

Endotoxins in Commercial Vaccines

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Twenty samples of commercial vaccines intended for administration to humans were assayed for the presence of bacterial endotoxins by using the *Limulus* amebocyte lysate test. Sixteen of the vaccines contained more than 0.1 ng of endotoxin per ml (which corresponds to 10^3 bacterial cell wall equivalents per ml in the undiluted vaccines). These results suggest that at some stage of preparation, the vaccines have contained varying amounts of gram-negative bacteria and may indicate the presence of other bacterial products as well. It might be useful to list the level of endotoxins, phage, and other contaminants on each vaccine lot to facilitate studies on any side effects of these contaminants. Selection of vaccine lots with the least endotoxin might reduce some of the adverse effects of vaccinations.

After the discovery and characterization of bacterial viruses in commercial sera (2, 8, 9, 18, 22) and vaccines manufactured with commercial sera (19, 24), we initiated studies for other bacterial products in sera and vaccines. Although serum used in vaccine production is filter sterilized, bacterial products such as nucleic acids, proteins, endotoxins, and viruses might pass through the filters.

The finding of live gram-positive bacteria, coliphages plating on *Escherichia coli* C3000, and/or bacterial endotoxins in 23 out of 24 lots of unfiltered calf sera collected by a "clean catch" method (22) indicated that even though gram-negative organisms could not always be cultured from the sera, their presence could at least be traced by the persistence of their specific phages and endotoxins.

The ability to monitor for some contaminants, such as bacterial viruses, may be hampered by the presence of bacteriocidal preservatives in commercial vaccines. It is also often unclear which bacteria host strains and growth conditions to use for the demonstration of contaminating viruses. The *Limulus* amebocyte lysate (LAL) assay for endotoxin provides a convenient alternative for screening biological products for current or preexisting gram-negative contamination.

A report detecting bacteriophages and endotoxin in certain commercial live-virus vaccines

(against polio, measles, mumps and rubella) demonstrates that the LAL assay for endotoxin can be used as a screen for prior contamination with gram-negative bacteria (19). The present study confirms these observations and extends them to include additional vaccines and similar parenteral products.

MATERIALS AND METHODS

Limulus Pyrotest kits were purchased from Difco Laboratories (Detroit, Mich.). They contained individual test tubes of lyophilized LAL, a pyrogen-free, distilled water negative control, and two positive controls containing, respectively, 0.5 and 5.0 ng of purified endotoxin per ml.

Some vaccines were donated by Wyeth; Merck, Sharp & Dohme; Smith, Kline & French Laboratories; Pfizer Inc.; Eli Lilly & Co.; Merrell-National; and Parke, Davis & Co. Other vaccines were purchased. All vaccines tested were intended for human administration.

Samples (200 μ l) of vaccine were withdrawn sterilely by syringe and injected directly into the test vials containing lysate. After mixing and 1 h of incubation at 37°C in a water bath, the tubes were gently inverted. Formation of a firm gel was designated as a positive result (5). A weak gel which could be broken by gently tapping was scored \pm , whereas a watery fluid was considered negative. When it was necessary to dilute the sample, serial dilutions were made with the negative control solution as diluent. Samples of the negative control were run through an identical mock dilution procedure to rule out contamination due to our manipulations. Undiluted vaccines which gave a negative reaction were tested for the presence of inhibitor by a "spiking" procedure (5): 20- μ l samples of the 5-ng/ml positive control were mixed with 180 μ l of vaccine, giving a final endotoxin concentration of 0.5 ng/ml, and then this mixture was tested. Because this

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concentration is close to the lower limit of resolution of the test, the presence of inhibitors would yield a negative result; a positive result would mean little or no inhibition. The vaccines tested in this manner all gave clear positives, with the exception of one polio vaccine which gave a weak positive, possibly reflecting a low level of inhibitor in that polio vaccine sample. Endotoxins are lipopolysaccharides, the lipid A moiety of which is chloroform soluble. Extraction of cholera and influenza vaccines with chloroform showed that the gelation agent present in the vaccines was chloroform soluble (17).

The LAL assay was also performed on five related products: diphtheria toxoid (courtesy of C. Hardegree, Bureau of Biologics, Food and Drug Administration) lot 40103-428; tetanus toxoid (Lederle Laboratories) lot 463-356; tetanus toxoid adsorbed and Purogenated (Lederle) lot 466-340; staphylococcus toxoid (Lederle) lot 463-314; and antirabies serum (Lederle) lot 460-228.

RESULTS

Of the 20 vaccines tested (including the swine flu sample not shown on the table), only three vaccines proved free of detectable endotoxin (Table 1). The volume of one smallpox sample was too small to test undiluted in our assay, whereas the other smallpox sample gave a \pm result which was interpreted as indicating small amounts of endotoxin (approximately 0.1 ng/ml). The high levels of endotoxin observed in the cholera and combined DPT vaccines most probably reflect their gram-negative origins and are inherent in the products per se (unless the biological activity of endotoxins could be destroyed while the antigenicity is retained). All of the other vaccines which showed some level of endotoxin did so in spite of the fact that no gram-negative organisms were deliberately involved in their manufacture. There were variations between some batches of the same type of vaccine from different lots or different manufacturers. All vaccines prepared in embryonated chick or duck eggs showed some level of contamination. This is probably due to contamination of such eggs with *Salmonella* (34). Of the 19 vaccines studied, 13 contained levels (≥ 1 ng/ml) sufficient to cause a febrile reaction in the rabbit pyrogen test.

The undiluted samples of antirabies serum, diphtheria toxoid and tetanus toxoid (both plain and adsorbed) gave \pm results. The staphylococcus toxoid result was negative.

DISCUSSION

The implications of endotoxin contamination are not yet fully understood. Adverse reactions ranging from mild fever to at least one fatal generalized Schwartzmann reaction (10, 14, 33) have been attributed to the presence of endo-

toxin in vaccines. However, it should be noted that the fatal Schwartzmann reaction occurred during the use of a typhoid vaccine for fever therapy and not immunization (33).

The febrile response to a vaccine is due to a number of factors, as indicated by the lack of correlation between human febrile response and endotoxin levels in influenza vaccines (1). On the other hand, adverse reactions to polysaccharide vaccines did correlate with endotoxin content as measured by the LAL assay and the rabbit test (14). Effects of endotoxins on the central nervous system, cardiovascular system, kidneys, liver, hypothalamus, and lymphatic system have been noted in experimental animal studies (15, 37). Most of these studies involved larger concentrations of endotoxins than we have observed in these vaccine studies; however, they may indicate some points of concern particularly in chronically ill or elderly individuals. A fatal additive effect of influenza vaccine and endotoxin has also been observed in guinea pigs (25). As a result of that observation, the federal government has set permissible levels of endotoxin for influenza vaccines only (25).

In view of these facts, it would obviously be desirable for clinicians to know the endotoxin levels of biological products intended for human administration.

The specificity and convenience of the LAL assay (5, 16, 26, 35) makes this an ideal rapid screening procedure for vaccine samples and other biomedical products. The test detects as little as 0.1 ng of endotoxin per ml (3), which corresponds to about 10^3 bacterial cell wall equivalents per ml (11). The commonly used rabbit test for endotoxin detects only 1 ng/ml and, furthermore, is subject to misinterpretation because pyrogenic vaccine components other than endotoxin also give positive results (4, 26), making it impossible to distinguish extraneous pyrogens from those inherent in the vaccine components themselves. Although the possibility that substances other than endotoxin might cause a positive LAL reaction cannot be rigorously excluded, we consider it to be unlikely because several studies have shown that antibiotics, normal plasma and its vasoactive components, calcium, hemoglobin, and various gram-positive products (28) fail to give any false positives with the LAL assay (3, 11, 17, 28, 29). Some investigators have reported false positives induced by proteins, synthetic polynucleotides (7), and purified gram-positive peptidoglycans (36). Thrombin also has been suggested as a possible source of false positive tests (7); however, more recent reports (28, 31) indicate that thrombin per se does not induce LAL coagula-

TABLE 1. Endotoxin levels detected in commercial vaccines by the LAL assay^a

VACCINE	MANUFACTURER	LOT NUMBER	PREPARATION	DILUTION									
				10 ⁰	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷		
Small Pox	Lederle	454-136	Chick embryo	*	-								
	Wyeth	305701	Calif lymph	±	-								
Rubeola	Lederle	439-161	Chick cell culture	-									
Rubella	Merck, Sharp & Dohme	1263V	Duck embryo cell culture	+	-								
Polio	Smith, Kline & French	4CO5R112	Rabbit kidney cell culture	-									
	Lederle	433-186	Monkey kidney cell culture	+	+	-							
Rabies	Lederle	442-258	Monkey kidney cell culture	+	+	±							
	Pfizer	503TD-11384-6	Human skin cell culture	-									
Influenza	Lilly	8FA70D	Duck embryo	+	±								
	Lilly	9PB83A	Chick embryo	+	+	+	-						
Mumps	Merrel	1156EK	Chick embryo	+	+	±							
	Parke-Davis	909515A	Chick embryo	+	+	+	±						
Rocky Mtn. Spotted Fever	Lederle	445-347	Chick embryo	+	+	+	±						
	Merck, Sharp & Dohme	0259V	Chick embryo cell culture	+	+	±							
D.P.T.	Lederle	433-154	Chick embryo	+	+	+	+	-					
	Lederle	469-249	Chick embryo	+	+	+	±						
Typhus	Lederle	451-122	Nutrient broth	+	+	+	+	+					
	Lederle	445-348	Chick embryo	+	+	+	+	+	+				
Cholera	Lederle	445-346	Nutrient broth	+	+	+	+	+	+				
			Endotoxin/ml	0.1 ng	1.0 ng	10.0 ng	100.0 ng	1.0 µg	10.0 µg	100.0 µg	1.0 mg		
			Bacterial equivalents/ml	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷	10 ⁸	10 ⁹	10 ¹⁰		

^a Undiluted vaccine not tested, as explained in the text. Symbols: +, firm gel; ±, loose gel; -, no gel.

tion. In fact, there is a greater likelihood of false negatives (26, 34) because some serum components, anticonosteroids, corticosteroids, and vaccine preservatives inhibit the reaction (16, 26). The presence of such inhibitors can be ruled out by the spiking procedure (described above).

Numerous studies (3, 6, 14, 20, 26, 32, 35) have been made to compare the efficiency and specificity of the LAL assay and the standard rabbit test. The consensus (26) at present appears to be that although neither test is ideal, there is generally a good agreement between them, with the LAL test being more sensitive, less expensive, and more convenient. The major disadvantages for the LAL assay were difficulty in standardizing different batches of lysate and the possibility of missing pyrogens other than gram-negative endotoxins. False negatives due to the presence of other chemicals, as noted above, can also interfere. For screening purposes where gram-negative contamination is suspected, however, the LAL assay certainly is more specific and sensitive. Although the rabbit test is still the standard test for pyrogens and the LAL assay has not yet been officially recognized, it is currently being used as an additional screen for influenza vaccines by the Bureau of Biologics, Food and Drug Administration. It is also being used to examine other parenteral products, including antitumor drugs (30), radiopharmaceuticals (20), drinking water (12), intravenous fluids (21), and intrathecal drugs (26). The reports of LAL tests generally are not included in the literature which accompanies the vaccine. We feel that the Bureau of Biologics should require that the endotoxin levels be indicated on all vaccine lots.

Where the observed endotoxins are an inevitable part of the vaccine production (as with cholera) or result from other procedures such as the inadvertent use of contaminated serum or eggs, it may be possible to eliminate them from the vaccines. The absence of endotoxin from at least three vaccines shows that this is not impossible. Various chemical methods of removing or inactivating endotoxin exist (23, 27), some of which might even be adapted to selectively remove the endotoxin from the antigenic determinants in the gram-negative vaccines themselves. For the viral vaccines, especially, the removal of endotoxin without loss of antigenicity may be economically feasible. A recent report from the Bureau of Biologics, Food and Drug Administration (27), describes a simple adsorption and elution technique which reduced the endotoxin levels of influenza vaccine 10- to 20-fold without significantly affecting its antigenicity and at the same time performing the nec-

essary concentration step in the production of the vaccine.

It is our opinion that the monitoring and reporting of endotoxins and other contaminants in vaccines might be useful in understanding some of the side effects observed in vaccine recipients. It would be useful for clinicians to have their laboratories measure the endotoxin levels in various vaccine lots from different companies using the LAL assay. Selection of the vaccines with the lowest endotoxin levels might help to avoid some of the adverse effects of vaccinations.

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