Statistical Determination of Endotoxin Content in Influenza Virus Vaccine by the Limulus Amoebocyte Lysate Test

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To determine laboratory-to-laboratory variability of the limulus amoebocyte lysate (LAL) test for evaluating endotoxin content in influenza virus vaccine, a collaborative study was designed. Participants were six influenza virus vaccine manufacturers and the Bureau of Biologics (BoB). Lysate lot 116, Reference Influenza Virus Vaccine for Endotoxin Assay E-1, and four test vaccines having different ratios of LAL activity relative to reference E-1 were supplied by the BoB. Each laboratory used its normal test procedure. All vaccines were coded. One pair (reference and test) of vaccines per day was tested for 4 days a week over a 4-week period. All data were analyzed at the BoB. The degree of variability experienced by testing laboratories was estimated by the study. This estimate did not conflict with experience gained from previous routine testing in any of the participating laboratories. A statistical approach to the evaluation of LAL data from testing influenza virus vaccine for endotoxin content was developed based upon the overall variation obtained from the collaborative study.

Influenza virus vaccine is produced by the inoculation of large numbers of embryonated chicken eggs to yield a single lot of vaccine. The harvesting and pooling of the allantoic fluid containing virus is done semiautomatically. It is not possible to detect and discard every egg that may contain bacteria. Therefore, the harvested fluid contains some amount of bacterial endotoxin. Purification procedures used to produce vaccine remove the endotoxin to varying degrees.

For several years, influenza virus vaccine manufacturers have been testing their vaccine for endotoxin content by using the limulus amoebocyte lysate (LAL) test. In 1974, a requirement was added to the *Food and Drug Regulations for Biological Products* (5) to test each lot for endotoxin content. The test procedure was not specified, but all manufacturers have been using the LAL test. Although this test is very sensitive for endotoxin (3, 4), an exacting technique is needed to obtain reliable results. Each individual performing the test must standardize the technique used so that no contaminating pyrogens will be introduced and so that dilution errors will be minimal. A procedure must be used to determine whether each test is within the expected limits of variation for the particular laboratory. Furthermore, the variation in a given laboratory must not be greater than that for other laboratories that will be testing the same products. A collaborative study was designed for six laboratories of

manufacturers producing influenza virus vaccine and the Bureau of Biologics in order to obtain a reliable estimate of the variability that may occur in each of these laboratories. The degree of variability from this study can be used to develop a statistical approach to the evaluation of data from LAL tests, comparing end points for a reference influenza virus vaccine to a test vaccine.

MATERIALS AND METHODS

The Bureau of Biologics' Reference Influenza Virus Vaccine for Endotoxin Assay lot E-1 was used in this study. This was a portion of a commercial lot of vaccine that had been tested clinically and was found to cause a low level of local reactions and no systemic reactions. The lowest dilution passing the pyrogen test (6) when tested at 0.5 ml per kg of rabbit body weight was 1:100. Relative to reference endotoxin lot 1b (*Klebsiella*), it contained 4.0 μ g of LAL activity per ml, which we have assumed to be endotoxin. Reference E-1 and four test vaccines having different percentages of endotoxin content relative to reference E-1 were provided to the participating laboratories. Using a similar vaccine with almost no endotoxin as diluent, reference E-1 was serially diluted in twofold increments to obtain these four test vaccines. This provided vaccines with identical properties except for endotoxin content. Thus the four test vaccines had hypothetical ratios of 0.125, 0.25, 0.5, and 1.0 relative to reference E-1. Thirty-two vials, each containing 1 ml of material, 16 vials of reference E-1, and 4 vials of each test vaccine were shipped to each participant at 4°C.

Limulus lysate lot 116 from Associates of Cape

Cod, Inc., Woods Hole, Mass., was used in this study. This lot had a sensitivity of 0.25 ng/ml relative to reference endotoxin lot 1b. Each participating laboratory was provided with enough of this lysate to complete the study. The freeze-dried lysate was stored at -20°C and shipped packed with dry ice. The lysate was rehydrated with 5.0 ml of sterile pyrogen-free distilled water just before use. Any unused rehydrated lysate was stored at -70°C and thawed only once.

This study was designed to test the endotoxin content of influenza virus vaccine in parallel with the reference vaccine over a fixed period of 4 weeks. One pair (reference and test) of vaccines per day was tested for 4 days a week over a 4-week period. During each week, all four test vaccines were tested. All vaccines were coded. Each vial had a code number from 1 to 32. Vials 1 and 2 were tested on day 1, 3 and 4 on day 2, and so on through vials 31 and 32 tested on day 16. Vials were coded independently for each of the seven participants.

Every test day, 0.2 ml of each vaccine was serially diluted in pyrogen-free water in tenfold increments from 10^0 to 10^{-4} . The test was done by adding 0.1 ml of each vaccine dilution to 0.1 ml of lysate in pyrogen-free tubes (10 by 75 mm) as described by Hochstein et al. (1). After gentle mixing, the tubes were placed in a 37°C noncirculating water bath and left undisturbed for 1 h. After the 1-h incubation period, the tubes were carefully removed from the water bath and read by inverting each tube. If a solid clot had formed, that dilution was recorded as positive; if no clot formed or if the clot broke when the tube was inverted 180° , that dilution was recorded as negative. Beginning with the last positive tenfold dilution, a series of five twofold dilutions (1:2 through 1:32) was made in pyrogen-free water. The LAL test as described above was repeated on these twofold dilutions.

Laboratories were provided with a form (Fig. 1) for recording the test results. Each laboratory followed the above procedure, but used its own method for making dilutions and used only one technician through the 4-week period. Laboratories were instructed to send the results to the coordinator on a weekly basis.

RESULTS

The results of each daily test were recorded directly on the daily laboratory work sheet (Fig. 1). It should be noted that the reading of each test was either positive or negative. A questionable gel was always recorded as negative.

Each laboratory did four replicate tests on each vaccine in parallel with reference E-1. Table 1 is a tabulation of the vaccine end-point dilutions for endotoxin content. Laboratories 2 and 6 had no replicate variation for vaccine 1; each test yielded a ratio of 0.1. Laboratory 5 had no replicate variation for vaccine 2, with a ratio of 0.25. For vaccine 3, laboratory 2 had no varia-

Date: _____ Laboratory No: _____

Week: _____ Day: _____ Technician Name: _____

Vial
Number

Vaccine Dilution

Undil.	10^{-1}	10^{-2}	10^{-3}	10^{-4}	
—*	1:2	1:4	1:8	1:16	1:32

Undil.	10^{-1}	10^{-2}	10^{-3}	10^{-4}	
—*	1:2	1:4	1:8	1:16	1:32

* Fill in appropriate tenfold dilution. Indicate reaction for every dilution tested as either + or -.

FIG. 1. Daily laboratory worksheet.

tion, with a ratio of 0.4. For vaccine 4, laboratory 3 was the most precise, with a ratio of 1.0.

Table 2 shows the geometric means (GM) of ratios by vaccines and laboratories. For vaccine 1, the GM ratio of laboratory 1 was closest to the hypothetical ratio of 0.125, and the ratio for laboratory 7 was the farthest from it. Laboratory 5 had the same GM ratio as the hypothetical value for vaccine 2. Ratios for laboratories 3 and 5 were close to the hypothetical value of 0.5 for vaccine 3. For vaccine 4, laboratories 3 and 7 had a GM ratio of 1.0, the same as the hypothetical value.

Ratios (T/R) obtained from Table 1 were converted to the logarithm to the base 2. The standard deviations (SD) were calculated for each of the four vaccines by laboratory (Table 3).

The variability of the $\log_2$ ratio of the endotoxin content of an influenza virus vaccine and reference E-1 was estimated in this collaborative study by the SD pooled over four vaccines and seven laboratories. The pooled SD was 0.66, with 83 df. This SD and the chi-square tables (7) were used to generate 99% upper limits on the SD of $\log_2$ ratio for sample sizes 4 through 28 in increments of 4 (Table 4).

The pooled SD 0.66 was again used to generate estimates of GM ratios for various sample sizes with the constraint that the 95% upper limit of this geometric mean be fixed at 1.0 (Table 5).

DISCUSSION

This collaborative study on the variability of the ratio of the endotoxin content of influenza

TABLE 1. *Influenza virus vaccine end-point dilutions^a for endotoxin content*

Laboratory no.	Vaccine															
	1 (0.125) ^b				2 (0.25)				3 (0.5)				4 (1.0)			
	1 ^c		2		3		4		1		2		3		4	
	T ^d	R ^d	T	R	T	R	T	R	T	R	T	R	T	R	T	R
	1	0.4	4	8	1	10	2	10	8	10	1	4	8	20	10	20

^a Multiply T and R values by 1,000 to obtain the reciprocal of end-point dilutions.^b Hypothetical ratio of endotoxin content with respect to reference E-1.^c Weeks.^d T, Test; R, reference.^e NT, No test.

virus vaccines and reference E-1 was designed in blocks of 4 days and 4 weeks because four replicates were desired on each of the four vaccines. To help eliminate any bias in reading the

TABLE 2. *GM ratios of vaccines for endotoxin content*

Laboratory no.	Vaccine			
	1 (0.125) ^a	2 (0.25)	3 (0.5)	4 (1.0)
1	0.13	0.24	0.45	0.84
2	0.10	0.14	0.40	0.95
3	0.12	0.18	0.56	1.00
4	0.20	0.53	0.60	0.95
5	0.11	0.25	0.46 ^b	0.71
6	0.10	0.17	0.27	1.13
7	0.24	0.15	0.60	1.00
Combined	0.13	0.21	0.46	0.93

^a Hypothetical ratio of endotoxin content with respect to reference E-1.^b Three replicates.TABLE 3. *SD of log₂ ratios by vaccine and laboratory*

Laboratory no.	Vaccine				Overall SD
	1 (0.125)	2 (0.25)	3 (0.5)	4 (1.0)	
1	0.47	0.96	0.70	0.50	0.69
2	0.00	0.58	0.00	0.16	0.30
3	0.50	0.57	0.57	0.00	0.47
4	1.05	0.92	0.50	0.16	0.75
5	0.16	0.00	0.19	0.58	0.32
6	0.00	0.50	1.61	0.57	0.89
7	1.07	0.96	0.50	0.82	0.86
Pooled	0.63	0.72	0.77	0.48	0.66

TABLE 4. *99% upper limit of SD of log₂ ratio*

Sample size (n)	Upper limit ^a
4	1.28
8	1.06
12	0.99
16	0.94
20	0.91
24	0.89
28	0.87

^a For conversion to log₁₀, multiply by 0.3.TABLE 5. *GM ratio of a sample size n with 95% upper limit fixed at 1.0*

Sample size (n)	GM ratio
4	0.68
8	0.76
12	0.80
16	0.83
20	0.84
24	0.86
28	0.87

tests, only a gel firm enough to maintain its shape when inverted was recorded as positive; everything else was negative.

The degree of variability experienced by the seven testing laboratories in the collaborative study was used to develop a statistical approach to the evaluation of endotoxin content data. The overall pooled estimate of the SD $\log_2$ ratio of the endotoxin content of influenza virus vaccine and reference E-1 is 0.66, with 83 df.

The degree of variability experienced by some laboratories was surprising. However, no laboratory was able to identify any factors on particular days that may have contributed to increased variability. No data were discarded in this study. It is important when assessing the true variability of an assay procedure that all values be included, even though there may be a temptation to exclude a few so-called aberrant results.

The data obtained from this study may be used to establish quality control procedures for testing the endotoxin content of each lot of influenza virus vaccine relative to the reference vaccine by the manufacturer of the vaccine. For this purpose, it is proposed that a minimum of 4 and a maximum of 28 replicate LAL tests be done on each lot of vaccine in parallel with reference E-1. The results of all tests performed should be used. The ratio of the endotoxin content of the test vaccine and reference E-1 should be transformed to $\log_2$, and then the SD and GM should be calculated. If the calculated SD is greater than the upper limit for the initial four replicates given in Table 4, the quality of the testing procedures of the laboratory is in doubt and four more replicate tests should be done. The SD of the log ratio should be recalculated, using the results of all tests performed. If after 28 replicate tests the SD is still above the value in the table for 28 tests, it must be concluded that the quality of testing by that laboratory is not within sufficient control to yield homogeneous values. The sources of variability should be determined and eliminated before any official testing can be performed.

If the SD for a given number of replicate tests is below the value in Table 4, the GM ratio may be assumed to be an accurate estimate of endotoxin content in the test lot relative to reference E-1.

If the GM ratio from the total number of tests having an acceptable SD is less than or equal to the value given in Table 5 corresponding to the number of tests performed, statistically there is at least a 95% chance that the test lot does not contain an endotoxin content greater than that in the reference vaccine. If it is greater than the value given in the table, another series of four

pairs of LAL tests (up to a maximum of 28) may be done, and the above steps on SD and GM should be repeated, using results from all tests.

CONCLUSION

The LAL test can be used for quantitating the content of endotoxin in influenza virus vaccines manufactured by current procedures. By testing a series of twofold dilutions of a product, an end point can be determined which may be expressed either in terms of the highest dilution yielding a positive reaction or in terms of endotoxin equivalents based on a reference endotoxin used to determine the sensitivity of the same lot of lysate used in the test. The endotoxin equivalent may be obtained by multiplying the highest dilution yielding a positive reaction by the minimum concentration of the reference endotoxin detectable by the lot of lysate used in the test (2). If a sample of any product containing a known amount of endotoxin is established as a reference, it may be titrated in parallel with each test lot of that product, and the ratio of the two end points can be used to evaluate the endotoxic purity of the product. This is a useful quality control measure for many bacterial vaccines and some viral vaccines such as influenza virus vaccine. The degree to which a laboratory may reproduce the ratio obtained for a given lot and the ability of another laboratory to confirm the ratio depends upon the variability of the individuals performing the tests.

By use of the figures in Table 4, testing that exceeds this degree of variation may be detected and invalidated. If the variability obtained in replicate testing is acceptable as judged by Table 4, equivalence of the GM to a ratio of 1 or less may be determined by the use of Table 5.

As data are collected from testing a given product within a laboratory, it should be possible to lower the upper limit of the standard deviation of the ratios and construct new Tables 4 and 5. This procedure provides a method for establishing a dynamic quality control mechanism for maintaining maximum precision in testing.

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LITERATURE CITED

1. Hochstein, H. D., R. J. Elin, J. F. Cooper, and E. B. Seligmann, Jr. 1972. Further developments of limulus amoebocyte lysate test. *Bull. Parenter. Drug Assoc.*, 26:153.
2. Jorgenson, J. H., J. C. Lee, and H. R. Pahren. 1976.

- Rapid detection of bacterial endotoxins in drinking water and renovated wastewater. *Appl. Environ. Microbiol.* 32:347-351.
3. Jorgenson, J. H., and R. F. Smith. 1973. Rapid detection of contaminated intravenous fluids using the *Limulus* in vitro endotoxin assay. *Appl. Microbiol.* 26:521-524.
 4. Levin, J., P. A. Tomasulo, and R. S. Oser. 1970. Detection of endotoxin in human blood and demonstration of an inhibitor. *J. Lab. Clin. Med.* June:903-911.
 5. Public Health Service. 1976. Food and drug regulations for biological products. Publ. no. 21 CFR 610.11a. Washington, D.C.
 6. Public Health Service. 1976. Food and drug regulations for biological products. Publ. no. 21 CFR 610.13(b). Washington, D.C.
 7. Snedecor, G. W., and W. G. Cochran. 1967. Statistical methods, 6th ed. Iowa State University Press, Ames.