test methods to address the limitations of the BET and RPT, but stressed the need for appropriate validation of any new method.

9.1 Summaries of *In Vitro* Pyrogen Test Methods and Data from Published and Unpublished Studies

As indicated in **Section 1.5**, NICEATM conducted an online literature search for relevant information on the proposed test methods using multiple databases (i.e., PubMed, SCOPUS, TOXLINE, Web of Science). This search revealed ten additional scientific publications that contained data from *in vitro* pyrogen product testing. These studies contained comparisons of the results obtained in an *in vitro* test method with those obtained in the RPT and/or BET (see **Tables 9-1** to **9-8**). These studies were not included in previous sections of the ICCVAM BRD because they used a different method or protocol, or because they lacked sufficient information for an evaluation of accuracy and reliability (e.g., an adequate validation study design was not included, a standardized reference pyrogen was not used). Summaries of these published studies and available data from the *in vitro* pyrogen methods are presented below.

9.1.1 Andrade et al. (2003)

The authors evaluated the utility of human PBMCs and diluted WB for *in vitro* pyrogen tests and compared the responses to those obtained in the BET and RPT for the same diverse sampling of parenteral pharmaceuticals and biological products (see **Tables 9-1** and **9-2**). Interference testing of each substance was performed with spikes of the international endotoxin standard WHO-LPS 94/580. These studies established an endotoxin detection limit

of 0.06 EU/mL for both *in vitro* assays, and the results were consistent with those from the BET and RPT. The authors concluded that both the PBMC and WB methods were comparable to the BET and the RPT in their ability to detect and quantify the presence of endotoxin. In addition, the WB test method was able to detect concentration-dependent IL-6 release on exposure of WB to non-endotoxin pyrogens and pyrogens from Gram-positive organisms (i.e., *Candida albicans* and *Staphylococcus aureus*).

Table 9-1 Results of Pyrogen Testing of Pharmaceutical/Biological Products in the Human PBMC Assay, the BET, and the RPT[1]

Product	Number of Batches[2]	PBMC (EU/mL)	BET (EU/mL)	RPT
Ampicillin - 1000 mg/5 mL A	1	<6	<0.06	Pass
Ampicillin - 1000 mg/5 mL A	1	<6	<0.06	Pass
Gentamycin - 80 mg/2 mL	2	<3	<0.06	Pass
Oxacillin - 500 mg/5 mL	2	<3	<0.06	Pass
Enoxaparin - 100 mg/mL	3	<1.2	<0.06	Pass
Insulin - 100 U/mL	2	<3	<0.06	Pass
Tenoxican - 40mg/2 mL	1	<6	<0.06	Pass
Metoclopramide - 10 mg/2 mL	4	<3	<0.06	Pass
Calcium folinate - 50 mg/5 mL	1	<2.4	<0.06	Pass
Ranitidine - 25 mg/mL	2	<6	1.2-2.4	Pass
Pantoprazol - 40 mg/10 mL	1	<3	<0.06	Pass
Human serum albumin - 20%	1	<4.8	0.48-0.96	Pass
Erythropoietin - 4000 IU/vial A	1	<1.2	0.48-0.96	Pass
Erythropoietin - 2000 IU/vial B	1	112 ± 10[1]	491-983	Fail
Erythropoietin - 4000 IU/vial C	1	<1.2	<0.06	Pass
recG-CSF - 200 μg/vial A	3	<0.6	<0.06	Pass
Saline solution - 0.9% A	1	<0.3	<0.06	Pass

Abbreviations: BET = Bacterial Endotoxin Test; CSF = Colony Stimulating Factor; EU/mL = Endotoxin units/mL; IU = international units; PBMC = Peripheral blood mononuclear cells; rec = Recombinant; RPT = Rabbit pyrogen test; U = units

[1]From Andrade et al. (2003)

[2]Batch results were combined; PBMC and BET study values represent a mean ± standard deviation value or consensus detection limits (n=3 donors; 4 replicates from each donor).

Table 9-2 Results of Pyrogen Testing of Pharmaceutical/Biological Products by the Human WB Culture Assay, the BET, and the RPT[1]

Product	Number of Batches[2]	WB Culture (EU/mL)	BET (EU/mL)	RPT
Dipyrone - 500 mg/mL	3	<24	<0.06	Pass
Amikacin - 500 mg/2 mL	2	<12	<0.06	Pass
Ampicillin - 1000 mg/5 mL A	1	<6	<0.06	Pass
Ampicillin - 1000 mg/5 mL A	1	<6	<0.06	Pass
Gentamycin - 80 mg/2 mL	2	<6	<0.06	Pass
Oxacillin - 500 mg/5 mL	2	<6	<0.06	Pass
Vancomycin - 500 mg/5 mL	2	<6	<0.06	Pass
Enoxaparin - 100 mg/mL	3	<0.6	<0.06	Pass
Heparin - 5000 IU/mL	2	<0.6	<0.06	Pass
Insulin - 100 U/mL	3	<6	<0.06	Pass
Ketoprofen - 100 mg/2mL	1	<6	<0.06	Pass
Diclofenac - 75 mg/3 mL	1	<12	<0.06	Pass
Tenoxicam - 40 mg/2 mL	2	<6	<0.06	Pass
Metoclopramide - 10 mg/2 mL	3	<3	<0.06	Pass
Cytarabine - 100 mg/5mL	1	<1.2	<0.06	Pass
Calcium folinate - 50 mg/5 mL	1	<0.6	<0.06	Pass
Ranitidine - 25 mg/mL	1	<6	1.2-2.4	Pass
Pantoprazol - 40 mg/10 mL	1	<6	<0.06	Pass
Furosemide - 10 mg/mL	2	<0.6	<0.06	Pass
rec-hGH - 4 IU/vial A	2	<0.2	<0.06	Pass
rec-hGH - 4 IU/vial B	1	12.4±2.5[1]	15.84-31.68	Pass
Human serum albumin - 20%	1	<2.4	0.48-0.96	Pass
Erythropoietin - 4000 IU/vial A	1	0.76	0.48-0.96	Pass
Erythropoietin - 2000 IU/vial B	1	141±2.8[1]	491-983	Fail
Erythropoietin - 4000 IU/vial C	1	<0.6	<0.06	Pass
recG-CSF - 300 μg/vial	3	<0.6	<0.06	Pass
Saline solution 0.9% A	2	<0.3	<0.06	Pass
Saline solution 0.9% B	1	44.8±5[1]	48-96	Fail
Glucose - 0.5%	1	2054±95[1]	1920-3840	Fail
Vitamin K - 10 mg/mL	2	<6	<0.06	Pass

Abbreviations: BET = Bacterial endotoxin test; CSF = Colony stimulating factor; EU/mL = Endotoxin units/mL; hGH = Human growth hormone; IU = International units; rec = Recombinant; RPT = Rabbit pyrogen test; WB = Whole blood; U = units

[1]From Andrade et al. (2003)

[2]Batch results were combined; PBMC and BET study values represent a mean ± standard deviation value or consensus detection limits (n= 3 donors; 4 replicates from each donor).

9.1.2 Bleeker et al. (1994)

This study measured IL-6 release from PBMCs as an indicator of pyrogenicity for *in vitro* safety testing of hemoglobin (Hb) solutions. The authors demonstrated that pure, polymerized Hb produced under aseptic conditions did not induce or inhibit IL-6 production, whereas production under non-aseptic conditions led to IL-6 release, which was also seen with the BET. Based on these findings, the authors concluded that IL-6 release from isolated PBMCs provides a sensitive indicator of endotoxin contamination in Hb solutions. The observed detection limit for endotoxin in Hb solutions (below 0.4 EU/mL) led the authors to suggest that this test method would be more sensitive to the presence of endotoxin than the RPT.

9.1.3 Carlin and Viitanen (2003)

Using WB and MM6-based *in vitro* pyrogen methods, this study evaluated the pyrogenic potential of a multivalent vaccine, Infanrix® (GlaxoSmithKline) that contains protein and polysaccharide components from both Gram-positive and Gram-negative bacteria. The five Infanrix® vaccines studied (e.g., Infanrix®, Infanrix® Hep B, Infanrix® polio, Infanrix® hexa, and Infanrix® polio Hib) contain Gram-positive bacterial components that are potentially pyrogenic but not detectable in the BET. IL-6 production in the WB/IL-6 test method varied among the seven donor blood samples in response to each of the five vaccines. Some donor samples produced a weak or no IL-6 release and others produced a large release (**Table 9-3**). However, IL-6 production from any single donor was similar for all vaccines when tested at various times. The variability in the magnitude of response to each vaccine among donors and the consistency of the response of any single donor was also seen when IL-1β was used as a marker. IL-6 release from WB was also examined following exposure to three concentrations of endotoxin standard (0.2, 2, and 20 pg/1.2 mL). All donor WB samples released IL-6 in a concentration responsive manner.

The IL-6 release from MM6 cells (**Table 9-4**) exposed to the five Infanrix® vaccines was measured using an ELISA and compared to the responses induced by three concentrations of endotoxin standard (0.2, 2, and 20 pg/1.2 mL) in three separate experiments. The MM6 cells produced minimal responses to the vaccines when compared to WB, but released significant amounts of IL-6 in response to high concentrations of endotoxin. However, IL-6 induction by two different endotoxin standards in MM6 cells was strongly attenuated (>80% inhibition) when either of two vaccines (Infanrix® and Infanrix® Hep-B) was present (data not included in **Table 9-4**). Based on these studies, the authors suggested that a BET or RPT result might not correlate with the human fever response one might expect in humans immunized with such vaccines, because the production of proinflammatory cytokines may be compromised by various components in the vaccine product, and because Gram-positive components in the vaccines would not be detected in the BET.

Table 9-3 IL-6 Production from WB after Exposure to Endotoxin or Five Infanrix® Vaccines[1,2]

Experiment (Blood Donor)	Endotoxin		Vaccine (Absorbance in ELISA; n=4[3])					
	Endotoxin (pg/1.2 mL)	Absorbance (ELISA)	Dilution (µL vaccine/1.2 mL	Infanrix®	Infanrix® Hep-B	Infanrix® Hexa	Infanrix® Polio	Infanrix® Polio Hib
1	0.2	0.47	0.03	0.945	1.052	1.069	0.869	1.082
	2	0.971	0.3	1.826	2.055	2.014	1.832	1.919
	20	1.116	3	2.826	2.587	2.638	2.609	2.2
2	0.2	0.001	0.03	0.149	0.256	0.231	NT	0.284
	2	0.127	0.3	0.869	0.847	1.095	NT	0.933
	20	0.764	3	1.998	1.986	2.187	NT	1.685
3	0.2	-0.007	0.03	0.005	0.037	0.009	0.007	0.208
	2	0.09	0.3	0.275	0.457	0.282	0.321	0.261
	20	0.811	3	0.941	1.057	0.795	1.284	1.325
4	0.2	0.006	0.03	0.056	0.053	0.028	0.088	0.104
	2	0.043	0.3	0.165	0.312	0.44	0.309	0.533
	20	0.458	3	1.229	1.489	1.476	1.181	1.242
5	0.2	0.043	0.03	-	0.071	-0.003	-0.003	0.011
	2	0.024	0.3	0.007	0.014	0.004	0.03	0.05
	20	0.435	3	0.042	0.164	0.008	0.08	0.12
6	0.2	0.013	0.03	-0.009	-0.018	-0.01	-0.022	0.012
	2	0.022	0.3	-0.007	-0.008	0.005	-0.019	-0.007
	20	0.569	3	0.132	0.411	0.042	0.132	0.188
7	0.2	0.036	0.03	-0.012	-0.012	-0.01	-0.014	0.07
	2	0.014	0.3	-0.01	-0.01	-0.012	-0.011	-0.013
	20	0.436	3	0.183	0.274	0.045	0.183	0.525

Abbreviations: ELISA = Enzyme-linked immunosorbent assay; Hep = Hepatitis; IL-6 = Interleukin-6; NT = Not tested; WB = Whole blood

[1]From Carlin and Viitanen (2003)

[2]WB was challenged with endotoxin standard or vaccine in pyrogen-free water to provide the final concentration and incubated overnight at 37°C.

[3]Duplicate samples were run in two separate experiments.

Table 9-4 IL-6 Production by MM6 Cells after Exposure to Endotoxin or Five Infanrix® Vaccines[1,2]

MM6 Batch	Endotoxin			Vaccine (Absorbance in ELISA; 250,000 MM6 cells); n=4[3]				
	Endotoxin (pg/1.2 mL)	Absorbance in IL-6 ELISA	Dilution (µL vaccine/ 1.2 mL	Infanrix®	Infanrix® Hep-B	Infanrix® Hexa	Infanrix® Polio	Infanrix® Polio Hib
1	0.2	-0.001	0.3	0.013	0.014	0.001	0.002	-0.001
	2	0.026	3	0.078	0.158	0.06	0.105	0.07
	20	0.383	30	0.054	0.052	0.053	0.106	0.089
2	0.2	-0.001	0.3	0.004	0.01	0.001	0.003	0.004
	2	0.025	3	0.033	0.062	0.019	0.037	0.032
	20	0.4	30	0.013	0.012	0.018	0.038	0.038
3	0.2	-0.009	0.3	-0.012	-0.017	-0.021	-0.014	-0.019
	2	0.03	3	0.019	0.05	0.01	0.043	0.026
	20	0.192	30	-0.018	-0.012	-0.007	0	0.005

Abbreviations: ELISA = Enzyme-linked immunosorbent assay; IL-6 = Interleukin-6; MM6 = Mono Mac 6

[1]From Carlin and Viitanen (2003)

[2]MM6 cells were stimulated with endotoxin standard or vaccine in pyrogen-free water to provide the final concentration and incubated overnight at 37°C.

[3]n = Duplicate samples were run in two separate experiments.

9.1.4 Carlin and Viitanen (2005)

This study provides support for the findings from a previous study (Carlin and Viitanen (2003) in which the authors demonstrated IL-6 release by a WB method in response to pyrogenic or spiked multivalent vaccine preparations that were inactive in the BET. It also confirms that IL-6 was released from WB of some, but not all donors. The present study demonstrates that IL-6 release in susceptible donors was caused by toxoids from Gram-positive diphtheria, and to a lesser extent, from tetanus bacterial components of the vaccines. The WB donors were studied for two years and their responses to the individual vaccines, whether responsive or non-responsive, were consistent. The responses of these donors to Gram-negative endotoxin or lipoteichoic acid (LTA) from Gram-positive bacteria were consistent and confirmed the findings of Fennrich et al. (1999) with respect to the consistency of responses among several hundred blood donors to endotoxin. The authors concluded that individual donor-specific differences in IL-6 release from WB exposed to the multivalent vaccines resulted from toxoids present in the diphtheria or tetanus component, and noted that these donor-specific responses to the vaccines were not observed in the BET.

9.1.5 Daneshian et al. (2006)

This study describes the development of a modification to the WB/IL-1β method termed AWIPT (Adsorb, Wash, *In Vitro* Pyrogen Test). The authors indicate that this modification is intended to increase sensitivity to the presence of endotoxin contamination by isolating endotoxin from WB. To accomplish this, the sample containing endotoxin (naturally occurring or spiked) is treated with human serum albumin (HSA) covalently linked to macroporous acrylic beads. The HSA-treated beads bind the endotoxin, which is subsequently eluted from the beads. The WB/IL-1β test method is therefore performed using a slightly modified protocol in which the diluted WB is incubated overnight with the sample in the bead suspension.

The results showed that HSA-coated beads bind endotoxin in a concentration-dependent manner (when spiked with 0, 25, 50, and 100 pg/mL LPS), but little or none was bound to unmodified beads. The test showed a detection limit of 25 pg/mL LPS (i.e., 0.25 EU/mL), which is less sensitive than the BET (3 pg/mL) and more sensitive than the RPT (50 pg/mL). IL-1β secretion in response to either LPS or LTA was generally higher using the AWIPT procedure, but the concentrations of LPS or LTA needed to induce a response were similar; thus the sensitivity of this test modification was comparable to that of the unmodified WB/IL-1β test method.

Daneshian et al. studied the kinetics of cytokine release from WB in response to a challenge with 2 pg/mL of endotoxin. IL-1β release in the AWIPT-treated samples lagged slightly behind that of the standard WB/IL-1β test in the 0 to 8 hr time period, whereas more IL-1β was produced in the AWIPT-treated samples in the 10 to 30 hr time period. Some immunomodulatory or toxic cancer drug samples tested in the WB/IL-1β method interfered with the WB/IL-1β assay and required a higher dilution (1/10 to 1/100) to detect IL-1β. Detection of endotoxin spiked into these test samples (measured as IL-1β release) generally occurred at lower dilutions in AWIPT than in the WB/IL-1β test method, suggesting that the interfering substances were removed by the procedure. For example, five dilutions (ranging from 1/3 to 1/316) of liposomal daunorubicin were spiked with 25 pg/mL of endotoxin and

detection of IL-1β was compared between the two methods. This cytokine was not detectable in the WB/IL-1β method (< 30% of the IL-1β released by endotoxin) at any drug dilution, whereas in the AWIPT, IL-1β was detected at drug dilutions of 1/32, 1/100, and 1/316 (>78% of the IL-1β released by endotoxin).

The authors concluded that the inclusion of endotoxin adsorption and washing steps in the WB/IL-1β method (i.e., the AWIPT) to remove potentially interfering substances improved the detection of pyrogenic contaminants in immunomodulatory and toxic cancer drug samples. They suggest that the AWIPT method offers an improvement for safety testing of products administered to patients, and for batch control in pharmaceutical processing.

9.1.6 Eperon et al. (1996, 1997)

Eperon and colleagues developed an *in vitro* test system for measuring pyrogenic substances using two clones derived from MM6 cells (Professor Ziegler-Heitbrock, University of Munich) and one from a THP-1 cell line (European Collection of Animal Cell Cultures, Porton Down, Salisbury, U.K.). These clones are reported to be more phenotypically stable over time with respect to their superior responsiveness to endotoxin than the parent cell lines. Endotoxin content was measured by the release of TNF-α using an immunoassay. These clones demonstrate high LPS sensitivity when non-pyrogenic fetal calf serum is used in the assay as a serum supplement. Enhanced expression of the cell-surface endotoxin receptor CD14 was obtained by pretreatment of the cells for two days with calcitrol. Purified endotoxin (i.e., LPS; smooth strain and rough mutant), other cellular components from Gram-negative or Gram-positive bacteria, and Mycobacteria were tested. The MM6 clones responded to these pyrogenic products in an order of potency of detection equivalent to that found in the RPT and similar to that observed in the BET (i.e., Gram-negative endotoxin > Gram-positive material > non-endotoxin pyrogens). The response of the THP-1 clone was similar to that of the MM6 clones, except that the THP-1 clone did not respond to diphosphoryl lipid A, a structural component of LPS.

Pyrogen testing of a panel of stable blood products, including albumin and Immunoglobulin G (IgG) for parenteral use, produced similar results in the RPT and MM6 or THP-1 clones when tested as received (i.e., free of detectable pyrogens). The products produced positive results when spiked with 20 EU/mL of endotoxin (**Table 9-5**), with a few exceptions. For example, in the cell-based test, there was one borderline but significantly positive result in an unspiked sample, representing a false positive result relative to the RPT. In the BET, 4 of 13 (31%) unspiked samples tested positive (i.e., false positive). The results suggest that the cell-based assays may produce fewer false positives than the BET.

When 10 bacterial and viral vaccine preparations were evaluated, the monocytoid cell-based test method (e.g., combined results from two experiments with each cell line) correlated well with the RPT (positive or negative for endotoxin) with the exception of one preparation that produced nearly 10-fold less TNF-α than the other samples, and was near the limit of detection. This result was not significantly different from the negative control (**Table 9-6**). The authors suggest that these cloned monocytoid cell-based test methods are valid *in vitro* alternatives for detection of endotoxin in commercial preparations, and produce results comparable to the RPT and BET.

Table 9-5 Pyrogenic Activity of Blood Preparations for Parenteral Use[1]

Preparation	Endotoxin Spike	RPT[2]	BET[3,4]	Cell Test[5,6]
IgG for i.v. use	20 EU/mL	+	+	+
	20 EU/mL	+	+	+
	-	-	-	-
	-	-	-	-
	-	-	-	-
	-	-	-	-
	-	-	-	-
	-	-	-	-
	-	-	-	-
	-	-	-	-
Albumin	20 EU/mL	+	+	+
	20 EU/mL	+	+	+
	-	-	-	-
	-	-	-	-
	-	-	-	-
	-	-	-	-
	-	-	-	-
Test threshold[7]		*Δt = 1.5 °C*	*300 pg/mL LPS*	*50 pg/mL TNF*

Abbreviations: BET = Bacterial endotoxin test; EU/mL = Endotoxin units/mL; IgG = Immunoglobulin G; i.v. = Intravenous; LPS = Lipopolysaccharide; RPT = Rabbit pyrogen test; TNF = Tumor Necrosis Factor

[1]From Eperon et al. (1997)

[2]n=3

[3]n=2

[4]Haemachem BET (St. Louis)

[5]n=4 [Note: Cell type not specified; author claims that the Mono Mac 6 or acute monocyte leukemia THP-1 cell lines are equally capable of endotoxin detection.]

[6]TNF induction was determined using a commercial TNF Enyme-linked immunosorbent assay.

[7]RPT threshold was obtained from the European Pharmacopeia; the threshold for the BET and cell-based test methods was considered to be equal to 2 standard deviations from the mean of a set of negative samples.

Table 9-6 Pyrogenic Activity of Vaccine Preparations[1]

Vaccine Preparation[2]	Batch[3]	Cell Test[4]	Pyrogenicity
IgG for i.m. use	A-1	n.d.[5]	-
	A-2	n.d.	-
Bacterial vaccines	B-1	10.8±0.3	+
	C-1	6.0±3.6	+
	D-1	1.4±1.8	-
Viral vaccines	E-1	n.d.	-
	E-2	n.d.	-
	F-1	n.d.	-
	F-2	n.d.	-
	G-1	21.2±3.2	+

Abbreviations: IgG = Immunoglobulin G; i.m. = Intramuscular; n.d. = Non-detectable
[1]From Eperon et al. (1997)
[2]Vaccine solutions were tested at 1/20 (v/v)
[3]Letters refer to distinct types of vaccine preparations; numbers to different lots
[4]Tumor necrosis factor (TNF)-α production in ng/mL±standard error of the mean (n=3) [Note: Cell type not specified; author claims that the Mono Mac 6 or acute monocyte leukemia THP-1 cell lines are equally capable of endotoxin detection.]
[5]No measurable quantity of cytokine was detected.

9.1.7 Marth and Kleinhappl (2002)

In 2000, Ticovac®, a thiomersal- and albumin-free tick-borne encephalitis (TBE) vaccine, was developed as a more immunogenic alternative to previous vaccines that also produced fewer side effects. Although the Austrian health authorities approved this vaccine, 779 cases of fever were reported in children less than 15 years of age, including a high incidence of febrile convulsions in children ages 2 and younger. To determine the cause of these fever reactions, Ticovac® was compared to FSME-Immun®, a TBE-vaccine that rarely resulted in febrile reactions, in an *in vitro* human WB assay that measured cytokine release (i.e., IL-1β, IL-6, IL-8, and TNF-α) as an indication of immune system activation. Ticovac®, which differs from FSME-Immun® only in the albumin component, induced high amounts of TNF-α ($P \leq 0.0001$) and lower amounts of IL-1β ($P \leq 0.05$) as compared to FSME-Immun®. The addition of 0.5 mg of albumin (i.e., the identical quantity of albumin in FSME-Immun®) to Ticovac® reduced the TNF-α induction significantly, resulting in TNF-α production that was similar to the level stimulated by FSME-Immun®. The incubation of Ticovac® with human WB resulted in an increase in TNF-α concentration after 4 hr (peaking at 15 hr) and returned to baseline levels by 27 hr. IL-1β release displayed a similar time course. This temporal response to Ticovac® correlated well with the progression of the clinical outcome (i.e., fever and convulsions in children 6 to 8 hr after the first immunization). Although the mechanism of cytokine production by Ticovac® is unknown, it is clearly linked to the absence of albumin, which is needed as a stabilizer to bind to the antigen of the vaccine. Thus, it was recommended that albumin be added to subsequently produced TBE-vaccines to inhibit nonspecific, excessive immunological reactions.

9.1.8 Martis et al. (2005)

The goal of this study was to establish the cause of 186 cases of aseptic peritonitis that occurred between 2001 and 2003 in peritoneal dialysis patients using an icodextrin-containing dialysate that met both European and USP standards. These patients were not febrile or toxic in appearance, but abdominal pain that was modest to absent and cloudy dialysate were common features. The authors conducted physical, chemical, and microbiological analyses on the recalled dialysate and calculated dose-response curves for IL-6 production in PBMCs from human donors and for sterile peritonitis in rats. Increased levels of IL-6 were identified in dialysis solutions of compliant batches (n=3), but not in non-complaint batches (n=2). Effluents from compliant batches also stimulated IL-6 release in the PBMC assay. Polymyxin B did not inhibit this response, suggesting that a lipopolysaccharide was not responsible for the increased IL-6 levels. When neither Gram-negative nor Gram-positive bacterial contamination was identified in the dialysates, the possibility of a non-endotoxin contaminant was considered. A Gram-positive bacterial cell wall component (i.e., peptidoglycan [PG]) was identified using a silkworm larvae assay in a significant number of dialysates. In the PBMC assay, IL-6 release increased with PG concentration in a dose-response manner. A microbial investigation revealed that the dialysates were contaminated with a Gram-positive organism (i.e., *Alicyclobacillus acidocaldarius*), which contains approximately 40% PG in its cell wall. In rat studies, intraperitoneal injection of icodextrin containing PG (0 - 5000 μg/mL) produced a dose-dependent inflammatory response as measured by an increase in TNF-α and IL-6 production. Subsequent PG contamination has been eliminated using more stringent filtration and carbon treatment steps in the manufacturing process, assaying for PG contamination with the silkworm larvae test, and measuring IL-6 production with the PBMC assay. The lack of aseptic peritonitis incidents that have occurred since the implementation of these additional detection processes support the concept that PG contamination of dialysate was responsible for the reported cases of aseptic peritonitis.

9.1.9 Pool et al. (1998)

This study describes a WB assay for the detection of pyrogens in blood products. IL-6 release from WB in response to endotoxin is used to define a pyrogenic response. This assay was highly responsive to *E. coli* endotoxin (i.e., the limit of detection of endotoxin was 1.25 EU/mL), and also responded to whole bacteria (*E. coli* and *Bacillus subtilis*). There was considerable variation in IL-6 levels released from WB between donors following exposure to endotoxin, but each donor response was always linear. The potential pyrogenicity of production batches of HSA, fibronectin (Fn), and stabilized human serum (SHS) solutions were evaluated using the WB method and compared to the BET and RPT. Spike recovery in batches of these samples varied between 90 and 116% for *E. coli* endotoxin, 74 to 111% for *B. subtilis*, and 61 to 99% for *E. coli* and the products tested did not interfere with the IL-6 assay system. Good correlations were found among the WB, BET, and RPT results (**Table 9-7**). Of 22 products tested, the WB assay and the RPT were in agreement (i.e., pass or fail) for all tests, while one sample was classified as negative in the BET, but positive in both the WB method and the RPT. The detection limit for endotoxin by the WB method was 1.25 EU/mL, which is lower than the established pyrogen cut-off level (as stated in the European Pharmacopeia) for the products under investigation (i.e., 2 EU/mL for HSA and SHS; 4.5

EU/mL for Fn). The authors concluded that the WB assay was able to detect both Gram-negative and a Gram-positive pyrogens and exhibited greater sensitivity to endotoxin than the RPT.

Table 9-7 Comparison of the WB test, BET, and the RPT for Detecting Pyrogens in Production Batches of Biological Products[1]

Product	Batch	WB (EU/mL)[2]	BET	RPT
Fibronectin - 0.5 mg/mL	Fn3195	<0.05	Pass	Pass
	Fn3296	<0.05	Pass	Pass
	Fn3596	1.28	Pass	Pass
Human serum albumin - 200 mg/mL	B274	29.4	Fail	Fail
	B291	<0.05	Pass	Pass
	B293	<0.05	Pass	Pass
	B294	<0.05	Pass	Pass
	B295	<0.05	Pass	Pass
	B296	<0.05	Pass	Pass
	B297	<0.05	Pass	Pass
	B298	1	Pass	Pass
	B299	1.1	Pass	Pass
	B300S	1	Pass	Pass
	B301	<0.05	Pass	Pass
	B302	>20	Pass[3]	Fail
Stabilized human serum - 50 mg/mL	SS349	0.7	Pass	Pass
	SS350	<0.05	Pass	Pass
	SS351	<0.05	Pass	Pass
	SS352	0.5	Pass	Pass
	SS353	<0.05	Pass	Pass
	SS354	0.6	Pass	Pass
	SS355	0.5	Pass	Pass

Abbreviations: BET = Bacterial endotoxin test; EU/mL = Endotoxin units/mL; RPT = Rabbit pyrogen test; WB = Whole blood

[1]From Pool et al. (1998)

[2]Result based on interleukin-6 secretion in human WB using an enzyme-linked immunosorbent assay calibrated to an *E. coli* endotoxin standard (Kabi Diagnostica).

[3]False negative relative to the RPT response

9.1.10 Taktak et al. (1991)

This paper summarizes the development of an *in vitro* pyrogen test method based on IL-6 release from MM6 cells. A detectable level of IL-6 was released in response to 2.5 pg/mL of endotoxin, yielding a level of sensitivity of 25 pg/mL when testing 5% HSA at a 1/10 dilution for the presence of endotoxin. Three batches of a therapeutic HSA that caused fever in humans were positive in the MM6/IL-6 method, whereas the same substances were negative in the BET and the RPT (**Table 9-8**). As in the BET, the samples required a 1/10 dilution to remove interfering substances. The assay had sensitivity equal to that of the BET (25 pg/mL) and 40-fold greater than the RPT (1000 pg/mL). The authors suggest that the MM6/IL-6 method represents an important alternative to the existing pyrogen tests and may be a more appropriate end-product test for the detection of pyrogens in parenteral products, such as HSA, that cannot be detected in the BET.

Table 9-8 Results of Pyrogen Testing of Batches of Therapeutic HSA Using the MM6/IL-6, BET, and RPT[1]

Batch of HSA	Endotoxin Quantitation by IL-6 Release (pg/mL)	Endotoxin Quantitation by the BET		RPT Result
		IU/mL	pg/mL[6]	
1[2]	97±2.3[3,4]	1.0-2.0	140-280	Pass
2[2]	30±2.8[4]	2.4-3.2	336-448	Pass
3[2]	31±2.3[4]	0.5-0.75	70-105	Pass
4	<25[5]	<0.24	<34	Pass
5	<25[5]	3.6-4.8[7]	504-762	Pass
6	<25[5]	<0.26	<36	Pass

Abbreviations: BET = Bacterial endotoxin test; HSA = Human serum albumin; IL-6 = Interleukin-6; IU = International units; MM6 = Mono Mac 6; RPT = Rabbit pyrogen test

[1]From Taktak et al. (1991)

[2]Batch of HSA used that caused fever in humans.

[3]Mean ± standard error of the mean

[4]Values are significantly different from subthreshold concentrations of endotoxin (<2.5 pg/mL; $p<0.001$).

[5]Values below the detection limit of the test system (25.0 endotoxin units/mL); preparations of HSA were tested at a dilution of 1/10, and 2.5 pg/mL endotoxin was the lowest concentration of endotoxin tested that evoked a significant release of IL-6.

[6]1.0 IU=0.14 ng for preparation used.

[7]False positive relative to RPT.

9.2 Conclusions from Scientific Literature Based on Independent Peer-Reviewed Reports and/or Reviews

An additional nine reports describing studies of cell-based *in vitro* pyrogen methods were obtained from the literature search described in **Section 1.5**. Although these reports did not include data on test substances that could be used in the performance analysis in **Section 6.0** and **Section 7.0**, they did evaluate the use of the *in vitro* pyrogen test methods for sensitivity to endotoxin (i.e., endotoxin detection limit), specificity of the response to endotoxin and/or non-endotoxin pyrogens (i.e., spectrum and relative potency of various pyrogens detected), and/or the impact of interfering substances. However, they did not compare results from the *in vitro* test methods to results from the RPT, BET, or human fever reaction. A summary of each study is presented below.

9.2.1 De Groote et al. (1992)

The authors measured the release of various cytokines (IL-1β, IL-6, TNF-α, IL-2, IFN- γ , and granulocyte-macrophage colony stimulating factor [GM-CSF]) in response to endotoxin or phytohemagglutinin (PHA) stimulation of WB and PBMC cultures. Endotoxin stimulated IL-1β, TNF-α, and IL-6 release, while PHA stimulated IL-2, IFN- γ , and GM-CSF release. There was a significant correlation between production of the three endotoxin-induced cytokines and the number of monocytes in the challenged culture, suggesting that monocytes are the major source of these cytokines: the other cytokines did not correlate with any of the cell types. The data also suggested that WB produced less variable levels of cytokines than PBMC on exposure to endotoxin. Consistent results were obtained with the WB test using more than 50 different blood donors. The authors suggest that WB is a more appropriate choice for studying cytokine production *in vitro* and its modulation by exogenous or endogenous factors, because natural cell-to-cell interactions are preserved, immune mediators are available, and cytokine levels obtained with PBMC were more variable.

9.2.2 Fennrich et al. (1999)

Fennrich and colleagues compared a commercially available human WB/IL-1β pyrogen assay (PyroCheck® from DPC Biermann, Bad Nauheim distributed by Millenia, U.K.) to the BET and RPT. There was a concentration-dependent IL-1β release in WB that was incubated with nitrocellulose filters containing live *E. coli* bacteria and *E. coli* killed by heat or by antibiotics. The authors also tested air conditioning filters from a veterinary sheep facility and identified filters to be contaminated with bacteria that were later confirmed by microbiological tests (the data and the identity of the organisms identified were not presented). The authors compared the PyroCheck®, BET, and the RPT (**Table 9-9**) and concluded that PyroCheck® is a simple, accurate test that detects a wider range of pyrogens than the BET.

Table 9-9 Comparison of the Application Spectra of the RPT, the BET, and the Human WB Assay (PyroCheck®)[1]

Test		Applications		
		PyroCheck®	BET	RPT
Pyrogens	Gram-negative	+	+	+
	Gram-positive	+	-	+
	Fungi	+	-	+
Product pyrogenicity	Biologicals	+	-	+
	Pharmaceuticals	+	+	+
	Medical devices	+	+[2]	-
	Air quality	+[2]	+[2]	-
	Blood products	+	-	-

Abbreviations: BET = Bacterial endotoxin test; RPT = Rabbit pyrogen test; WB = Whole blood
[1]From Fennrich et al. (1999)
[2]Based on preliminary data

9.2.3 Hansen and Christensen (1990)

This study compared the results from PBMC exposed to endotoxin or ultraviolet light-killed *S. aureus* as an index of pyrogenicity, and then compared these results to the BET and the RPT. The authors used human PBMC obtained from heparinized peripheral blood and measured IL-1-like material in culture supernatants by evaluating co-mitogenic activity on PHA-stimulated murine thymocytes (measured in units of IL-1β where 1 unit is defined as the concentration that gives 50% of the maximal incorporation of ^{3}H-thymidine in the thymocyte assay). The endpoint is referred to as an IL-1-like material because other cytokines such as IL-2, IL-6, and TNF-α may also stimulate the proliferative response of the thymocytes. When exposed to endotoxin, PBMC secreted cytokines in a concentration-dependent manner that provided a limit of detection of 200 pg/mL of endotoxin. In comparison, the BET can normally detect 10 to 100 pg/mL of endotoxin, while the RPT can detect 500 pg/mL. Therefore, the PBMC procedure had a level of detection of endotoxin 2.5-fold lower than that of the RPT and 2-fold higher than the BET. The PBMCs also responded with greater sensitivity to the Gram-positive pyrogen *S. aureus* (10^5 cells/mL), which was not detected in the BET (10^9 cells/mL). Based on these results, the authors proposed that the PBMC test be used as an alternative *in vitro* test to the BET and RPT.

9.2.4 Hartung and Wendel (1996)

The authors stimulated human WB with various inflammatory agents to release endogenous cytokines (i.e., IL-1β, TNF-α) and inflammatory mediators (i.e., prostaglandin E_2) as an *in vitro* method for the detection of pyrogenic materials. Cytokines were released in a concentration-dependent manner following exposure to endotoxin or LTA. Heat-killed Gram-positive bacteria (*S. aureus*) or components of these organisms (i.e., muropeptides, LTA, enterotoxins, streptolysin O) and plant mitogens such as phorbol myristate acetate and PHA also produced a cytokine response. Higher concentrations (three orders of magnitude) of the Gram-positive pyrogens were needed to elicit a response as compared to Gram-negative pyrogenic material.

Studies to determine the variability among the responses of different donor WB samples were also performed. Only two of the 18 donor samples released IL-1β in response to 1 pg/mL of endotoxin, but all responded to 10 pg/mL endotoxin. The release of IL-1β from the WB samples of 45 individual donors exposed to 100 ng/mL of endotoxin was also consistent. Based on these results, the authors suggested using the WB/IL-1β test method as an *in vitro* alternative to the RPT.

9.2.5 Moesby et al. (1999)

Moesby and colleagues compared pyrogen testing using MM6 cells, isolated PBMC, and the BET. LPS and ultraviolet (UV) light-killed Gram-negative *Staphylococcus typhimurium* or Gram-positive *S. aureus* produced concentration-dependent increases in IL-6 production in MM6 or PBMC cultures. PBMC, but not MM6 cells, were able to differentiate UV-irradiated yeast (*C. albicans*) and mold (*Aspergillus niger*) pyrogens, as evidenced by statistically significant increases in IL-6 production. The BET can detect Gram-negative endotoxin, but not Gram-positive endotoxin or LTA (the pyrogenic component of Gram-positive bacteria),

and it may weakly detect yeast or viral pyrogens that the MM6 assay could not detect. Therefore, the authors suggest that pyrogen testing using MM6 cells would be a useful supplement to the BET for the detection of both Gram-negative and Gram-positive bacteria.

9.2.6 Nakagawa et al. (2002)

Nakagawa and colleagues describe an *in vitro* pyrogen test system based on proinflammatory cytokine release from a sub-clone of MM6 cells (i.e., MM6-CA8) and compare this response to a human WB culture system and the RPT. Similar to MM6 cells, MM6-CA8 were developed for superior reactivity to both endotoxin and PG. The MM6-CA8 cells release IL-6, TNF-α, and IL-1, but in greater quantities than MM6 cells in the range of 1 to 1000 pg/mL of endotoxin (up to 4-fold greater) or to 1 to 1000 ng/mL PG (up to 10-fold greater) compared to MM6 cells. The range of responses of human WB to the various pyrogens was similar to that of the MM6-CA8 cells. The relative potencies of the various pyrogens in the RPT were similar to those of the cytokine-induction potencies in the WB and MM6-CA8 methods, except for polyinosinic:polycytidylic acid, which was reported to be 10,000-fold more potent as a pyrogen injected in rabbits when compared to humans. The authors conclude that these results suggest MM6-CA8 cells can detect a variety of pyrogens using IL-6 as the marker, and that these responses are highly relevant to the prediction of human fever reactions.

9.2.7 Pool et al. (1999)

This article describes a method to differentiate between endotoxin and non-endotoxin pyrogens when testing HSA solutions in a WB culture assay. Detection limits for four Gram-positive (*Bacillus stearothermophilus*, *B. subtilis*, *Micrococcus luteus*, and *S. aureus*) and four Gram-negative bacteria (*E. coli*, *Kleibsiella pneumoniae*, *Proteus vulgaris*, and *Pseudomonas aeruginosa*) were expressed as the number of whole bacteria required to produce a pyrogenic response equal to that of 1.25 EU/mL endotoxin. *B. stearothermophilus* and *E. coli* produced concentration-dependent increases in IL-6 production. The cationic antibiotic Polymyxin B, which inhibits the binding of endotoxin to the CD14 receptor, produced concentration-dependent inhibition of IL-6 release following exposure to 10 EU/mL endotoxin in the WB assay at concentrations up to 1 EU/mL and completely inhibited IL-6 release at concentrations above 2 EU/mL. In contrast, Polymyxin B had no effect on IL-6 release following exposure to *B. subtilis*. These data suggest that Polymyxin B may be useful for differentiating endotoxin and non-endotoxin pyrogenic contaminants. The data also suggests that binding of endotoxin to Polymyxin B (e.g., by linkage to an affinity column) may be used in the depyrogenation process.

9.2.8 Poole et al. (2003)

This paper describes a rapid single-plate *in vitro* test for the presence of pyrogenic substances based on monocyte activation. The assay uses polyclonal antibodies to IL-6 or TNF-α cytokines, coated and stabilized onto 96-well plates. Monocytoid cells (e.g., PBMC, MM6 or THP-1 cells), endotoxin standard (LPS), test sample, and a second biotinylated antibody specific for the cytokine (e.g., either IL-6 or TNF-α) are incubated for 2 to 4 hr in the antibody-coated wells. An ELISA for one of the cytokines is then performed on the washed plate. IL-6 is preferred and provides a limit of detection of 0.015 EU/mL with PBMC, 0.05

EU/mL in MM6 cells, and 0.03 EU/mL with diluted WB. The amount of TNF-α released in WB in response to endotoxin was approximately 50 to 70% lower than IL-6, but was released earlier (i.e., 2 vs. 4 hr). The amount of IL-6 released on exposure to endotoxin tended to be greater in this single plate test when compared to the traditional two-plate test (i.e., in which the supernatant from one plate is transferred to a second plate for the ELISA) using PBMCs, MM6 cells, THP-1 cells, or WB. The authors report that this single plate assay using IL-6 release as the endpoint can be completed in 5 hr, and that this time could be reduced to 3 hr using TNF-α as the endpoint (because it is released earlier from the cells). The authors also suggest that this single plate test method is readily adaptable to high-throughput assays.

9.2.9 Schindler et al. (2004)

The authors optimized conditions for use of cryopreserved human WB in pyrogen testing to obviate the need for fresh WB. The release of IL-1β from fresh and Cryo WB collected from five donors was used as the measure of endotoxin presence. Challenge with 0.5 or 1.0 EU/mL endotoxin resulted in IL-1β release in bloods from all donors, although kinetic studies suggested that IL-1β release was delayed one hr in the cryopreserved samples. Cryopreservation did not appear to alter the spectrum of detectable pyrogens or immune stimuli when results were compared to that of fresh WB, and no cytokine release was measured in materials that fresh WB did not respond to. Seven clinical-grade (i.e., endotoxin-free) parenteral products spiked with 0.5 EU/mL of endotoxin revealed that there was less interference in Cryo WB than in fresh WB based on lower minimal interference dilutions that were always at or below the MVD for each product. The data showed that a broad variety of drugs could be tested for pyrogenic contaminants using Cryo WB while maintaining the ELC established in the various Pharmacopeias.