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Effect of Aluminum on TNF-α Secretion from Murine RAW264.7 Cells for Endotoxin Detection in Hepatitis B Vaccines

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Abstract The rabbit pyrogen test and *Limulus* amoebocyte lysate (LAL) assay have been used to detect endotoxins present in vaccines. Currently, the rabbit pyrogen test is used to detect endotoxins in hepatitis B (HB) vaccines, even though the HB surface protein, which is the active ingredient, is overexpressed in and purified from eukaryotic cells that lack these endotoxins. Although the LAL clot assay is sensitive and reliable and can be used to replace the rabbit pyrogen test, its reaction is limited by the lack of responsiveness to the Gram-positive bacterial components. Furthermore, aluminum hydroxide in the HB vaccine can interfere with the LAL assay. In contrast, macrophages can detect the endotoxin as well as other pyrogens, and secrete TNF-α. Therefore, this study was undertaken to examine the possibility of replacing the animal tests with a more efficient TNF-α secretion assay. With this in mind, we determined if aluminum hydroxide in the HB vaccines affects the TNF-α secretion assay. HB vaccines and the HB protein solutions spiked with lipopolysaccharide (LPS) produced the same level of dose-dependent TNFα secretion and temperature increase in rabbits, indicating that aluminum hydroxide in the HB vaccine does not interfere with the pyrogenic response in rabbits, nor does it interfere with TNF-α secretion. In addition, the TNF-α assay was found to be more sensitive than the LAL assay, and correlated well with the pyrogen test and the LAL assay. These results suggest that the TNF-α assay in RAW264.7 cells is a good substitute for the current pyrogen assays that are used for detecting LPS in HB vaccines as well as in other vaccines containing aluminum.

Key words: LAL clot assay, hepatitis, vaccine, endotoxin, TNF-α, pyrogen

The bacterial cell wall components that are released during an infection, such as lipopolysaccharides (LPS), lipopeptides, peptidoglycans, and teichoic acids, induce fever and septic shock. The LPS molecule consists of a polysaccharide, a core oligosaccharide, and a highly conserved lipid A portion, which is responsible for the toxic pyrogenic properties of LPS [5, 7]. Therefore, the detection of LPS in parenteral preparations of vaccines is essential for quality control. The rabbit pyrogen test is broadly accepted for the detection of endotoxin. However, for ethical and economic reasons, an *in vitro* alternative, the *Limulus* amoebocyte lysate (LAL) assay, has been widely used, but does not exactly correlate with the *in vivo* pyrogenic activity [11]. Furthermore, the lack of reaction of the LAL to the pyrogenic substances from Gram-positive bacteria and the limited supply of *Limulus* (horseshoe crabs) mean that an alternative method is needed. Therefore, an *in vitro* method capable of responding to the bacterial components has been proposed to detect pyrogens in monocytoid or macrophage cells [15, 16, 19, 23, 30]. Since a bacterial infection stimulates the host macrophages to release inflammatory cytokines, macrophages can be regarded as being functional analogues of *Limulus* amoebocytes, and macrophages or their related cell lines can be used as endotoxin indicators [17, 19, 23, 30]. The human and mouse monocytoid cells are highly sensitive to lipopolysaccharide (LPS) for the production of the tumor necrosis factor-alpha (TNF-α), and substantial amount of TNF-α is secreted by the stimulated monocytoid cells [2]. However, the TNF-α induction in human cells suffers from a considerable discrepancy in the hands of different researchers (primary cell cultures) and from low sensitivity to LPS (cell lines) [3, 4, 8, 13, 14, 21, 24, 26–28]. Therefore, a sensitive *in vitro* assay using murine RAW264.7 cells was developed, and the induction of TNF-α in RAW264.7 cells correlated with the pyrogenicity in rabbits [31].

Aluminum, which is a vaccine adjuvant, has been shown to cause inflammation. The aluminum induces inflammatory nodules in aluminum-sensitized adults revaccinated for HB [9]. In rats, low aluminum doses produce marked

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portal inflammation that correlates with the duration of exposure and the amount of aluminum accumulated in the liver [10]. In addition, the level of TNF-α secreted was increased as a result of exposure to aluminum in human glioblastoma cells [6]. Therefore, colloidal aluminum seems to serve as a pro-inflammatory substance. However, the ability of aluminum to induce the secretion of TNF-α in the murine macrophage cell line, RAW264.7, has not been examined.

The hepatitis B (HB) vaccine contains colloidal aluminum and the HB surface protein, which is the active ingredient (used at 20 μg/ml per dose). Since the HB surface protein is currently overexpressed and purified from eukaryotic cells such as Chinese hamster ovary cells or yeast that do not have any endotoxin sources, the chance of endotoxin contamination is very low, if the HB vaccine were prepared by good manufacturing practices. It was previously demonstrated that the rabbit pyrogen test correlates well with the LAL assay, when the samples were appropriately diluted [22]. Since the application of the LAL assay to HB vaccines is hindered by the presence of aluminum and is limited by the insensitivity of the LAL assay to bacterial components [11], this study examined the effect of aluminum on TNF-α secretion. In addition, the sensitivity of the RAW264.7 cells towards the LPS in the presence of aluminum was compared with those of the LAL test and rabbit pyrogen test. The RAW264.7 TNF-α assay was found to be more sensitive to LPS detection than the LAL assay and correlated well with the pyrogen test and the LAL assay. These results suggest that the TNF-α assay in RAW264.7 cells is a good substitute for the current pyrogen assays for the detection of LPS in HB vaccines as well as in other vaccines containing aluminum.

MATERIALS AND METHODS

Reagents

A panel of HB vaccine preparations, which were overexpressed and isolated from yeast, and aluminum hydroxide gel (Alhydrogel™, Brenntag Biosector, Frederikssund, Denmark) were kindly provided by the LG Biotech., Iksan, and Green Cross Vaccine Co., Yong-In, Korea. The HB protein and vaccines were pretested and found not to induce any pyrogenic response in rabbits.

LAL Test

The LAL reagents, endotoxin-free water, endotoxin-free pipette tips, and endotoxin-free test tubes were purchased from Charles River (Charleston, U.S.A.). The control standard endotoxin LPS from the *E. coli* strain O55:B5 was obtained from Charles River Endosafe™ (Lot No. EX23232) and was used as the reference LPS in the LAL assays. The LAL clot assay was performed in duplicate in test tubes to which 0.1 ml of the HB vaccine sample and 0.1 ml of the LAL reagent (Charles River Endosafe™, Charleston, U.S.A.) were added. After 1 h of incubation at 37°C, the test tubes were examined by 180° inversion for the presence of a stable solid clot, which was considered to be a positive result. The sensitivity of the clotting assay was defined to be the lowest concentration of LPS that still produced a positive LAL reaction. A LAL clot sensitivity of 0.06 EU/ml was confirmed by serial dilutions prior to use.

Estimation of Endotoxin Recovery

The recovery of the added LPS was measured in order to evaluate the validity of the assay. The method principally relies upon the determination of the recovery of the endotoxin added exogenously to the HB proteins and vaccines. The level of endotoxin recovery was determined as described above, except that the standard curve was established in pyrogen-free water. The endogenous endotoxin levels of all the samples were subtracted from the recovery data.

Rabbit Pyrogen Test

New Zealand male white rabbits (Samtako, Korea), weighing approximately 2 kg, were used. The animals were housed in individual cages in an animal room at 20°C and allowed free access to food and water. The animals were fasted for 24 h prior to the experiments. The rabbit pyrogen test was performed at Green Cross Vaccine Co., Yong-In, Korea, according to the standards of the US Pharmacopoeia. A US Pharmacopoeia-grade reference standard endotoxin LPS from the *E. coli* strain O113 (Charles River, Cat. No. 23550, 10,000 EU/vial) in sterile phosphate-buffered saline was injected into the marginal ear vein at a dose of 1 ml/kg of body weight. All glassware, needles, and syringes were pyrogen-free. The rectal temperatures were recorded automatically using a digital temperature recorder (PyroMon™ System, Ellab Co., Roedovre, Denmark). This probe measured the rectal temperature continuously for 30 min before and for 5 h after injecting the pyrogen. The recordings were made on unrestrained animals at an ambient temperature of 20°C. The results are expressed as averages of the differences between the initial and highest temperatures recorded within 3 h of the injection.

Cell Cultures and Treatment with LPS

Murine macrophage RAW264.7 cells and human monocyte HL60 cells were cultured in RPMI medium containing 10% fetal bovine serum supplemented with 100 μg of streptomycin/ml and 100 U of penicillin/ml (complete RPMI medium). For the differentiation of the HL-60 cells, dimethylsulfoxide was added to the culture media to a final concentration of 1.3%, which was followed by 48 h of incubation.

LPS (from *E. coli* O111:B4, Sigma) was reconstituted under sterile conditions. Ultrapure LPS (10 ng/ml) exceeded the sensitivity limits of the assay. The cell cultures were treated with LPS for 24 h. The culture medium was harvested for analysis at the end of the experimental period(s).

Determination of TNF-α

The amount of TNF-α was determined using an enzyme-linked immunosorbent assay (ELISA) kit for a mouse TNF-α or a human TNF-α (Mono/Mono BD OptEIA kit, BD Biosciences) according to the manufacturer's instructions. Briefly, the RAW264.7 cells were seeded into a 12-well plate at 2×10^5/2 ml of medium/well, and various concentrations of LPS were added to the culture. After 24 h of incubation, the medium was removed and spun in a Beckman centrifuge at 13,000 rpm for 10 min, and the supernatant was used for TNF-α determination. For each group, the mean of three wells was expressed with standard deviation (SD).

Cell Proliferation Assay

In order to determine the effect of aluminum on cell proliferation, the RAW264.7 cells were cultured in the presence of various concentrations of aluminum or the preservative for 24 h, and the level of cell proliferation was determined using a CyQuant proliferation assay kit (Molecular Probe, Cat No. P36930) according to the manufacturer's instructions.

Statistics

The data are presented as mean±SD. The student's *t*-test was used for statistical analysis. $P<0.05$ was considered significant.

RESULTS

Evaluation of Cell Lines for Endotoxin Detection by TNF-α Secretion

Since the human monocytic cell line Mono Mac 6 (MM6) and myelomonocytic cell line U-937 cells were found to secrete different levels of TNF-α depending on the experimental conditions [4, 8, 13, 27, 28], we employed the human monocyte cell line, HL-60, and the mouse macrophage cell line, RAW264.7, to examine the secretion of TNF-α after spiking with 0–10 pg/ml of LPS. However, no correlation was found between the level of TNF-α secreted and the amount of LPS spiked in the human HL-60 cells (data not shown). In addition, the HL-60 cells did not show any reproducibility even after a treatment with a differentiation agent, 1.3% (v/v) dimethyl sulfoxide (DMSO), for 48 h (data not shown). In contrast, the mouse macrophage RAW264.7 cells demonstrated a good correlation between the amount of LPS spiked and the level of TNF-α secreted (Fig. 1). Furthermore, the TNF-α assay could detect <2 pg/ml of LPS, which could not be detected by the LAL assay, demonstrating that the TNF-α assay is more sensitive than the LAL assay. In addition, the HB vaccine containing aluminum resulted in a higher level of TNF-α secretion than the HB protein without aluminum (Fig. 1). Therefore, the RAW264.7 cells were adopted for the following TNF-α secretion assays.

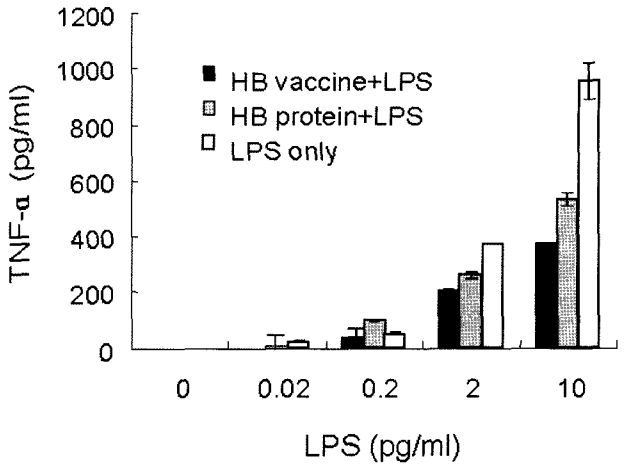

Fig. 1. Secretion of TNF-α from the RAW264.7 cells spiked with LPS.
RAW274.7 cells containing the HB vaccine were spiked with the indicated concentrations of LPS (pg/ml) and incubated for 24 h. Subsequently, the amount of TNF-α secreted from the RAW264.7 cells was determined by ELISA. All experiments were carried out in quadruplicate. Results from three independent experiments are presented as means±SD.

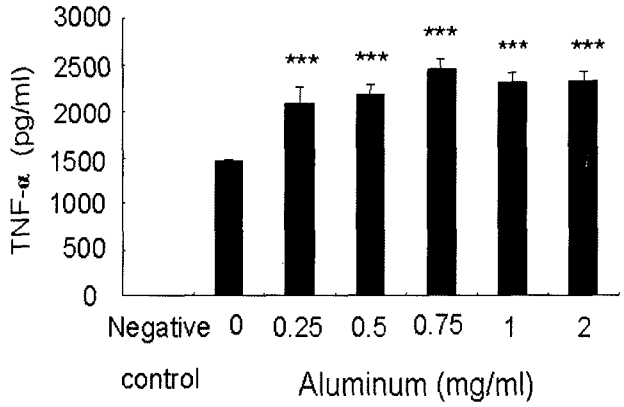

Fig. 2. Effect of aluminum on TNF-α secretion from the RAW264.7 cells.
Amount of TNF-α secreted from the RAW264.7 cells was determined after adding the aluminum concentrations indicated and 10 ng/ml of LPS to the culture. The RAW264.7 cells not spiked with either LPS or aluminum served as the negative control. All experiments were carried out in quadruplicate. Results from two (negative control) and four (aluminum-treated group) independent experiments are presented as means±SD (*** $P<0.0001$ relative to the no aluminum control).

Table 1. Comparison of the TNF-α secretion assay with the pyrogen test and the LAL assay in the HB vaccine after spiking with LPS[a].

Spiked LPS	Pyrogen test	LAL test		TNF-α secretion assay	
		HB protein	HB vaccine[b]	HB protein	HB vaccine
10 EU/ml (2 ng/ml)	+	+	+	+	+
5 EU/ml (1 ng/ml)	+	+	+	+	+
2.5 EU/ml (0.5 ng/ml)	-	+	+	+	+
1.25 EU/ml (0.25 ng/ml)	-	+	+	+	+
0.06 EU/ml (0.012 ng/ml)	-	+	±	+	+
0.03 EU/ml (0.006 ng/ml)	-	+	-	+	+
0.015 EU/ml (0.003ng/ml)	-	-	-	+	+

[a]The samples were spiked with the specific concentrations of LPS and diluted. The level of LPS recovery was determined by the LAL clot assay as described in Materials and Methods. The results are based on two independent duplicate experiments. Symbols + and - represent the positive and negative results, respectively, and in the case of the TNF-α assay, the detection limit was determined by the limit of the ELISA kit commercially available.
[b]Positive response could be obtained only after appropriate dilutions of the samples.

Effect of Aluminum on TNF-α Secretion

In order to determine if aluminum did not interfere with the TNF-α secretion in RAW264.7 cells, various concentrations of aluminum in the presence or absence of 10 ng/ml of LPS were added to the culture of the RAW264.7 cells. As shown in Fig 2, aluminum significantly increased the secretion of TNF-α, but not dose-dependently.

The pyrogenicity of the rabbits after the LPS spiking of either the HB vaccine or HB protein was examined in order to determine whether the TNF-α assay in RAW264.7 cells correlates with the pyrogen test. The HB vaccine and HB protein were supplemented with endotoxin, and the rabbit pyrogenicity was then determined. Supplementation of the 2.5 endotoxin unit (EU) showed negative results in both the HB vaccine and HB protein. However, supplementation of either 5 or 10 EU resulted in positive results in both the HB vaccine and the HB protein (Table 1). These results are in good agreement with previous results that demonstrated that aluminum hydroxide did not significantly interfere with the pyrogenicity of the endotoxin *in vivo* when the HB vaccines comprising aluminum were spiked with 2.5, 5, or 10 EU/ml LPS, and the increase of temperature in the 5 EU/ml group was similar to that in the 10 EU/ml group [22]. In order to further corroborate the TNF-α secretion in the RAW264.7 cells at the pyrogenic LPS dose, the HB vaccine or HB protein was challenged with 2.5, 5, and 10 EU/ml of LPS, and the amount of TNF-α secreted was determined. As expected, LPS strongly induced TNF-α secretion from the RAW264.7 cells (Table 1).

The correlation between the TNF-α secretion assay and the LAL assay was further confirmed by performing a LAL clot analysis after LPS spiking of either the HB vaccine or the HB protein. The LAL assay gave a positive response at concentrations of 2.5, 5, and 10 EU/ml of LPS. However, at lower LPS doses (<0.06 EU/ml or 12 pg/ml) where the TNF-α assay could detect LPS, the LAL assay gave negative results. In addition, the HB vaccines comprising aluminum caused significant interference with the LAL assay and resulted in a negative or a pseudo positive response (Table 1). Therefore, it was confirmed that the TNF-α assay was more sensitive than either the rabbit pyrogen test, which has a detection of 5 EU/ml, or the LAL assay, which has a detection limit of 0.06 EU/ml. In addition, the results demonstrated that the TNF-α assay correlated well with both the pyrogen test and the LAL assay. Furthermore, these results indicate that aluminum does not interfere with the TNF-α assay under the experimental conditions used.

Since aluminum increased the level of TNF-α secretion in human glioblastoma cells [6], we further investigated this possibility in the mouse RAW264.7 cells. The aluminum-stimulated cell proliferation due to proinflammatory property of aluminum was further checked by adding various concentrations of aluminum to the RAW264.7 culture. As seen in Fig. 3, aluminum decreased the level of cell proliferation, but not dose-dependently: At 0.5 mg/ml aluminum, which is the concentration currently used in

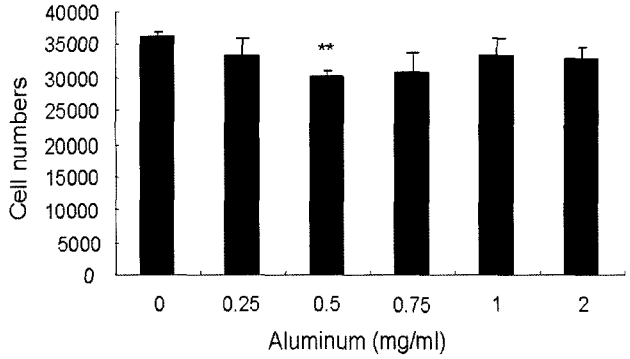

Fig. 3. Effect of aluminum on proliferation of the RAW264.7 cells.
To determine the effect of aluminum on cell proliferation, the RAW264.7 cells were cultured in the presence of various aluminum concentrations for 24 h, and the level of cell proliferation was determined by the proliferation assay kit. All experiments were carried out in quadruplicate. The results from two independent experiments are presented as a mean±SD (** $P<$ 0.001 relative to the negative control).

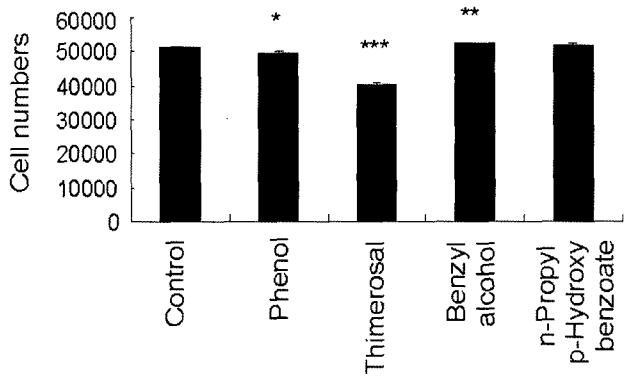

Fig. 4. Effect of preservatives on proliferation of the RAW264.7 cells.

To determine the effect of preservatives on cell proliferation, the RAW264.7 cells were cultured with the preservatives (0.5% phenol, 0.01% thimerosal, 2% benzyl alcohol, or 0.02% propyl p-hydroxybenzoate) for 24 h, and the level of proliferation was determined using the proliferation assay kit. The RAW264.7 cells without any preservatives served as the negative control. All experiments were carried out in quadruplicate. The results from two independent experiments are presented as a mean±SD (* $P<0.05$, ** $P<0.001$, *** $P<0.0001$ relative to the negative control).

the HB vaccines, the number of cell decreased by 17%, compared with the control, whereas the level of TNF-α secretion was increased. The result indicates that the increase of TNF-α secretion was due to the stimulation of the RAW264.7 cells by aluminum.

Effect of Preservatives on TNF-α Secretion

Currently, several preservatives are added to parenteral drugs. In order to check the effect of these preservatives on TNF-α secretion, the amount of TNF-α secreted was determined after incubating the RAW264.7 cells for 24 h in the presence of the preservatives currently used and with 0.5 mg/ml of aluminum. However, the level of TNF-α secretion was unaffected (data not shown), even though the level of cell proliferation was significantly affected, depending on the preservatives used; 2% (v/v) benzyl alcohol increased the level of cell proliferation significantly, whereas both 0.5% phenol and 0.01% thimerosal decreased the level of cell proliferation. On the other hand, cell proliferation was unaffected by 0.02% (v/v) propyl p-hydroxy benzoate. These results indicate that, at a specific concentration of the preservatives, TNF-α secretion in the RAW264.7 cells could be standardized and be used for endotoxin detection.

DISCUSSION

The nature and composition of biopharmaceuticals comprising recombinant proteins has been well characterized, and the lack of endotoxin is normally confirmed by a rabbit pyrogen test. Although the rabbit pyrogen test is being gradually superseded by the LAL assay, the rabbit pyrogen tests are still carried out around the world, where the LAL assay cannot be applied. However, the rabbit test is costly and not quantitative. In addition, the LAL assay can give false-negatives with certain products, can overestimate the pyrogen content of other products, and does not detect pyrogens other than bacterial endotoxins (LPS), such as Gram-positive exotoxins, viruses, and fungi [23, 25]. Therefore, this study was undertaken to evaluate the correlation between the *in vivo* rabbit test, the *in vitro* LAL test, and the TNF-α secretion assay from mammalian cells, and check the feasibility of substituting the *in vivo* test with the *in vitro* TNF-α assay. The human monocytoid HL-60 cells exhibited discrepancy and lack of reproducibility, whereas the mouse macrophage RAW264.7 cells exhibited high sensitivity to LPS and produced a dose-dependent level of TNF-α secretion, similar to a previous report [12]. Therefore, the TNF-α assay in the RAW264.7 cells could be used to detect endotoxins in biopharmaceuticals.

In addition, this study compared the ability of the TNF-α assay with the rabbit pyrogen test and the LAL assay to detect endotoxin in HB vaccines. The results demonstrate that 1) aluminum does not interfere with the TNF-α assay and the TNF-α assay is a more reliable method for the detection of endotoxin in HB vaccine than either the pyrogen test or the LAL assay; 2) there is a good correlation between the TNF-α assay and the rabbit pyrogen test for the detection of endotoxin in the HB vaccines, indicating that the *in vivo* and *in vitro* tests have a good correlation; and 3) the TNF-α assay is more sensitive than the *in vivo* rabbit pyrogen test (detection limit >5 EU/ml/kg body weight) and the *in vitro* LAL assay (LAL clot detection limit <0.06 EU/ml). The sensitivity of the rabbit pyrogen test in this study matched with that reported in a previous study [29]. The TNF-α assay may be a simpler and more cost-effective test for quantitatively detecting endotoxin in the HB vaccines.

The LAL clot assay relies on the principle that endotoxin causes the extracellular coagulation of the blood (hemolymph) of a horseshoe crab, *Limulus polyphemus* [18]; The endotoxin triggers the conversion of the preclotting enzyme into a clotting enzyme, which subsequently converts coagulogen into a coagulin gel. The LAL clot assay detects this gelation after incubating the samples for 1 h. During this step, aluminum can interact/adsorb to any component(s) required for the gel clot [1]. On the other hand, the TNF-α assay relies on the innate immune response that is triggered by foreign immunogenic components such as endotoxins; LPS and other molecular structures, which are expressed by microbes that bind the Toll-like receptors (TLRs). The subsequent activation of the TLRs initiate an inflammatory cascade that attempts to clear the offending pathogen and set in motion a specific adaptive immune response, which comprises the secretion of cytokines such as TNF-α as

a part of the self-defense mechanism in mammals [20]. Therefore, the TNF-α assay could respond to foreign materials that do not trigger a clot reaction of the LAL reagent. Thus, the TNF-α assay, in theory, appears to be superior to the LAL assay.

In conclusion, the aluminum adjuvant in vaccines did not interfere with the *in vitro* TNF-α assay for pyrogenic substances detection, and there was a close correlation between the sensitive LAL assay and the TNF-α assay, and between the pyrogen test and the TNF-α assay. These results clearly show that the TNF-α assay could be a substitute for the pyrogen test in the detection of endotoxin in the HB vaccine as well as in other parenteral biopharmaceutical products.

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