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An investigation of the possibility to replace the rabbit pyrogen test by an *in vitro* test

A.M.Gommer, L.A.M. Donders

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National Institute of Public Health and the Environment, P.O. Box 1, 3720 BA Bilthoven, The Netherlands
Telephone: 030-2749111, Telex: 47215 rivm nl, Telefax: + 31 30 2742971

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Abstract

The classical test for the detection of pyrogenic contaminations in pharmaceuticals is the rabbit pyrogen test. The most frequently occuring and most important pyrogenic contamination in biological and biotechnological pharmaceutical preparations are bacterial endotoxins, originating from the cellular wall of Gram-negative bacteria. There is an alternative in vitro test for the detection of these bacterial endotoxins available, the so-called LAL-test. However, due to some drawbacks, e.g. interfering factors in some pharmaceuticals, this LAL-test cannot always be applied as a suitable alternative for the rabbit pyrogen test. At the Department of Biologicals of the Laboratory for Medicines and Medical Devices experiments were performed to determine the suitability of a so-called "cytokine release test" as an alternative for the rabbit pyrogen test. Cells of the monocyte cell-line MonoMac-6 were incubated in the presence of several vaccines and blood products, and the cytokine (IL-6 and TNFα) release promoting activity of these vaccines and blood products was determined. The results of the cytokine release test were then compared with the results of both the LAL-test and the rabbit pyrogen test. Regarding some bacterial vaccines both the cytokine release test and the LAL-test are considered to be suitable alternatives for the rabbit pyrogen test. With respect to blood products it was concluded that one should be very cautious in replacing the rabbit pyrogen test by the cytokine release test. This conclusion is based on both the results of tests performed on blood products spiked with endotoxin and the results of tests performed on batches that have been demonstrated to be positive in the rabbit pyrogen test. It is shown that the cytokine release test can be used to detect pyrogenic activity caused by pyrogenic substances other than endotoxins, such as cytokines themselves.

Samenvatting

Parenteraal toe te dienen geneesmiddelen, d.w.z. geneesmiddelen die d.m.v. injectie, infusie of implantatie aan het menselijk lichaam worden toegediend, dienen steriel en vrij van pyrogene verontreinigingen te zijn. Pyrogene verontreinigingen zijn verontreinigingen, zoals virussen, schimmels, steroïden, polynucleotiden en bepaalde synthetische toevoegingen aan geneesmiddelen, die leiden tot een koortsreactie. De meest voorkomende pyrogene verontreinigingen in biologische en biotechnologische geneesmiddelen zijn bacteriële toxinen. Met name endotoxinen afkomstig van Gram-negatieve bacteriën zijn bekend om hun pyrogene eigenschappen.
Voor veel parenteraal toe te dienen geneesmiddelen, met name die welke in een volume groter dan 15 ml moeten worden toegediend, dient partijgewijs te worden aangetoond dat zij niet pyrogeen zijn (Europese Farmacopee 3e editie, monografie 0520). De klassieke test voor de detectie van pyrogene activititeit is gebaseerd op vergelijking van de rectale temperatuur van konijnen, voor en na toediening van een te testen substantie.
Circa twintig jaar geleden is een in vitro test op bacterieel endotoxine ontwikkeld, die voor veel geneesmiddelen kan dienen als alternatief voor de pyrogenentest: de zogenaamde LAL-test (Limulus Amoebocyten Lysaat test).
De LAL-test kent echter een aantal beperkingen, waardoor het niet voor alle parenterale geneesmiddelen geschikt is als alternatief voor de pyrogenentest.

Op de afdeling Biotechnologische Geneesmiddelen (BTG) van het Laboratorium voor Geneesmiddelen en Medische Hulpmiddelen (LGM) is onderzoek verricht naar de mogelijkheid een alternatieve *in vitro* pyrogenentest te ontwikkelen, die is gebaseerd op de *in vitro* productie van cytokines door een humane monocyten cellijn (MonoMac-6). De resultaten van dit onderzoek zijn in dit rapport weergegeven.

De resultaten van het onderzoek wijzen er op dat voor een aantal bacteriële vaccins zowel de test op bacterieel endotoxine als de bepaling van cytokine-afgifte door humane monocyten een goed alternatief kunnen vormen voor de pyrogenentest in konijnen.
Voor een aantal bloedproducten wijken de resultaten van de pyrogenentest, de test op bacterieel endotoxine en de test gebaseerd op cytokine-afgifte door humane monocyten dusdanig van elkaar af dat een vervanging van de klassieke pyrogenentest door één van de mogelijke alternatieve testen niet wordt aanbevolen.
Tenslotte wijst het onderzoek uit dat de test gebaseerd op cytokine-afgifte door monocyten gebruikt kan worden voor het detecteren van pyrogene activiteit veroorzaakt door pyrogene substanties anders dan endotoxine. Een voorbeeld voor dergelijke pyrogene substanties zijn cytokines zelf.

Summary

For many pharmaceuticals that are administered parenterally it is required that absence of pyrogenic contaminations has to be shown. Pyrogenic contaminations may induce inflammatory reactions, resulting in fever and vascular effects.

The classical pyrogen test is based on the comparison of the rectal temperature of rabbits, before and after the administration of the test substance.
About twenty years ago an in-vitro alternative for the rabbit pyrogen test was developed, that is called the "bacterial endotoxin test" or "LAL-test". The LAL-test has some drawbacks, making it unsuitable as an alternative for the rabbit pyrogen test for a number of pharmaceutical preparations.
In this report experiments are described that were performed in order to determine the suitability of a so-called "cytokine release test" to completely replace the rabbit pyrogen test. Human monocytes were incubated in the presence of a pharmaceutical substance to be tested and the amount of either IL-6 or TNFα released was used to estimate the pyrogenic activity of the substance. In order to be able to express the results of the cytokine release test in endotoxin units (EUs), an endotoxin preparation was used as reference in the cytokine release test.

Both a number of vaccines and blood products were tested by means of the cytokine release test, the LAL-test and the rabbit pyrogen test. The results of the cytokine release test are compared with the results of the LAL-test and the rabbit pyrogen test. The results of the experiments show that the value of the cytokine release test as an alternative to the rabbit pyrogen test depends on the type of product tested. In determining the suitability of the cytokine as a pyrogen test it should be kept in mind that as far as vaccines are concerned the pyrogen test is merely used as a consistency test and not as a safety test. In contrast, for most blood products the pyrogen test is generally used as a safety test. This has to do with the fact that vaccines are generally administered intramusculary and the blood products concerned are administered intravenously.

For Typhoid polysaccharide vaccine the LAL-test is considered to be a suitable alternative to the rabbit pyrogen test.
As is the case for Typhoid polysaccharide vaccine, for Meningococcal polysaccharide vaccine the LAL-test is shown to be a possible alternative for the rabbit pyrogen test. Pneumococcal polysaccharide vaccine, on the other hand, contains polysaccharides originating from Gram-positive bacteria. Since the LAL-test can only detect endotoxins originating from Gram-negative bacteria, for this vaccine the cytokine release test is considered to be a more suitable alternative to the rabbit pyrogen test than the LAL-test.

For Influenza vaccine it is shown that the use of different lysates in the LAL-test may cause discrepancies in the results of this test. This observation calls for a better understanding of what is really causing the LAL-reaction and if the LAL-test result reflects the pyrogenic activity of the substance tested.

As mentioned above, for blood products the rabbit pyrogen test is considered to be a safety test rather than a consistency test. In this report it is shown for human albumin solutions, varicella immunoglobulin, antithrombin III concentrate, normal immunoglobulin and tetanus immunoglobulin that one should be very cautious in replacing the rabbit pyrogen test by the cytokine release test. This conclusion is based on both the negative results of cytokine release tests performed on blood products spiked with endotoxin and on batches that have been demonstrated to be positive in the rabbit pyrogen test.
Furthermore, for most of these products the LAL-test is not considered to be a suitable alternative for the rabbit pyrogen test, as these products contain factors such as blood proteins that may interfere with the LAL-test.
Therefore it can be concluded that for most blood products no adequate alternative for the rabbit pyrogen test is available.

Experiments with an Interleukin-2 preparation have shown that the cytokine release test can well be used to detect pyrogenic activity caused by pyrogenic substances other than endotoxin, such as cytokines themselves. It is foreseen that, as the number of cytokine products grow, a test on pyrogens will become more important and more often used. For this kind of products the cytokine release test may proof to be a good alternative for the rabbit pyrogen test.

1. Introduction

1.1. Pyrogen testing in pharmaceuticals

Parenterals, i.e. sterile pharmaceutical preparations that are intended for administration by injection, infusion or implantation into the human body must be sterile and free of pyrogenic contaminations. Pyrogenic contaminations may induce inflammatory reactions, resulting in fever and vascular effects. Pyrogens include bacterial, viral and fungal components, steroids, polynucleotides and certain synthetic additives in pharmaceuticals. The most frequently occuring and most important pyrogenic contaminations in biological and biotechnological pharmaceutical preparations are bacterial toxins.
In particular endotoxins originating from Gram-negative bacteria are known for their pyrogenic activity. Bacterial endotoxins are components of the cellular wall of Gram-negative bacteria and resistant to steam sterilisation. As the bacteria of which they are originating, endotoxins are present in air, water and food. Because of the omnipresence, the relative heat resistance and the possible physiological effect, the detection and avoidance of endotoxin contamination is of great importance to the manufacturers of parenterals. For many parenterals, particularly those dosed in a volume greater than 15 ml, absence of pyrogens has to be shown for each batch (2).
The classical pyrogen test is a biological assay in animals. It is based on the comparison of the rectal temperature of rabbits, before and after the administration of the test substance. Rabbits can only be used once for testing substances containing non rabbit proteins. Therefore many rabbits are used for the pyrogen test on (human) protein containing preparations, in particular blood products.

About twenty years ago an in vitro alternative for the rabbit pyrogen test, the so-called "bacterial endotoxin test" or "LAL-test" was developed. LAL is an abbreviation of Limulus Amoebocyte Lysate, an extract of the blood cells (amoebocytes) of the horseshoe crab Limulus Polyphemus. The interaction between endotoxin and a clotting enzyme present in the amoebocytes causes a clotting of the lysate, which can be used as indicator for the presence of endotoxin. The LAL-test has a number of drawbacks, preventing it from use as an alternative for the pyrogen test for <u>all</u> pharmaceutical substances:

1) Different from the rabbit pyrogen test, the principle of the LAL-test is not based on the detection of pyrogens, but on the detection of endotoxins (6). Other pyrogenic substances are not detected. The LAL-test has a limited physiological relevancy, i.e. the test is not based on mechanisms that play a role in the initiation of inflammation in humans.
2) Some pharmaceuticals interfere with the LAL-response and cannot be tested in a LAL-assay.

3) From an animal wellfare point of view the LAL-test is not an ideal alternative to the rabbit pyrogen test, as for the collection of the lysate crabs have to be captured and bled.

1.2. An alternative pyrogen test

Generally, inflammatory responses in humans are initially mediated by the presence of circulating endogenous mediators, that are produced by activated blood monocytes in response to exogenous factors. Endogenous mediators that are pyrogenic (i.e. "endogenous pyrogens") comprise a variety of cytokines, such as Interleukin-1 (IL-1), Interleukin-6 (IL-6) and Tumor Necrosis Factor (TNFα). Taktak et al. (1991) have demonstrated that the amount of IL-6 produced by MonoMac-6 cells is related to the amount of endotoxin present in the culture medium of the cells (8). At the Department of Biologicals of the Laboratory for Medicines and Medical Devices a pyrogen test is developed which is based on the in vitro production of cytokines, in particular Interleukin-6 (IL-6) and Tumor Necrosis Factor α (TNFα), produced by cultured human monocytes. As primary blood cells, originating from several donors, may differ in their sensitivity to pyrogens (4,5,7), a cell-line is preferred to obtain a well standardized test. The monocyte cell-line used is the MonoMac-6 cell-line, developed by Ziegler-Heitbrock at the University of München (9, 10).

The objective of the experiments described in this report is to determine the suitability of the so-called "cytokine-release test" to replace the rabbit pyrogen test. In short, monocytes were incubated in the presence of different concentrations of endotoxin and the quantitative relationship between cytokine release and the concentration of endotoxin was determined. In addition, the cytokine release promoting activity of several pharmaceuticals that are routinely tested by the rabbit pyrogen test was examined. The results of the cytokine release test are compared with the results of the LAL-test and the rabbit pyrogen test. In most experiments described in this report the IL-6 release was determined and in these cases the cytokine release test is referred to as the IL-6 release test. In some experiments decribed, the release of TNFα was determined. In these cases the cytokine release test is referred to as the TNFα release test.

2. Materials and Methods

2.1. Samples

Reference preparation for endotoxin:

* Biological Reference Preparation for endotoxin, BRP lotno. 2; 800 IU/ml; obtained from the European Pharmacopoeia (Council of Europe)

Vaccines:

* Typhoid polysaccharide vaccine
* Pneumococcal polysaccharide vaccine
* Haemophilus type B conjugate vaccine
* Meningococcal polysaccharide vaccine
* Influenza vaccine (surface antigen, inactivated)

Blood products:

* Human albumin solution
* Human varicella immunoglobulin
* Human antithrombin III concentrate
* Human normal immunoglobulin for intravenous administration
* Human tetanus immunoglobulin

Others:

* Interleukin-2

2.2. Cytokine release test

In the cytokine release test monocytes are incubated overnight in the presence of different concentrations of endotoxin and/or the samples to be tested. After the incubation period the supernatants of the cell cultures are harvested. The amount of IL-6 and/or TNFα present in the supernatants are determined by ELISA.

The monocytes used in the test are cultured in Iscove's Modified Dulbecco's Medium (IMDM) with Glutamax-1, to which 100 units Penicillin, 100 µg Streptomycin and 10 % Fetal Bovine Serum is added.

The materials used in the cytokine-release test are:

* MonoMac 6 cells kindly provided by dr.Ziegler-Heitbrock (University of München)
* Iscove's Modified Dulbecco's Medium (IMDM) with Glutamax-1; Life Technologies; order no. 31980-022

* Myclone Super Plus Fetal Bovine Serum (mFBS); Life Technologies; order no. 10081-065
* Penicillin/Streptomycin, 10.000 units/ml Penicillin and 10.000 µg/ml Streptomycin; Life Technologies; order no. 5145-014
* Pyrogen-free water for injections; NPBI; order no. D1217
* 96-wells cell-culture microtiterplates (flat-bottom); Costar; order no. 3596

2.2.1. Preparation of the samples to be tested

If necessary, samples are diluted. In order to adapt the samples to the incubation conditions 20 µl of the samples are added to the wells of a microtiterplate and incubated for 30 minutes at 37 °C, 5 % CO_2 and H_2O saturated. After incubation 130 µl of complete medium (IMDM + 2 % mFBS + 100 units/ml penicillin/streptomycin) is added to each well.

2.2.2. Preparation of the cell suspension

The cell culture is centrifuged for 8 minutes at 400 g. After removal of the supernatant the cells are resuspended in a small volume (ca. 2 ml) of complete medium (+2 % mFBS + penicillin/streptomycin) and counted using a Coulter Counter. The cell suspension is than diluted with completed medium to obtain a cell concentration of 200.000 cells per 50 µl. Finally 50 µl cell suspension is added to each well of the microtiterplate mentioned in 2.2.1. The final cell concentration in the wells is now $1x10^6$ cells per ml (total volume in the wells is 200 µl).

2.2.3. Incubation

If the TNFα release is determined, the time of incubation of the monocytes with the samples to be tested is 4 hours at 37 °C, 5 % and H_2O saturated. If the IL-6 release is determined, the incubation time is 24 hours at the conditions mentioned above.

2.2.4. Collection of the supernatants

After the incubation period and before the supernatants are harvested, the morphology of the cells is determined visually by microscope. Stimulated cells tend to coagulate, while non-stimulated cells have a round shape and do not coagulate. Then the

supernatants are transferred to another microtiterplate and stored at -20 °C, until assay of IL-6 or TNFα by ELISA.

2.3. IL-6 ELISA

The IL-6 levels in the supernatants of the cell culture are determined by using a sandwich ELISA (c.f. figure 1).

Materials:

* Human Interleukin-6 ELISA kit; Central Laboratory of the Netherlands Red Cross Blood Transfusion Service; order no. M1916
* Carbonate buffer, pH 9.6;
* Phosphate Buffered Saline (PBS), pH 7.2;
* 3,5,3',5'-tetramethylbenzidine (TMB)/ethanol solution (60 mg TMB in 10 ml ethanol);
* Washing buffer (PBS + 0.005 % Tween 20);
* Sodium acetate, pH 5.5;
* destilled water;
* H_2O_2 30 %;
* Sulphuric Acid 2M;

Method:

A flat bottom microtiterplate is coated with anti human IL-6 diluted in carbonate buffer and is incubated overnight at room temperature. Then 100 µl of the IL-6 reference or of the samples (supernatants) to be tested are added in various dilutions. After 1 hour incubation at room temperature 100 µl anti human IL-6 biotin-conjugate is added and one hour later 100 µl streptavidin-HRP-conjugate. Following 30 minutes of incubation 100 µl of substrate solution is added. Approximately 15 minutes after the addition of the chromogenic substrate the reaction is stopped by the addition of 100 µl of 2M sulphuric acid and absorbance is read at 450 nm.

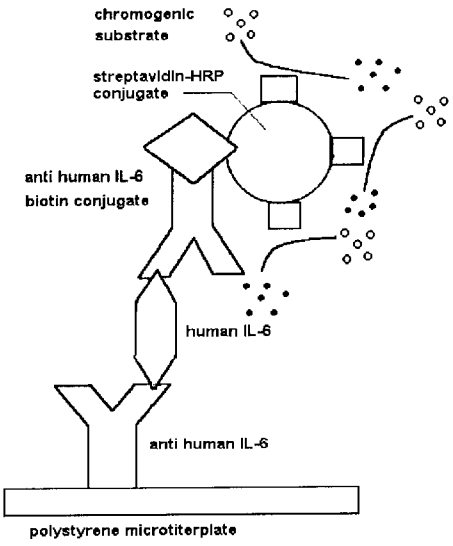

Figure 1: schematic presentation of the IL-6 ELISA

2.4. TNFα ELISA

The TNFα levels in the supernatants of the cell culture are determined by using a sandwich ELISA (c.f. figure 1).

Materials:

* Human TNFα ELISA kit; Central Laboratory of the Netherlands Red Cross Blood Transfusion Service; order no. M1923
* Carbonate buffer, pH 9.6;
* Phosphate Buffered Saline (PBS), pH 7.2;
* 3,5,3',5'-tetramethylbenzidine (TMB)/ethanol solution (60 mg TMB in 10 ml ethanol);
* Washing buffer (PBS + 0.005 % Tween 20);
* Na-acetate, pH 5.5;
* destilled water;
* H_2O_2 30 %;
* Sulphuric Acid 2M;

Method:

A flat bottom microtiterplate is coated with anti human TNFα diluted in carbonate buffer and is incubated overnight at room temperature. Then 100 µl of the TNFα reference and of the samples (supernatants) to be tested are added in various dilutions. After 1 hour incubation at room temperature 100 µl anti human TNFα biotin-conjugate is added an one hour later 100µl streptavidin-HRP-conjugate. Following 30

minutes incubation 100 µl of a chromogenic substrate is added. Approximately 15 minutes after the addition of the chromogenic substrate the reaction is stopped by the addition of 100 µl of 2M sulphuric acid and absorbance is read at 450 nm.

2.5. LAL-test

The endotoxin concentration of test samples is determined using a semi-quantitative LAL gel-clot test (European Pharmacopoeia , 3rd edition, Supplement 1998: monograph 2.6.14.)

Material:

* Limulus Amoebocyte Lysate (FDA-licensed; sensitivity 0.125 EU/ml); Sanbio (Endosafe), order no. AE015; Bioproducts (pyrogent), order no. N184
* Biological Reference Preparation for endotoxin, BRP lot no. 2; 800 IU/ml; obtained from the European Pharmacopoeia (Council of Europe)
* Water for injection (pyrogen-free); NPBI; order no. D1217
* Chlorophenol red (pH 4.8-6.4) 0.1 %; Merck; order no. 3024
* Bromthymolblue (pH 5.8-7.6) 0.25 %; Merck; order no. 103026
* Microtiterplate (flat bottom); Titertek; order no. 77-172-05

Method:
Prior to incubation at room temperature samples were vortexed. The freeze-dried lysate is reconstituted with 5.2 ml water. The endotoxin reference preparation is diluted with water to 1 IU/ml (= 1 EU/ml) and added to the wells of a microtiterplate in duplicate. Each sample is added to the wells in triplicate. Subsequently the contents of the wells are diluted further in the plate by twofold dilution steps. One replicate of each sample dilution is spiked with the reference for endotoxin at a concentration of 2λ (λ is the sensitivity of the lysate = 0.125 EU/ml). After dilution of the reference preparation and the samples and after spiking of the samples, lysate is added to each well in a 1 : 1 volume-ratio and the plate is covered and incubated for 1 hour at 37 °C. After incubation a colour solution (pH-indicator: Chlorophenol red or bromthymol blue) is added to each well.
Based on the spreading of the colour solution in the wells, a positive or negative result (clotting and no clotting respectively) is obtained. The endotoxin-concentration of the samples is calculated by multiplying the highest dilution showing a positive result by the sensitivity of the lysate. If the spiked sample dilution shows a positive result (clot) the LAL-reaction is not inhibited and the result is valid.

2.6. Rabbit pyrogen test

Pyrogenicity of test samples is determined using the rabbit pyrogen test (European Pharmacopoeia, 3 rd edition, 1997: monograph 2.6.8.). The requirement "pyrogenfree" is specified as a maximum allowed rise in body temperature in response to intravenous administration of a product-specific dose per kg bodyweight.

Materials:

* computerized temperature measurement system (developed and produced at RIVM)
* pyrogen free saline; NPBI; order no. H1001

Experimental animals:

* Rabbits, Elco (specific pathogen free), 1.5-3.5 kg; Harlan-Cpb

Method:

During a period of 90 minutes the bodytemperature of three rabbits per sample is monitored. During this so-called calibration period the temperature of the rabbits is determined three times at intervals of 30 minutes.The initial temperature of each rabbit is determined by taking the mean of two temperature readings, recorded for that rabbit at an interval of 30 minutes in the 40 minutes immediately preceding the injection of the sample to be examined. After injection of the sample to be examined the recording of the temperatures is continued for another 3 hours. The maximum temperature of each rabbit is the highest temperature recorded for that rabbit in the 3 hours after the injection. The difference between the maximum temperature and the initial temperature of each rabbit is taken as response.

3. Results

Both the LAL-test and the cytokine release test are relative assays, i.e. the reponse to the sample to be tested is compared to the response to a known amount of endotoxin. In contrast, the rabbit pyrogen test is an absolute assay in which no reference preparation is used. In this chapter the results of the three types of tests are compared and the extend to which they correlate is determined. In order to make it possible to compare the results of the three types of test, these results are all expressed in endotoxin units (EU's). These are units that are normally used in the LAL-test. In the cytokine release test different dilutions of the endotoxin standard preparation are used to obtain an endotoxin dose/IL-6 response curve. By doing so, the results of the test can be expressed in EU's, as will be explained further in the next paragraphs. Though the rabbit pyrogen test is an absolute assay it is possible to express the results of this test in EU's too, because it is known that the sensitivity of the rabbits to endotoxin is approximately 1 ng endotoxin (corresponding to 10 EU) per kg bodyweight (1). This will also be explained further in the next paragraphs when discussing the results of the tests conducted.

3.1. Vaccines

3.1.1. IL-6 release versus LAL-test results

Table 1 and 2 show the results of the IL-6 release test, performed on Typhoid polysaccharide vaccine, Pneumococcal polysaccharide vaccine, Haemophilus type B conjugate vaccine and Meningococcal polysaccharide vaccine. Both the incubation of the monocytes with the test samples and the ELISA on the supernatants are performed in duplicate. In each test a blanc was included, consisting of pyrogen free water for injection or culture medium. In none of the tests the blanc resulted in a detectable IL-6 reponse.

Table 1: Results of the IL-6 release test on Typhoid polysaccharide vaccine

IL-6 release test							
			IL-6-concentration (pg/ml)				
Sample	Dilution used in the test	Endotoxin concentration used in test	exp. 1	exp. 2	mean	S.D.	V.C.
BRP, lot 2 (800 IU/ml)	10	80	1611	1107	1359	356.4	26.2
BRP, lot 2 (800 IU/ml)	100	8	1544	651	1097.5	631.4	57.5
BRP, lot 2 (800 IU/ml)	1000	0.8	406	213	309.5	136.5	44.1
BRP, lot 2 (800 IU/ml)	10000	0.08	0	20	10	14.1	141.4
Typhoid vaccine	31		1177	1357	1267	127.3	10.0
Typhoid vaccine	310		1108	621	864.5	344.4	39.8
Typhoid vaccine	3100		256	150	203	75.0	36.9
Typhoid vaccine	31000		n.d.	0	0	-	-

Table 2: Results of the IL-6 release test on Pneumococcal polysaccharide vaccine, Haemophilus type B conjugate vaccine and Meningococcal polysaccharide vaccine

IL-6 release test							
			IL-6 concentration (pg/ml)				
Sample	Dilution used in the test	Endotoxin concentration used in test	exp. 1	exp. 2	mean	S.D.	V.C.
BRP, lot 2 (800 IU/ml)	10	80	1242	1693	1468	319	22
BRP, lot 2 (800 IU/ml)	100	8	729	1027	878	211	24
BRP, lot 2 (800 IU/ml)	1000	0.8	115	168	142	38	27
BRP, lot 2 (800 IU/ml)	10000	0.08	0	0	0	0	-
Pneumococcal vaccine	10		193	486	340	207	61
Pneumococcal vaccine	50		39	62	51	16	32
Haem.b.conj. vaccine	10		232	786	509	392	77
Haem.b.conj. vaccine	20		10305	21418	15862	7858	50
Meningococcal vaccine	50		383	654	519	192	37
Meningococcal vaccine	500		34	69	52	25	48
Meningococcal vaccine	5000		0	0	0	0	*

The formula of the best fitting dose-reponse curve can be defined by: 'y = a0 +a1*exp(-x/a2)', where y is the IL-6 concentration and x is the endotoxin concentration and where a_0, a_1 and a_2 vary from one test to another. Figure 2 shows a typical dose-response curve.

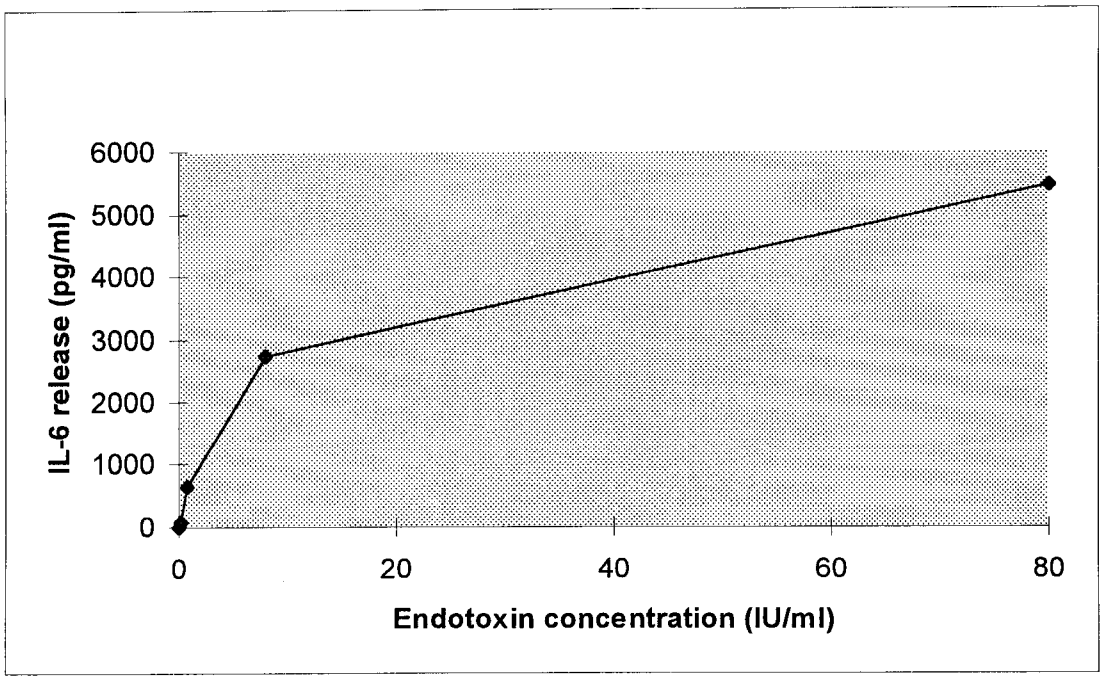

Figure 2: Typical dose response curve of the IL-6 release by MonoMac-6

Table 3 and 4 show the results of the IL-6 release test and the LAL-test expressed in endotoxin units (EU's) per ml.

Table 3: Results of the IL-6 release test on Typhoid polysaccharide vaccine, expressed in endotoxin units per ml (EU/ml)

IL-6 release test								
Sample	EU/ml LAL-test	Dilution in the IL-6 release test	experiment 1			experiment 2		
			IL-6 conc. pg/ml	EU/ml in diluted sample	EU/ml in undiluted sample	IL-6 conc. pg/ml	EU/ml in diluted sample	EU/ml in undiluted sample
Typhoid vaccine	2500	31	1177	3.1	96	1357	> 80 *	-
Typhoid vaccine	**2500**	310	1108	2.7	**837**	621	7.4	**2294**
Typhoid vaccine	2500	3100	256	0.1	310	150	0.5	1550

* outside dose-response curve

Tabel 4: Results of the IL-6 release test on Pneumococcal polysaccharide vaccine, Haemophilus type B conjugate vaccine and Meningococcal polysaccharide vaccine, expressed in endotoxin units per ml (EU/ml)

IL-6 release test								
Sample	EU/ml LAL-test	Dilution in the IL-6 release test	experiment 1			experiment 2		
			IL-6 conc. pg/ml	EU/ml in diluted sample	EU/ml in undiluted sample	IL-6 sample pg/ml	EU/ml in diluted sample	EU/ml in undiluted sample
Pneum.vaccine	**25**	10	193	1.2	**12**	486	2.6	**26**
Pneum.vaccine	25	50	39	0	0	62	0	0
Haem.type b.conj.	**12.5**	10	232	1.5	**15**	786	5.2	**52**
Haem.type b.conj.	12.5	20	10305	>> 80*	-	21418	>> 80*	-
Mening.vaccine	4000	50	383	3.1	155	654	4	200
Mening.vaccin	4000	500	34	0	0	69	0	0

*outside dose-response curve

From these data it can be deduced that the results from the LAL-test and the IL-6 release test correlate well for the products Typhoid and Pneumococcal polysaccharide vaccine and for the Haemophilus type B conjugate vaccine. However, the extend to which the results of both tests overlap depends on the dilutions of the samples used in the cytokine release test. The dilutions giving the best match are 1:310 for Typhoid vaccine and 1:10 for Pneumococcal and Haemophilus type B conjugate vaccine (results printed in bold) respectively. For the other product dilutions (resp. 1:31 and 1:3100 for Typhoid polysaccharide vaccine, 1:50 for Pneumococcal polysaccharide vaccine and 1:20 for Haemophilus type B conjugate vaccine) and for a batch of Meningococcal polysaccharide vaccine there is no clear correlation between the results obtained with the IL-6 release test and the LAL-test. Remarkably, incubation of the monocytes with a 1:20 dilution of the Haemophilus vaccine results in a much higher IL-6 reponse than incubation with the 1:10 dilution. This response even exceeds the response to incubation of the monocytes with the highest endotoxin concentration. To exclude that this effect is caused by interference of the vaccine with the IL-6 ELISA a dilution series of IL-6 is made in dilution buffer, 1:10 and in 1:100 diluted vaccine. The dilutions of IL-6 in dilution buffer are then assayed in the IL-6 ELISA and compared

with the IL-6 dilutions made in diluted vaccine. The results of these comparisons are shown in Table 5.

Table 5: Comparison of the IL-6 assay (ELISA) in dilutionbuffer and in diluted Haemophilus type B conjugate vaccine

Results of human IL-6 (reference preparation) assay (ELISA-CLB)		
IL-6 in buffer; pg/ml	IL-6 in 1:10 diluted Haem.type B conj.vaccine; pg/ml	IL-6 in 1:100 diluted Haem.type B conj.vaccine; pg/ml
750	572	417
250	253	165
83.3	80.6	58.3
27.8	27.2	18.8
9.3	9.2	6.8
3.1	2.8	2.3
1.0	0.4	0.8
0	0	0

It appears that the estimation of IL-6 in diluted vaccine does not differ more than a factor 2 from the estimation of IL-6 in dilution buffer. On basis of these results it is concluded that the remarkable IL-6 response observed for Haemophilus type B conjugate vaccine cannot be ascribed to interference of the product with the IL-6 ELISA and therefore reflects the real IL-6 response of the cells to this vaccine.

3.1.2. IL-6 release versus pyrogen test

Table 6 shows the results of various products in the pyrogen test. Also the doses used in the pyrogen test are shown in the table. These doses are the doses used in the routine batch release pyrogen tests of the products. The same batches of the products have been tested in the IL-6 release test and the LAL-test (cf. Table 1 to 4). Administration of the products to rabbits resulted in a small rise in the body temperature of the individual rabbits, but the total temperature rise of the three rabbits did not exceed 1.2 °C, a temperature rise that is generally accepted as a significant pyrogenic response (European Pharmacopoeia, 3 rd edition, 1997: monograph 2.6.8.).

Table 6: Results of the rabbit pyrogentest on Pneumococcal polysaccharide vaccine, Haemophilus type B conjugate vaccine and Meningococcal polysaccharide vaccine

Rabbit pyrogentest					
Product	Test dilution	Volume	Dose	Individual temp.rises (°C)	Total temp.rise (°C)
Pneum.vaccine	1:40	1 ml/kg	1.25 µg/ml	0.4 - 0.4 - 0.3	1.1
				0.5 - 0.4 - 0.3	1.2
Haem.type b.conj.	1:1000	1 ml/kg	0.030 µg/ml	n.d.	n.d.
Mening.vaccine	1:40.000	1 ml/kg	0.005 µg/ml	0.5 - 0.3 - 0.2	1.0

For a batch of Meningococcal polysaccharide vaccine it was investigated whether the results of the cytokine release test correlated better to the results of the rabbit pyrogen test or to the results of the LAL-test. Meningococcal polysaccharide vaccine was chosen for this experiment mainly for logistical reasons. The batch was tested in the three types of test. The results of these tests are shown in Table 7.

Table 7: Results of different tests performed on a batch of Meningococcal polysaccharide vaccine

Rabbit pyrogen test			LAL-test	IL-6 release test		TNFα release test	
dilution	individual temperature rise	total temp. rise	EU/ml	dilution	pg/ml	dilution	pg/ml
1:25	1.0 - 0.6 - 0.2 °C	1.8 °C	500	1:10	1681	1:10	720
1:50	0.4 - 0.9 - 0.7 °C	2.0 °C		1:20	1214	1:20	448
1:100	0.2 - 0.7 - 0.3 °C	1.2 °C		1:40	689	1:40	0

Based on the results of the rabbit pyrogen test, the calculated pyrogenicity of the Meningococcal polysaccharide vaccine is approximately 1000 EU/ml, as the sensitivity of the rabbits to endotoxin is about 1 ng endotoxin (corresponding to 10 EU) per kg bodyweight. Injection of 1 ml of a 1:100 dilution of the vaccine per kg bodyweight results in a total temperature rise of 1.2 °C, which is a minimum pyrogenic response in terms of the standards of the European Pharmacopoeia. Therefore the pyrogenic activity of the undiluted vaccine corresponds to about 100 * 10 EU/ml = 1000 EU endotoxins/ml. This result correlates well with the value of the endotoxin concentration obtained using the LAL-test.

Based on the results of the IL-6 release test the pyrogenic activity of the Meningococcal polysaccharide vaccine corresponds to about 70 EU/ml. Based on the results of the TNFα release test the pyrogenic activity of this vaccine corresponds to about 80 EU/ml. Using the cytokine (both IL-6 and TNFα) release test, a response of the vaccine is obtained which is more than ten times lower than the response that was expected on the basis of the responses in the rabbit pyrogen test and the LAL-test.

3.1.3. LAL-test on Influenza vaccine

For a monovalent bulk of Influenza vaccine a discrepancy was observed in the results of the LAL-test, using lysates from two different sources (Endosafe and Pyrogent lysate). Table 8 shows the results of the LAL-test using both lysates. The endotoxin concentration determined using Endosafe lysate was 20 times higher than the endotoxin concentration using Pyrogent lysate.

Table 8: Results of the LAL-test on a monovalent bulk of Influenzavaccine

Results of LAL-test (IU/ml)				
Sample	Endosafe lysate (exp. 1)	Endosafe lysate (exp. 2)	Bioproducts lysate (exp. 1)	Bioproducts lysate (exp. 2)
monovalent Influenza-bulk	1000	1000	< 125	50

In order to find out whether the high concentrations determined with Endosafe lysate were due to the pyrogenic activity of the bulk, a dilution of the bulk was tested in the rabbit pyrogen test, as well as in the IL-6 release test.
A dilution of 1:50 in pyrogen free saline was intravenously injected in three rabbits at a dose of 1 ml per kg. This dose corresponds to 20 EU endotoxin per kg as estimated on basis of the result of the LAL-test using Endosafe lysate. In addition, the endotoxin reference preparation was diluted with pyrogen free saline, to obtain a concentration of 20 EU/ml, and injected intravenously in three rabbits at a dose of 1 ml per kg, corresponding to a dose of 20 EU endotoxin per kg. Table 9 shows the results of the rabbit pyrogen test on the diluted monovalent Influenza vaccine bulk and the endotoxin solution.

Table 9: Results of the rabbit pyrogen test on a monovalent bulk of Influenza vaccine

Rabbit pyrogen test on monovalent bulk of Influenza vaccine		
	monovalent bulk	endotoxin ref. preparation
dilution/concentration	1:50	20 EU/ml
testdose	1 ml per kg	1 ml per kg
individual temp.rises	0.0 - 0.1 - 0.3 °C	0.6 - 0.9 - 0.7 °C
total temp.rises	0.4 °C	2.2 °C

It appears that the endotoxin concentration of the monovalent bulk of Influenza vaccine, as estimated in the LAL-test using Endosafe lysate, does not correspond to the pyrogenic activity of this bulk in rabbits.

The Influenza vaccine bulk was tested in the IL-6 release test at 10 and 100 fold dilutions. No IL-6 release could be detected after 24 hours of incubation with the monocytes.

3.2. Blood products

In two different ways it was investigated whether or not pyrogens in blood products can be detected by means of the cytokine release test.

Blood products were spiked with endotoxin to ensure whether there are factors within the products that interfere with the response of the monocytes to endotoxin. In addition, various batches of blood products known to be pyrogenic in rabbits were assayed in the cytokine release test.

3.2.1. Spiking experiments

The following blood products were spiked with different concentrations of the endotoxin reference preparation:

* Human Immunoglobulin solution 5 %
* Human Immunoglobulin solution 20 %
* Human normal immunoglobulin for intravenous administration
* Human tetanus immunoglobulin

Pyrogen free water samples spiked with the same endotoxin concentrations as the products tested were used as controls. The results of these spiking tests are shown in Table 10 to13. Samples of Albumin solution 5 % and Albumin solution 20 % were spiked with different concentrations of endotoxin. It appeared that the IL-6 release in the Albumin solutions is at least as high as the IL-6 release in the controls. It can therefore be assumed that using the IL-6 release test, 100 % of the endotoxin spike in the Albumin solutions is recovered (cf. Table 10 and 11).

Table 10: Results of the IL-6 release test on spiked Albumin solution 20%

Spikingsexperiments in Albumin solution 20%				
endotoxin-concentration (IU/ml)	ÌL-6 response (pg/ml)			
	Experiment 1		Experiment 2	
	water	Albumin 20%	water	Albumin 20%
80	2674	3472	8545	12731
40	2351	4444	4299	6701
20	1844	3239	3396	7284
10	1323	1698	2777	5243
5	865	1317	1991	3804
2.5	504	1035	1556	2245
1.3	388	623	983	1594
0.6	100	225	603	751
0	0	0	0	0

Table 11: Results of the IL-6 release test on spiked Albumin solution 5%

Spiking experiments in Albumin solution 5%				
endotoxin-concentration (IU/ml)	IL-6 response (pg/ml)			
	Experiment 1		Experiment 2	
	water	Albumin 5%	water	Albumin 5%
80	6751	7178	6441	5049
40	7202	4999	3849	7110
20	4055	5191	3040	5905
10	2482	4113	2252	4156
5	1596	4116	1618	4658
2.5	1077	3857	1294	3797
1.3	595	2005	677	2640
0.6	254	646	274	1086
0	0	0	0	0

In contrast, spiking of a batch of Human immunoglobulin for intravenous adminstration with different concentrations of endotoxin resulted in an IL-6 release that was significantly lower compared to the IL-6 release in the controls (cf. Table 12). This resulted in a five times higher detection limit of endotoxin when it was dissolved in the Immunoglobulin than when it was dissolved in water (2.5 IU/ml in product and 0.6 IU/ml in water).

Table 12: Results of the IL-6 release test on spiked Human normal immunoglobulin for intravenous administration

Spiking experiments in Human normal immunoglobulin for intravenous administration		
endotoxin concentration (IU/ml)	IL-6 response (pg/ml)	
	water	Imm. intraven.
80	8761	n.d.
40	5681	4439
20	4299	2655
10	3336	1299
5	2390	457
2.5	1627	210
1.3	740	0
0.6	343	0
0.3	0	0
0.15	0	0
0	0	0

For a batch of Human tetanus immunoglobulin the detection limit for endotoxin was at least a factor 10 higher compared with detection limit of endotoxin in water (1.3 IU/ml and 0.15 IU/ml respectively). The same shift in detection limit was observed when the release of TNFα in response to endotoxin was measured (20 IU/ml and 1.3 IU/ml respectively). The detection limit for endotoxin depended on the type of cytokine determined. Using TNFα the detection limit for endotoxin was about ten times higher compared to IL-6 (cf. Table 13).

Table 13: Results of the IL-6 and TNFα release test on spiked Human tetanus immunoglobulin

Spiking experiments in Human tetanus immunoglobulin				
endotoxin-concentration (IU/ml)	IL-6 response (pg/ml)		TNFα response (pg/ml)	
	water	Antitet. imm.	water	Antitet. imm.
80	5896	-	341	-
40	4247	1490	279	175
20	3999	677	1450	57
10	3020	268	161	0
5	2172	121	285	0
2.5	1209	30	68	0
1.3	600	14	66	0
0.6	287	0	0	0
0.3	119	0	0	0
0.15	37	0	0	0
0	0	0	0	0

3.2.2. Batches that gave a positive result in the rabbit pyrogen test

Batches of different types of blood product, that had been previously demonstrated to be positive in the rabbit pyrogen test by the manufacturer, were tested in the cytokine release test. These products tested were:

* Human albumin solution
* Human varicella immunoglobulin
* Human antithrombin III concentrate

These products are produced by different manufacturers.

Rabbit pyrogen test

The batches tested have been previously rejected by the manufacturer on basis of the European Pharmacopoeia requirements with regard to the absence of pyrogenic activity (rabbit pyrogen test). The doses used in the rabbit pyrogen test and the response measured in this test are shown in Table 14.

Table 14: Results of the rabbit pyrogen test on Human albumin solution, Human varicella immunoglobulin en Human antithrombin

Results of the rabbit pyrogen test		
Product	dose	response
Albumin solution	3 ml/kg	6 rabbits: 7.1°C
Varicella immunoglobulin	1 ml/kg	6 rabbits: 6.4°C
Antithrombin	1 ml/kg	12 rabbits: 7.0°C

The response observed for the batch of Albumin solution, a total temperature rise of 7.1°C in 6 rabbits, is significant as the pass/fail limit included in the European Pharmacopoeia is a total temperature rise of 4.3°C using 6 rabbits.
The response observed for Human varicella immunoglobulin, a total temperature rise of 6.4°C in 6 rabbits, is significant as well. Finally, the response observed for Human antithrombin, a total temperature rise of 7.0°C in 12 rabbits, is lower but still significant. The pass/fail limit of Ph.Eur. is 6.6°C when 12 rabbits are included in the test.

As mentioned earlier in this report, rabbits show a pyrogenic response to 1 ng endotoxin per kg bodyweight. This corresponds to 10 EU (= endotoxin units) per kg bodyweight. Therefore, in view of the fact that the batches have shown a pyrogenic response in the rabbit pyrogen test, their pyrogenic activity at least equals 10 EU/dose/kg. The pyrogenic activity of the batch of Albumin solution equals 10 EU per 3 ml, as the dose used in the rabbit pyrogen test is 3 ml per kg bodyweight. The pyrogenic activity of the batch of Human varicella immunoglobulin and the batch of Human antithrombin equals 10 EU per ml, as the dose used in the rabbit pyrogen test for these two products is 1 ml per kg bodyweight.

LAL-test

The pyrogenic batches were also tested in the bacterial endotoxin test (LAL-test) and the results are shown in Table 15.

Table 15: Results of the LAL-test on a batch of Human albumin solution, Human varicella immunoglobulin and Human antithrombin

Results of the LAL-test on pyrogenic blood products		
Sample	Endosafe lysate (EU/ml)	Bioproducts lysate (EU/ml)
Albumin solution	40	10
Varicella immunoglobulin	40	40
Antithrombin	< 0.125	< 0.125

Both for the Albumin solution and the sample of Varicella immunoglobulin a good correlation is observed between the results of the LAL-test and the results of the rabbit pyrogen test. In view of the LAL-results, the dose of the batch of Albumin solution that was administered to the rabbits (= 3 ml/kg) equals 30 to120 EU per kg bodyweight (3 ml/kg x 10-40 EU/ml). The dose of the Varicella immunoglobulin sample equals 40 EU per kg bodyweight (1 ml/kg x 40 EU/ml).Therefore for both products a pyrogenic response in the rabbits could be expected based on the results in the LAL-test. The results of the LAL-test on Antithrombin do not correlate with the results of the rabbit pyrogen test. Based on the results in the LAL-test no pyrogenic response could be expected in the rabbit pyrogen test, as the dose used in the pyrogen test equals less than 0.125 EU/ml per bodyweight. This means that the pyrogenic response observed in the rabbit pyrogen test can not be ascribed to the presence of endotoxin in the Antithrombin sample.

Cytokine release test

In Table 16 the results of the IL-6 and TNFα release test are shown. Incubation of the monocytes with the sample of Varicella immunoglobulin and Antithrombin resulted in a non-significant, but detectable release of IL-6 after 24 hours of incubation (35 and 32 pg IL-6 per ml respectively). Both for the batch of Varicella immunoglobulin and the batch of Antithrombin a much higher IL-6 response was expected, because the pyrogenic activity of these batches in the rabbit pyrogen test was approximately 10 EU/ml. Based on this pyrogenic activity in rabbits an IL-6 response of approximately 1000 pg/ml was expected. Different from the IL-6 response, the TNFα response correlates well with the results of the rabbit pyrogen test. Incubation of the monocytes with the batch of Varicella immunoglobulin resulted in a significant release of TNFα during 4 hours of incubation (278 pg TNFα per ml).

Table 16: Results of the cytokine-release test on Human albumin solution, Human varicella immunoglobulin and antithrombin

Cytokine release test on pyrogenic products			
Product	Test dilution	IL-6 response (pg/ml)	TNFα-response (pg/ml)
Albumin solution	1:10 1:100 1:1000	0 0 0	0 0 0
Varicella immunoglobulin	1:10 1:100 1:1000	35 0 0	278 0 0
Antithrombin	1:10 1:100 1:1000	32 0 0	0 0 0

When compared with the TNFα release of the monocytes during incubation with the endotoxin reference preparation, the calculated pyrogenic activity of the batch of Varicella immunoglobulin in the TNFα release test is approximately 25 EU/ml. This is comparable with the results obtained in the rabbit pyrogen test (10 EU/ml).

Table 17 shows an outline of the results of the different tests conducted on the Albumin solution and the samples of Varicella immunoglobulin and Antithrombin.

Table 17: Outline of the results of different tests on Human albumin solution, Human varicella immunoglobulin and Human antithrombin

Schematic presentation of test results				
Sample	Rabbit pyrogen test	LAL-test	IL-6 release test	TNFα release test
Albumin solution	+	+	-	-
Human var. imm.	+	+	(+)	+
Antithrombin	+	-	(+)	-

3.3. Other products

In Table 18 the results of the IL-6 release test on a batch of Interleukin-2 are shown. Interleukin-2 is known to provoke pyrogenic reactions in man

Table 18: Results of the IL-6 release test on Interleukin-2

IL-6 release test						
		IL-6-concentration (pg/ml)				
Sample	Dilution in the IL-6 release test	experiment 1	experiment 2	mean	S.D.	C.V.
BRP, lot 2 (800 IU/ml)	10	1611	1107	1359	356.4	26.2
BRP, lot 2 (800 IU/ml)	100	1544	651	1097.5	631.4	57.5
BRP, lot 2 (800 IU/ml)	1000	406	213	309.5	136.5	44.1
BRP, lot 2 (800 IU/ml)	10000	0	20	10	14.1	141.4
IL-2	10	563	143	353	297.0	84.1

Again, the formula of the best fitting dose-response curve can be defined by: 'y = a0 +a1*exp(-x/a2)', where y is the IL-6 concentration and x is the endotoxin concentration. Using this formula, the IL-6 reponse to incubation with each sample dilution is expressed in endotoxin units (EU's) per ml. The results are shown in Table 19, as well as the results of the LAL gel-clot test. The IL-6 releasing activity of the batch of Interleukin-2 is higher than could be expected based on the results obtained in the LAL-test. Therefore it appears that the IL-6 releasing activity cannot be ascribed to endotoxin. This could be expected from the intrinsic pyrogenic activity of Interleukin-2.

Table 19: Results of the IL-6 release test on Interleukin-2, expressed in EU/ml

Il-6 release test								
Sample	EU/ml LAL-test	Dilution in the IL-6 release test	experiment 1			experiment 2		
			IL-6 conc. pg/ml	EU/ml in diluted sample	EU/ml in undiluted sample	IL-6 conc. pg/ml	EU/ml in diluted sample	EU/ml in undiluted sample
IL-2	<0.125	10	563	0.8	8	143	0.4	4

Table 20 shows the dilutions, doses and effects of Interleukin-2 in the rabbit pyrogen test.

Table 20: Results of the rabbit pyrogen test on Interleukin-2

Rabbit pyrogen test				
Product	Test dilution	Dose (ml/kg)	Individual temp.rises (°C)	Total temp.rise (°C)
IL-2	1:6	1.9 - 2.7 - 2.5	0.6 - 0.5 - 0.6	1.7

Administration of the sample of Interleukin-2 to the rabbits resulted in a clear pyrogenic response. As mentioned earlier, rabbits show a pyrogenic response to 1 ng endotoxin per kg bodyweight, corresponding to 10 EU per kg bodyweight. The mean dose administered to the rabbits was 2.4 ml per kg bodyweight of a 1:6 dilution of the sample of Interleukin-2. In view of the fact that the sample of Interleukin-2 resulted in a pyrogenic response in the rabbit pyrogen test, the pyrogenic activity of the sample at least equals (10 EU/2.4 ml x 6 =) 25 EU/ml. Therefore the sample of Interleukin-2 appears to be more reactive in the rabbit pyrogen test than in the IL-6 release test.

4. Discussion

This report describes the results of tests performed to investigate the suitability of a so-called cytokine release test as an alternative for the rabbit pyrogen test. Endotoxin was used as a model substance, stimulating the monocytic cell-line Mono Mac 6 to release the cytokines Interleukin-6 (IL-6) and Tumor Necrosis Factor α (TNFα).
Monocytes were incubated in the presence of different concentrations of endotoxin and the quantitative relationship between cytokine release and endotoxin concentration was determined. Also the cytokine release promoting activity of several pharmaceutical substances was examined.

The results of the cytokine release test were compared with the results of the bacterial endotoxin (LAL-) test and the rabbit pyrogen test. In order to accomplish this, all results were expressed in endotoxin units per ml (EU/ml), originating from the LAL-test. In the cytokine release test different dilutions of a endotoxin standard preparation were used to obtain an endotoxin dose/IL-6 response curve. Though the rabbit pyrogen test is an absolute assay, the results of this test were also expressed in EU's, based on the knowledge that rabbits are sensitive to a minimum of approximately 1 ng endotoxin (corresponding to 10 EU) per kg bodyweight.

4.1. Vaccines

A number of vaccines was tested conducting the IL-6 release test, the rabbit pyrogen test and/or the LAL-test. These vaccines are listed below.

* Typhoid polysaccharide vaccine
* Pneumococcal polysaccharide vaccine
* Haemophilus type B conjugate vaccine
* Meningococcal polysaccharide vaccine
* Influenza vaccine (surface antigen, inactivated)

4.1.1. Typhoid and Pneumococcal polysaccharide vaccine

The good correlation between the results of the LAL-test and the results of the IL-6 release test indicates that the IL-6 response of the Mono Mac 6 cells to incubation with Typhoid polysaccharide vaccine and Pneumococcal polysaccharide vaccine can be ascribed to endotoxin present in these vaccines.

4.1.2. Haemophilus type B conjugate vaccine and Meningococcal polysaccharide vaccine

For Haemophilus type B conjugate vaccine and Meningococcal polysaccharide vaccine, no correlation was observed between the results of the LAL-test and the IL-6 release test. The IL-6 release in response to incubation with the batch of Haemophilus type B conjugate vaccine largely depends on the test dilutions of this vaccine. After incubation of the cells with a 1:10 dilution of the vaccine almost no IL-6 reponse could be detected, whereas incubation with a 1:20 dilution of the vaccine resulted in an enormous IL-6 response. The basis of the deviant dose-response curve of this vaccine remains to be clarified. The IL-6 release in response to incubation with a batch of Meningococcal polysaccharide vaccine was much smaller than could be expected on basis of the results obtained in the LAL-test. It therefore appears that the high endotoxin concentration found in this vaccine has no indicative value for the IL-6 release of the Mono Mac 6 cells in response to incubation with the vaccine. However, it is shown that the result of the LAL-test on Meningococcal polysaccharide vaccine correlates well with the response observed in the rabbit pyrogen test. Therefore it is recommended to replace the rabbit pyrogen test on the investigated Meningococcal polysaccharide vaccine by the LAL-test.

4.1.3. Influenza vaccine

In the LAL gel-clot test high endotoxin concentrations were observed in a monovalent bulk of Influenza vaccine, using Endosafe lysate as the LAL-reagent. Using Pyrogent lysate the results of the LAL gel-clot test were a factor 20 lower. The bulk of Influenza vaccine was also tested for its pyrogenic activity in the rabbit pyrogen test and in its IL-6 stimulating activity in Mono Mac 6 cells. The results of the rabbit pyrogen test indicate that the endotoxin concentration estimated using Endosafe lysate is no indication for the pyrogenic activity of the monovalent bulk of Influenza vaccine. Also, no correlation is found between the endotoxin concentration estimated using the Endosafe lysate and the IL-6 releasing activity of the bulk. The low endotoxin concentrations observed using Pyrogent lysate in the LAL-test are in line with the results of both the IL-6 release test and the rabbit pyrogen test.

4.2. Blood products

In two different ways it was investigated whether or not pyrogens can be detected in blood products using the cytokine release test.

4.2.1. Spiking experiments

Various blood products were spiked with endotoxin, after which the recovery of the spiked endotoxin was determined. The blood products that were included in the spiking experiments were:
* Human albumin solution
* Human normal immunoglobulin for intravenous administration
* Human tetanus immunoglobulin

From the results of spiking experiments it is concluded that the IL-6 release test can be used as a test to detect endotoxin in Human albumin solution. Howevere, both Human normal immunoglobulin for intravenous administration and Human tetanus immunoglobulin seem to contain IL-6 release inhibiting factors, that have a negative effect on the detection limit of endotoxin in this test.

4.2.2. Rabbit pyrogen test versus LAL-test versus cytokine release test

In addition to the spiking experiments, different tests were performed on batches of blood products that had been demonstrated to be positive in the rabbit pyrogen test by the manufacturer of these products.These blood products were:
* Human albumin solution
* Human varicella immunoglobulin
* Human antithrombin

As far as the products Human albumin solution and Human varicella immunoglobulin are concerned the results of the LAL-test and the rabbit pyrogen test were in good agreement. Based on the endotoxin concentration estimated by means of the LAL-test the pyrogenic response, that had been observed in the rabbits, could be expected. In contrast, the results of the IL-6 release test do not confirm the results of either the LAL-test or the rabbit pyrogen test. Therefore the IL-6 release test cannot be considered to be a suitable alternative to the rabbit pyrogen test as far as these products are concerned. Apparently this test does not detect the endotoxin in these products responsible for the pyrogenic response of rabbits.

In contrast to the findings described above, the results of the rabbit pyrogen test and the LAL-test on the batch of Human varicella immunoglobulin are confirmed by the TNFα release test. So it seems that the release of TNFα by the monocytes can be used as a measure for the amount of endotoxin present in the batch of Human varicella immunoglobulin.

As far as a batch of Human antithrombin is concerned the result of the LAL-test does not confirm the pyrogenic activity of this batch in the rabbit pyrogen test. Probably this can be attributed to the fact that the pyrogenic activity of this batch of Human antithrombin is not caused by endotoxin but by another pyrogenic factor present in the batch. The pyrogenic activity of the batch in the rabbit pyrogen test is not confirmed by the results of the IL-6 release test. Therefore, neither the LAL-test nor the cytokine release test seems to be a suitable alternative to the rabbit pyrogen test.

4.3. Other products

As can be expected the cytokine release test can also be used to detect pyrogens other than endotoxin. In this study a sample of Interleukin-2, the administration of which resulted in a pyrogenic response in rabbits, also resulted in a significant IL-6 release. The IL-6 reponse was somewhat lower than could be expected on basis of the results of the rabbit test.

5. Conclusions

The experiments described in this report show that the suitability of the cytokine release test as an alternative for the rabbit pyrogen test depends very much on the type of pharmaceutical tested.

5.1. Vaccines

In general it must be kept in mind that as far as vaccines are concerned the pyrogen test is merely used as a consistency test and not as a safety test. Therefore the limits set for the pyrogen content are generally based on emperical data rather than on safety limits. Furthermore, when the vaccine concerned contains components of Gram-positive bacteria, the rabbit pyrogen test cannot be replaced by the LAL-test without hesitation, because the LAL-test cannot detect pyrogens other than endotoxin originating from Gram-negative bacteria.

5.1.1. Typhoid and Pneumococcal polysaccharide vaccine

The IL-6 release test may be a suitable alternative to the rabbit pyrogen test as far as Typhoid and Pneumococcal polysaccharide vaccine are concerned, but also the LAL-test seems to be a suitable alternative. In fact, in the European Pharmacopoeia the rabbit pyrogen test for Typhoid polysaccharide vaccine has already been replaced by the LAL-test (monograph 1997: 1160). However, for Pneumococcal polysaccharide vaccine the rabbit pyrogen test is still included in the European Pharmacopoeia (monograph 1997: 0966). The main reason is that the vaccine is formulated on basis of polysaccharides originating from Gram-positive bacteria, that may have intrinsic pyrogenic activity. Therefore the LAL-test, which can only detect endotoxins originating from Gram-negative bacteria, is not considered to be a suitable alternative to the rabbit pyrogen test. As it is known that monocytes do not only release cytokines in response to endotoxin, but also in response to (components of) Gram-positive bacteria, the cytokine release test may be a more acceptable alternative to the rabbit pyrogen test than the LAL-test.

5.1.2. Meningococcal polysaccharide vaccine

It is concluded that for Meningococcal polysaccharide vaccine the LAL-test is a possible alternative to the rabbit pyrogen test. For the product investigated there

appears to be a rather good correlation between the endotoxin concentration determined by the LAL-test and the pyrogenic activity in the rabbit pyrogen test.

5.1.3. Influenza vaccine

The discrepancy found in the results of the LAL-test on Influenza vaccine when using lysates from two different sources indicates the need for an improved test. The possibility of using the cytokine-release test as an alternative to the LAL-test must be further explored.

5.2. Blood products

In contrast to what is the case with vaccines, the pyrogen test is generally used as a safety test on blood products rather than as a consistency test. Therefore the doses used in the rabbit pyrogen test and the endotoxin limits applied in the LAL-test are chosen to guarantee that the product tested will not cause a pyrogenic response in humans when injected intravenously. Generally, the most important pyrogenic contamination in blood products is endotoxin. Because of interfering factors such as blood proteins, for most blood products the LAL-test is not a suitable alternative to the rabbit pyrogen test.
However, for some blood products (for example FactorVIII) the LAL-test is already applied while in the monograph of the European Pharmacopoeia (1997: 0275) the rabbit pyrogen test is still required.

5.2.1. IL-6 versus TNFα release test

The finding of a pyrogenic batch of Human varicella immunoglobulin causing only a slight IL-6 release but a significant release of TNFα by monocytes indicates that, in some cases, focussing at the release of TNFα instead of IL-6 may result in a better correlation between the cytokine release test and the rabbit pyrogen test.

5.2.2. Cytokine release test as an alternative to the rabbit pyrogen test

Both the results of the cytokine release test on blood products spiked with endotoxin and the results of the test on batches that have been demonstrated to be positive in the rabbit pyrogen test show that one should be very cautious in replacing the rabbit pyrogen test, without a thorough validation.

5.3. Other products

Experiments with Interleukin-2 have shown that the cytokine-release test can well be used to detect pyrogenic activity caused by pyrogenic substances other than endotoxin, such as cytokines. As the number of cytokine products grows, the pyrogen test will become more important and will be more often used. The experiments with Interleukin-2 show that the cytokine release test is probably a good alternative to the rabbit pyrogen test for this type of products.

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National Institute of Public Health and the Environment, Bilthoven
- Dr.J.G.C. van Amsterdam
- Drs.P.M.J.M. Jongen
- Drs.H.H.M. Tummers

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam

- Prof.Dr.L.A. Aarden

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the authors

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Mailing List

1	J.Verhoeff, psychiater, Hoofdinspecteur voor de Gezondheidszorg
2	dr.H.J. Schneider, Directeur-Generaal VWS
3- 12	Platform Alternatieven voor Dierproeven
13	Voorzitter van de Gezondheidsraad
14	drs.P.H.Vree, Hoofdinspecteur voor de farmacie en de medische technologie
15	prof.dr.L.A. Aarden; Central Laboratory of the Netherlands Red Cross Blood Transfusion Service
16	dr.H.W.L. Ziegler-Heitbrock; University of München
17	Netherlands Centre Alternatives to Animal Use
18	Nederlandse Vereniging voor Proefdierkunde
19	European Department for the Quality of Medicines (EDQM)
20	Depot Nederlandse Publikaties en Nederlandse Bibliografie
21	Directie van het Rijkinstituut voor Volksgezondheid en Milieu
22	Coördinatiepunt Alternatieven voor Dierproeven
23	dr.ir.G. de Mik
24	dr.ir.J.F. van Sonderen
25	drs.I. Hegger
26	drs.H.H.M. Tummers
27	drs.P.M.J.M. Jongen
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