Pertussis Vaccine Testing for Freedom-from-Toxicity

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ABSTRACT

PITTMAN, MARGARET (National Institutes of Health, Bethesda, Md.), AND CLAIRE B. COX. Pertussis vaccine testing for freedom-from-toxicity. Appl. Microbiol. **13**:447–456. 1965.—The results of 9.5 years of official testing of vaccines containing pertussis vaccine, plain or adsorbed with alum, Al(OH)₃, or AlPO₄, are reported. Toxicity was evaluated by weight changes in mice at 3 and 7 days after injection and intercurrent deaths. Toxicity was encountered during the early use of AlPO₄ in pertussis vaccine products, with a special product and quadruple-antigen vaccines. Throughout the study Al(OH)₃ products, few in number, were the least reactive of the adsorbed products. The slopes of regression lines of graded-dose responses reflected variations in reactivity of "nontoxic" vaccines. The U.S.-prescribed test is discussed relative to the (i) reactivity in children, (ii) causes of toxicity, (iii) other assays for pertussis vaccine toxicity, and (iv) the use of a reference vaccine in the toxicity test.

Bordetella pertussis is unique among infectious bacteria in its marked ability to modify biological responses. (See reviews, Pittman, 1957; Kind, 1958.) Hence, there has been special concern that pertussis vaccine be as free as possible from reactive factors and yet provide adequate protection against whooping cough. Among the known reactive substances are a heat-labile dermonecrotic toxin, an endotoxin which is common to all gram-negative bacteria and specific and nonspecific sensitizing factors, including the histamine-sensitizing factor (HSF). Although not proven, HSF has been suggested as being a contributor to the neurological symptoms sometimes observed in whooping cough and after vaccination. Fortunately, encephalopathy after vaccination is relatively rare.

At a Round Table Conference on Pertussis Immunization, Prague, Czechoslovakia, 1962, as well as at a Pertussis Vaccine Symposium, Bethesda, Md., 1963, emphasis was placed on untoward reactions that may be induced by pertussis vaccine in man, the lack of a clear definition of the nature of the cause of these reactions, and the need for laboratory tests that would be precisely correlated with the chemical and antigenic properties of the components of the vaccine and the correlation of these assays with untoward reactions in children.

The purpose of this report is to give the experience of the Division of Biologics Standards (DBS) in toxicity testing of products containing pertussis vaccine and to discuss the causes of toxicity, the relation of laboratory assays to un-

toward reactions in children, and proposed changes to provide better precision in the toxicity test.

Methods of assay for toxicity were studied during the development of U.S. requirements for pertussis vaccine more than 15 years ago. With use of experimental vaccines, it was found that loss of rabbit dermonecrotic toxicity of the vaccine was correlated with weight gain in mice (Pittman, 1952). In general, when 1.0 opacity unit (OU) failed to induce dermonecrosis in rabbits, mice injected intraperitoneally with 10 OU weighed no less than initially at the end of 72 hr and more than initially at the end of 7 days, and without intercurrent deaths. (The opacity of the U.S. Opacity Standard was adjusted to be equivalent to the opacity of aged pertussis vaccines containing 10,000 million bacteria per milliliter as determined by direct count. A freshly prepared suspension of bacteria of equivalent density contains about one-third fewer bacteria. The opacity of a vaccine is measured within 2 weeks after harvest of the bacteria and usually within 48 hr.) These findings were the basis for the U.S. test for freedom-from-toxicity in which mice are used as the test animal. Three test doses have been prescribed. From 1949, when the U.S. requirements for pertussis vaccine were adopted, to 1953, the test was performed with 7.5 OU of the bulk bacterial suspension. In 1953 the dose was changed to a specified portion of the total human immunizing dose (THD) and in 1961 to a specified portion of the single human dose (SHD).

The results of the toxicity tests performed in the Division from February 1954 to August 1963 are reported in this paper. During this period, several summaries and analyses of results were prepared to provide information for the manufacturers to point up the influence of $AlPO_4$ on mouse toxicity and to substantiate a revision of the test. The results of these analyses covering periods of 1.5, 5, and 1 years and a subsequent 2-year period following the 1961 revision of the test are presented. Particular attention is given to the relative effect of the different aluminum adjuvants on toxicity and to the experience with the 1961 revised test, which, because of more stringent specifications, has influenced greater variation in assay results between DBS and manufacturers.

MATERIALS AND METHODS

Mice. Ten male mice of the NIH-BS strain, each weighing 14 to 16 g, were used per test or per group in a test.

Vaccines. The vaccine samples were from final lots of single or multiple antigen products containing pertussis vaccine, submitted for release, in support of license application or obtained on annual inspection of an establishment. Inspection sample results were included only in the data obtained between February 1954 and August 1955 (first period). At the beginning, 15 establishments held a U.S. license for manufacture of pertussis vaccine; at present there are 12. Confidential nature of the results precluded identification of the manufacturers. Further, changes have been made in preparation of some products, and others are not extant.

The plain vaccines (nonadsorbed) contained 40 to 75 OU per THD, and the adsorbed, 30 to 48 OU. For the past several years, only one plain product has contained more than 65 OU. Some products contained only pertussis vaccine (P), others contained diphtheria toxoid (DP), but the majority contained both diphtheria and tetanus toxoids (DTP), whereas a few contained poliomyelitis vaccine (DTP-P). The latter will be designated specifically. A large majority of the products contained an aluminum salt adjuvant: alum, $Al(OH)_3$, or $AlPO_4$. The term adsorbed is used irrespective of whether an antigen was adsorbed to or precipitated by the adjuvant.

Procedures of the test. The test was performed as prescribed in Minimum Requirements: Pertussis Vaccine, May 1953, and revision, August 1961. The mice, having had free access to food and water, were weighed as a group immediately prior to intraperitoneal (ip) injection of the vaccine and at the end of 72 hr and 7 days. In addition, the group weight was observed at the end of 24 hr and in the first period at the end of 14 days. Also, in the first period the plain vaccine was injected intravenously (iv) into a second group of mice. The vaccine diluted in saline was injected in 0.5-ml volume. Control mice were given 0.5 ml of saline. From 1953 to August 1961, the dose for adsorbed vaccine was one-fifth THD, and for plain vaccine, one-tenth THD; each dose usually contained 6 to 9 OU. After August 1961, the test dose was one-half SHD for either type; each dose of adsorbed vaccine contained approximately 5 to 8 OU, and the plain vaccine up to 10 OU. (The bulk bacterial suspension was tested by the manufacturer at a dose of 10 OU.) The average weight change per mouse was calculated from the difference between group weights at the time of injection and at the observed period. Deaths were recorded at 7 days and also at 14 days in the first period analyzed.

A vaccine was accepted as free from toxicity if at the end of 72 hr the group weight of the mice was no less than initially, and at the end of 7 days, preceding the 1961 revision, the group weight was greater than initially and there were no vaccine-related deaths, and after the 1961 revision, the average mouse weight gain was no less than 3.0 g and mortality was no greater than 5%.

If a lot failed to pass on the first test, the test(s) was repeated. All results were used to determine acceptance or rejection of the lot. Repetitions are reflected in the results given below where the average number of mice per lot exceeded 10.

Graded dose response. Groups of mice were given graded doses of a vaccine and observed as for the single-dose test. Dose-regression curves were calculated for the 7-day weight gains by the formula $y = a + bx$ with a log transformation of dose.

RESULTS

Saline control mice. Table 1 gives the yearly average weight gains and deaths of the control mice (the first period covered 18 months) and the averages for each of the four periods analyzed. From 1954 to 1960 the weight gains remained relatively constant. From 1960 to 1963 the mice showed a greater gain each succeeding year. The increase in the 7-day weight gain from the first to the last period, 3.96 versus 6.06 g, was significant.

Toxicity results from February 1954 to August 1955. After the revision of the toxicity test in 1953, some lots of a few manufacturers failed. The new test dose contained no more and sometimes fewer bacteria than formerly. To find the cause, all lots of vaccine submitted for 18 months were tested for toxicity. There were 280 lots, 74 plain and 206 adsorbed; 23 of each type were inspection samples. $AlPO_4$ had been in use only a short time. The plain vaccines were injected iv as well as ip. D. G. Evans (*personal communication*) had used the iv route with some of the British field trial vaccines (Medical Research Council 1956, 1959).

The combined results of the tests of the lots of plain vaccines of all manufacturers, with the two

TABLE 1. *Weight gain of saline control mice during 9.5 years of toxicity testing*

Dates	No. of tests	No. of mice	Avg wt gain (g)				Deaths	
			24 hr	72 hr	7 days	14 days	7 days	14 days
From 9 February 1954 to 16 August 1955	58	577	0.39	1.63	3.96	6.99	4 (0.69%)	6 (1.04%)
1956*	29	290	0.36	1.85	4.43	7.5	0	0
1957	24	240	0.44	1.75	4.25	7.4 (8)†	0	0
1958	27	270	0.48	1.76	4.50	—	0	—
1959	33	330	0.33	1.77	4.49	—	0	—
1960	34	340	0.45	1.84	4.69	—	1 (0.29%)	—
5 Years average	147	1,470	0.41	1.80	4.49	7.49 (37)†	1 (0.07%)	0
1961	32	320	0.62	2.20	5.30	—	1 (0.31%)	—
1962	36	359	0.74	2.58	5.99	—	2 (0.56%)	—
1963	29	290	0.68	2.64	6.13	—	0	—
2 Years average	65	649	0.71	2.61	6.06	—	2 (0.31%)	—

* The time period begins on 16 August of the preceding year and runs until 16 August of the year given.

† Number of tests which differ from number observed at 7 days is enclosed in parentheses.

TABLE 2. *Influence of route of injection on response of mice to plain vaccines containing pertussis vaccine**

Route of injection	No. of lots	No. of mice	Avg wt change (g)				Deaths	
			24 hr	72 hr	7 days	14 days	7 days	14 days
ip	74	810	−0.79	1.04	3.55	6.96	14 (1.73%)	26 (3.21%)
iv	74	800	−0.37	0.90	2.71	6.11	27 (3.38%)	51 (6.38%)

* Period of observation was from February 1954 to August 1955. Test dose was one-tenth THD.

routes of injection, are given in Table 2. Each average weight change was calculated from the sum of the average weight change per manufacturer's product. The average gain per product at the end of 7 days after ip injection ranged from 2.2 to 4.7 g. For only 2 of 14 products was the average less than 3.0 g; these were 2.2 and 2.77 g. The iv injection had a greater adverse effect than the ip injection. At 24 hr, the ip mice showed a greater loss; thereafter the iv mice weighed less and mortality was two times greater at 7 and 14 days.

Table 3 shows that the adjuvants differed in effect on the number of deaths. AlPO$_4$ was the most harmful. Table 4 gives the results for each manufacturer's adsorbed product that was being made at that time. The data show that some products containing the same amount of adjuvant were similar in toxicity, whereas others were different, that concentration of an adsorbent in a single product significantly affected toxicity (last column of the table), and that adsorbents differed in influence on mouse toxicity. Alum products in general and the Al(OH)$_3$ products

TABLE 3. *Influence of adjuvants on death of mice**

Adjuvant†	No. of lots	No. of mice	Deaths			
			7 days		14 days	
			No.	Per cent	No.	Per cent
Alum.........	108	1,160	5	0.43	15	1.29
Al(OH)$_3$......	19	220	3	1.36	5	2.27
AlPO$_4$........	79	1,060	39	3.68	62	5.85

* Period of observation was from February 1954 to August 1955. Test dose was one-fifth THD.

† Adjuvant in P, DP or DTP.

did not differ in toxicity. AlPO$_4$ products were more toxic. Within a product, toxicity was related to concentration of AlPO$_4$, but it was not, necessarily, between products. For example, Code 2 product containing 9.70 to 9.73 mg alum equivalent of AlPO$_4$ per SHD was no more toxic than Code 11 product containing 4.40 to 4.51 mg, and less toxic than Code 7 which contained

TABLE 4. *Influence of adjuvants on weight and death of mice**

Manufac-turer code	Alum equivalent (mg/SHD)	No. of lots	No. of mice	Avg wt change (g)				Deaths		Wt change regression	
				24 hr	72 hr	7 days	14 days	7 days	14 days	Grouped	Scatter graph
	Alum										
11	2.04– 2.98	4	40	−0.75	1.37	4.5	7.1	0	0		
	3.03– 3.99	7	70	−0.98	0.97	4.21	6.67	0	0	+†	+
	4.03– 5.13	5	60	−1.10	0.88	3.8	6.44	0	1		
3	2.4 – 2.5	4	40	−1.20	0.85	3.2	4.72	0	0	Low range	
4	2.5 – 2.7	6	60	−1.28	0.75	3.98	6.7	0	1	−	−
	3.4 – 3.75	5	50	−0.80	1.06	3.88	6.5	0	0		
6	2.05– 3.35	4	40	−0.70	1.12	3.78	7.62	0	0	−	−
	3.5 – 4.1	4	50	−0.70	1.21	4.34	6.91	1	1		
8	1.36– 5.39	4	40	−1.19	0.66	3.39	5.98	0	0	±	−
	7.55–11.89	3	40	−1.28	0.45	3.68	5.87	1	1		
10	8.0 – 9.0	2	20	−1.22	0.92	4.12	6.42	0	0	Low range	
12	2.5 – 3.5	10	100	−0.97	1.06	3.79	6.99	0	1	Low range	
13	1.63– 5.93	13	150	−1.03	0.8	3.35	6.95	2	4	−	−
	6.07– 8.79	15	150	−1.03	0.73	3.13	6.41	1	5		
15	0.65– 1.97	5	50	−0.9	1.34	4.3	7.34	0	0	Low range	
	Al(OH)₃										
3	3.63– 3.82	9	100	−0.7	1.24	4.05	7.13	1	3	+	+
	7.2 – 7.5	4	40	−1.25	0.68	3.8	6.78	0	0		
	AlPO₄										
1	1.73– 2.7	16	170	−1.04	0.76	3.51	6.60	1	2	+	−
	3.2 – 7.62	5	80	−1.00	0.48	2.93	5.76	11	12		
2	9.70– 9.73	14	150	−1.18	0.74	3.55	6.96	1	1	+	+
	13.6	13	170	−1.2	0.17	2.85	6.14	12	16		
7	2.1 – 2.94	15	200	−0.84	0.82	3.16	6.18	6	11	Low range	
11	4.40– 4.51	2	30	−0.82	1.52	3.42	7.18	0	1	Low range	
13	7.03– 9.68	5	110	−1.38	0.77	3.88	7.08	3	9	Low range	

* Period of observation was from February 1954 to August 1955.

† Indicates significant difference in weight change relative to concentration of adjuvant in the product.

only 2.1 to 2.94 mg. The AlPO₄ toxicity problem was resolved largely by reducing the adjuvant content in the individual manufacturer's products. Thereafter, AlPO₄ vaccines were no more toxic than other adsorbed vaccines. This is shown below in subsequent analyses.

Toxicity results from 1955 to 1963. The analyzed data for the 8 years were divided in periods of 5, 1, and 2 years. The last period covered 2 years of experience with the latest revision of the test. Not all, but a large percentage of, lots submitted were tested. Lots of products which consistently passed the toxicity test were examined slightly less often than those showing some irregularity in toxicity. Hence, data, if biased, would be toward greater mouse reactivity. Besides weight changes and deaths, dose response regression curves of plain vaccines and of each type of adsorbed product were plotted.

The 7-day weight gains after injection of plain vaccines for the three periods were 3.8, 4.65, and 4.52 g, respectively. Figure 1 shows that the increases in weight gains paralleled the increases of the control mice except for the last 2-year period. The latter reflected the change in the test dose from one-tenth to one-sixth THD (one-half SHD). Mortality also reflected this change. In 1954 to 1955 the mortality was 1.73%; from 1955 to 1961, 2.21%; and from 1961 to 1963, 4.04%.

Table 5 shows that weight gain and death of mice that received P, DP, or DTP containing any one of the three adjuvants were similar except for one alum product. The lack of toxicity of the AlPO₄ vaccines, obtained after adjustments in concentration of AlPO₄, are in contrast to those given in Tables 3 and 4. The new alum product which was under development in the latter part of the 5-year period was significantly more mouse-reactive than the customary alum
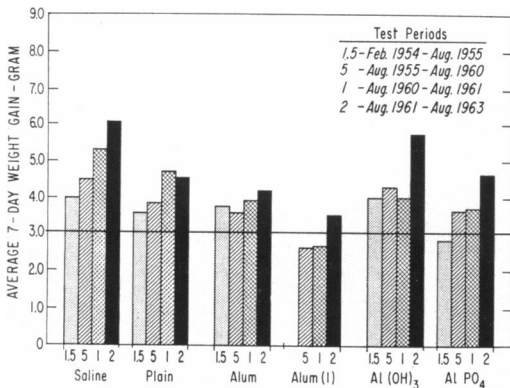
FIG. 1. *Effect of plain and adsorbed vaccines on average 7-day weight gain of mice in four test periods covering 9.5 years. Alum (1) designates a special adsorbed product.*

FIG. 2. *Average weight gains and deaths of mice induced by varying doses of seven lots of vaccine; 50 mice per dose per lot were used, and each point on a line represents 350 mice.*

TABLE 5. *Influence of adjuvants and poliomyelitis vaccine on weight gain of mice**

Adjuvant	No. of lots	No. of mice	Avg wt change (g)			Deaths
			24 hr	72 hr	7days	
						%
Alum†	107	1,590	−1.24	0.55	3.59	3.33
	7‡	340	−0.96	0.48	2.63	12.06
Al(OH)₃†	31	420	−1.05	1.02	4.28	2.86
AlPO₄†	276	3,850	−1.16	0.74	3.60	3.32
Alum or AlPO₄§	77	1,410	−1.46	0.51	3.15	4.96

* Period of observation was from 1955 to 1960. Test dose was one-fifth THD.
† Adjuvant in P, DP or DTP.
‡ A new product under development.
§ Adjuvants in DTP-P.

products (Table 5). Toxicity was marked by slow weight gain after 3 days and late deaths. During the last 2-year period the weight gains were satisfactory.

As a whole, the DTP-P lots were more toxic than those of the other products. Certain lots were unsatisfactory for release.

Figure 1 gives an overall picture of the pertussis vaccine products, except DTP-P, for the four periods studied. It is of special interest to note that the average weight gains for all types of products (except for the AlPO₄ products in the 1.5-year period and the special alum product) prior to the reduction in test dose in 1961 would have satisfied the 1961 specified weight gain of 3.0 g. Weight gains for the customary alum products have remained remarkably constant.
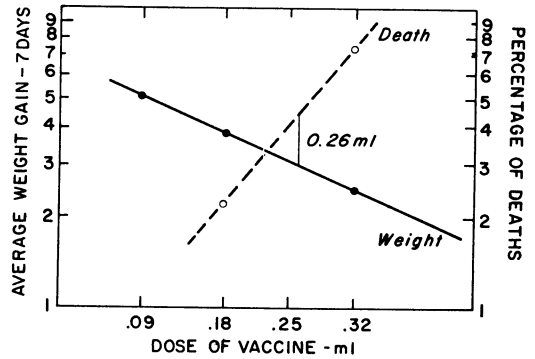
In the last period, the Al(OH)₃ and AlPO₄ products have shown an increase in weight which, no doubt, was influenced by the decrease in test dose, 0.3 to 0.25 ml, and an increase in the growth rate of the mice as evidenced by weight gain of the control mice (Table 1). Al(OH)₃ products have been the least reactive.

Graded dose response. The sensitivity of mice to small changes in test doses of borderline toxic vaccines is illustrated by the regression dose curves in Fig. 2, which were calculated from data given by Gerwe (1961). The intersection of the weight line and 3.0 g is at 0.26 ml and the mortality is 4.3%. A vaccine of this reactivity would be acceptable. However, a vaccine so near the lower limit of acceptability would need to be tested with 50 or more mice in order to prevent rejection of a nontoxic vaccine or acceptance of a toxic vaccine. Fortunately, very few lots submitted for release are of borderline toxicity.

In 1962–63, graded dose responses of each manufacturer's product were determined. No fewer than three lots per product were tested, with use of three doses and 30 mice per dose per lot. The weight gains at 7 days for similar products were combined and plotted (Fig. 3). The lines of the four types of products were parallel. The individual products, however, showed differences in reactivity (Fig. 4). Three products did not induce graded responses. This indicated no reactivity at the test doses. The other products gave varied graded responses. The steepest slopes indicating greatest toxicity were obtained with two AlPO₄ products. With each product the weight gain was greater than 3.0 g not only at
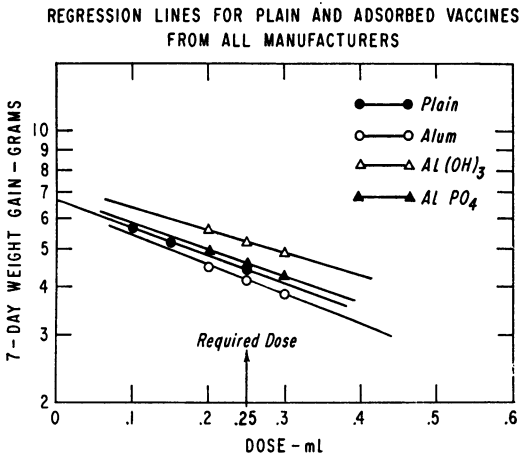
REGRESSION LINES FOR PLAIN AND ADSORBED VACCINES
FROM ALL MANUFACTURERS

FIG. 3. *Dose-response regression lines calculated from the combined results obtained with all plain and adjuvant pertussis products, respectively.*
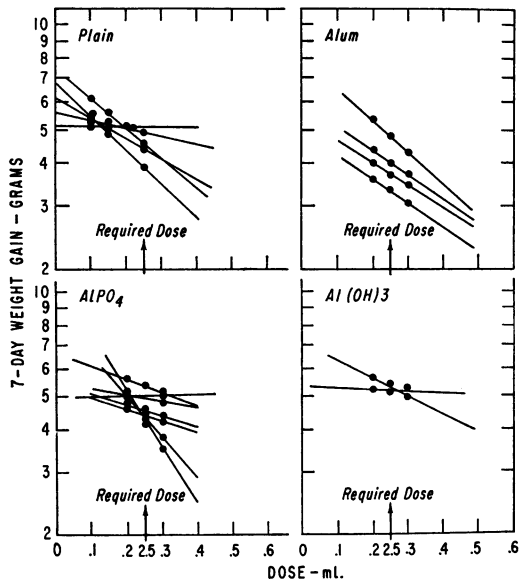

FIG. 4. *Dose-response regression lines of individual products calculated from the combined results of not less than three lots per product.*

FIG. 5. *Test results of individual lots of two products plotted against the median lines of lots of the respective products for three periods of testing.*

the 7-day weight gains obtained with consecutive lots of the product, after computation of the regression curve, were plotted on the chart. Charts for two products are shown in Fig. 5. It is obvious that the lots of these two products were not of borderline toxicity. Other products showed similar results. In the past three years, only three lots have been rejected because of toxicity. Two were plain vaccines; the other was AlPO$_4$-adsorbed.

DISCUSSION

It has been shown in this report that within our laboratory reproducible results in toxicity testing have been obtained and that the test has differentiated certain products with mouse toxicity greater than that of the products in general. Without doubt, the test has contributed to the control of toxicity of pertussis vaccine. However, with the 1961 revision, which specified more stringent requirements for freedom-from-toxicity, some difficulty has been encountered in reproducibility of results between laboratories. For background to discuss the significance of the toxic factors and toxicity assays of pertussis vaccine and their relation to untoward reactions in children, and the methods that might increase reproducibility of results between laboratories, a brief resumé of pertinent information on *Bordetella pertussis* toxicity is given.

In the preparation of pertussis vaccine there is a delicate balance between detoxification and

the required dose of 0.25 ml but also at 0.3 ml, the required dose before August 1961.

Consistency among lots of a product. The median 7-day weight gains for the lots of each product for the periods 1955 to 1960 and 1960 to 1961 were plotted as straight lines on a chart. For the period 1961 to 1963, a third line, which represented the weight gain at the 0.25-ml dose level on the regression curve, was drawn. Then
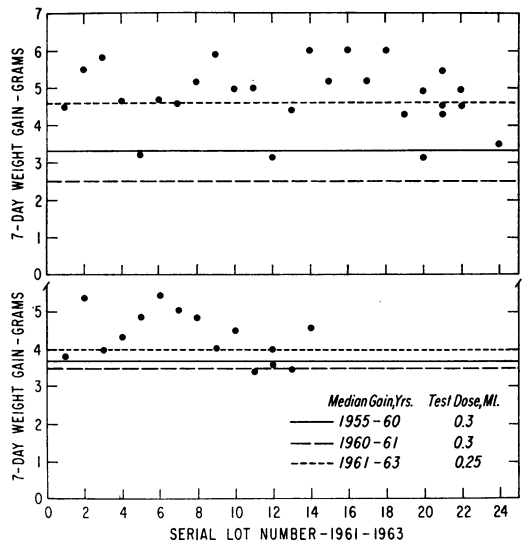
preservation of potency. Control of toxicity would be facilitated if there were satisfactory answers to the following questions: (i) Is mouse-measured toxicity correlated with untoward reactivity in children? (ii) What are the best methods to produce a vaccine of minimal reactivity for children? (iii) What laboratory assays best reflect toxicity? (iv) Does HSF play a role in reactivity? (v) How can variability in toxicity test results between laboratories be controlled?

Information on the relation of animal assays to reactions in children is inadequate. Cohen (1963) reported two instances in which the mouse test reflected a difference in the reactivity of vaccines for children. Toxic reactions occurred more frequently both with AlPO$_4$-adsorbed DTP (16 OU/SHD) than with plain P (20 OU/SHD), and with plain vaccine prepared with bacteria grown in a fluid medium than with plain vaccine prepared with bacteria grown on solid medium.

Using two vaccines that passed the toxicity test, J. A. Bell and M. Pittman observed that the percentage of children with temperature equal to or greater than 101.6 F was slightly higher with a vaccine that showed in mice gradations in weight-gain response related to size of test dose than with another vaccine in which response was not graded (Reported by M. Pittman, Prague Conference, 1962).

On the other hand, the Research Committee on Pertussis Vaccine, Japan, failed to show a correlation between results of several laboratory tests (but different from the U.S. test) and temperature rises in children measured 24 hr after injection (reported by J. Kaneko, Prague Conference, 1962).

Production of satisfactory pertussis vaccine is influenced by the interplay of many factors: bacterial strain(s), medium, method of detoxification, preservative, adjuvant, and inherent factors within a laboratory. A few selected but not inclusive references are cited. Bacterial strains vary in protective antigenicity (Kendrick, Updyke, and Eldering, 1949). There is inadequate information on protective activity of strains that differ in toxicity and the influence of these strains on toxicity of final product. Cohen's observations on the greater toxicity of vaccines prepared from bacteria grown in a liquid medium versus bacteria grown on a solid medium were cited above. The former was also more potent. Age of bacteria at time of harvest, no doubt, has an effect on the permeability of the cell wall, thereby influencing toxicity. Detoxification by heating for 30 to 60 min at 56 C is more effective than heating for several days at 37 C (Pittman, 1952; Cohen, 1963). Besides effect on dermo-

necrotic toxin, the heating at 56 C may inhibit deleterious enzymatic activity. Vaccine heated at 35 C and preserved with Merthiolate retained potency better than did unpreserved vaccine (Gardner, 1964).

Recent work has shown that pertussis vaccine in DTP-P preserved with benzethonium chloride is unstable in potency (Olson, Eldering, and Graham, 1964; Gardner, 1964). This surface-acting preservative, no doubt, contributed to the greater toxicity of DTP-P reported in this paper and by Pittman (1962) by favoring the leaching of the toxin from the bacterial cell. It is well known that alkalinity favors lysis and thereby promotes toxicity. Niwa, Yamadeya, and Kuwajima (1964) reported the influence of various substances on leakage and inhibition of leakage.

Merthiolate apparently acts as a stabilizer of potency (Gardner, 1964). It is the most effective preservative known for pertussis vaccine. In its presence vaccines stored at 4 C gradually decrease in toxicity. This, however, may require several months (e.g., Pittman, 1952, and others). Formalin is effective in detoxification, but it can have an adverse effect on potency (Pittman, 1952; Joó, Pusztai, and Juhász, 1961; *unpublished results*). However, with precaution, it can be used without apparent deleterious effect (Pekárek and Moheská, 1961).

Results in this report show that aluminum salt adjuvants differ in their effect on the mouse toxicity of pertussis vaccine. AlPO$_4$ has caused the greatest difficulty. Differences within a manufacturer's product, but not necessarily between manufacturers' products, were related to the amount of adjuvant present. Cohen (1963) observed that AlPO$_4$ vaccine, although low in reactivity in children, was more so than plain vaccine. Joó et al. (1961) showed that the strong alkalinity that arises when the AlPO$_4$ gel is produced *in statu nascendi*, as contrasted with use of preformed AlPO$_4$ gel, affects potency. The alkalinity, which may reach pH 14, would favor toxicity.

Differences in potency from lot to lot of a manufacturer's product (e.g., Cohen, 1963) and lack of reproducibility between manufacturers using the same procedure show that there are unknown factors in the production of pertussis vaccine (*personal observations*).

In addition to the U.S. toxicity test, other tests have been reported. Dermonecrotic (labile) toxin has been determined by skin testing of rabbits and suckling mice (Katsampes, Brooks, and Bradford, 1942; Andersen, 1952). In a comparison of the suckling mice test with the intraperitoneal injection of adult mice (U.S. test), Andersen (1958) found that the latter not only

acted as a measure of the effect of the known toxin but also of less well defined toxic substances which cause a decrease in resistance of mice to histamine and a homologous sensitization.

In the early work of the National Institute of Public Health in the Netherlands, toxicity was measured by response of guinea pigs to intraperitoneal injection of vaccine; this was followed by a guinea pig subcutaneous test (Cohen, 1963). Cohen reported, however, that the U.S. test was more sensitive in discovering traces of toxin.

Pekárek and Řezábek (1959) and J. Pekárek et al. (Prague, 1962) determined toxicity by titration of vitamin C clearance from rat adrenals. Results correlated with rat foot edema. Maximal reactions of both tests were obtained at 4 hr. Results of these tests also correlated with LD_{50} of the vaccine, but not necessarily with rabbit skin reactivity. The supernatant liquid of a fluid culture which caused decrease in vitamin C had no effect on the rabbit skin, whereas the sedimented bacterial cells innocuous in the stress test caused rabbit skin necrosis. We consider that the latter difference was due, at least in part, to the availability of the toxin. The toxin in the bacteria would not be readily available for the stress measurement, whereas it would be liberated from the bacteria localized in the skin. These authors liken the reactivity measurement by vitamin C depletion to an endotoxin effect, and emphasized that this test does not measure the allergic type of reactivity. Their work brings to focus the complexity of the reactivity of *B. pertussis*.

Kurokawa et al. (1962*a*, *b*) and Kurokawa, Ishida, and Kuratsuka (1963) developed a mouse test in which the estimation of the toxicity of a vaccine is made by comparison with a reference preparation, and toxicity is expressed relative to the reference. Vaccines showing a difference by their proposed criteria for acceptability showed no difference in reactivity in children (Research Committee on Pertussis Vaccine, Japan, reported by J. Kaneko, Prague, 1962). The difference measured by their mouse test may have been that between relatively atoxic vaccines, and the difference between proposed relative values for designating toxic and nontoxic vaccines may have been too small.

Pyrogenicity testing has been used with experimental preparations (Billaudelle et al., 1960; Barta, 1963). Correlation with human reactivity has not been reported.

The role of the HSF in reactivity of children remains an enigma. This factor is closely associated with the protective antigen. Munoz (1963) considered that the two form an antigen complex. However, in certain preparations the two factors appear in different proportions (Pittman, 1951, and Prague 1962; Cohen, 1963). Dolby (1958) has claimed separation. All commercial vaccines, however, do have HSF, and the question arises, should there be a prescribed maximal level of acceptability? By the specification of an upper limit on the estimated potency of a vaccine, the amount of HSF in U.S. vaccines has, to some extent, been restricted.

In spite of limitations of an absolute test without a base line of reference, the lack of agreement between DBS and the manufacturers in the assessment of toxicity has been limited largely to individual products and then only with lots of borderline toxicity. As a whole, vaccines have been and are well above the lower limit of acceptability. However, with the 1961 revised requirements, the limitations of an absolute test have become more prominent.

Mice of different strains vary or change in response to the vaccine. With the same dose of a common vaccine, but with different strains of mice, seven manufacturers observed mortality ranging from 4 to 43% (H. D. Piersma, Prague, 1962). With NIH-BS mice we obtained 2% mortality (*unpublished data*). Andersen (1952), Kurokawa et al. (1962*a*), and others reported variations in toxicity response of different strains of mice. Mice with a high degree of toxin susceptibility also respond poorly to immunization (Pittman, 1962). That a strain of mouse may change in weight response was shown in this report by a significant increase in the rate of growth of the saline control mice. Such a change would tend to influence the acceptance of vaccines when a specified weight gain is required. Whether or not this change was due to improved air-conditioned quarters in 1960 or a change in diet is not known. Cohen (1963) found food to be a significant factor.

Although the need for a toxicity reference vaccine has been obvious for some time, its selection has been deterred by the lack of knowledge of what toxic substances should be present. Should the vaccine contain thermolabile toxin, endotoxin, sensitizing reactive factors, and, if so, in what proportion should each be present?

All vaccines induce a slight weight loss which is usually regained within 3 days. Thereafter, with some vaccines, there is a steady increase in weight. With a few, weight gain is slow or nil and late deaths occur. The latter type of reaction is different from that induced by other gram-negative bacterial vaccines when compared on an equivalent endotoxin basis. This reaction may be caused by a sensitizing factor. C. G. Culbertson

(*personal communication*), using germ-free and conventional mice, found that death in the latter is associated with intestinal ulcers. He recommended termination of the test at 3 days. However, late deaths occur only with certain lots and may be an important indication of undesirable stress or sensitizing factors.

Another deterring factor in the selection of a reference preparation has been the lack of information on the correlation of laboratory assay results with untoward reactions in children, and, also, what toxic component is responsible for human reactivity. Nevertheless, two vaccine preparations have been dried, and their toxic properties are being characterized by different assay procedures.

It is anticipated that a cooperative field study will be initiated after a base line of reference has been established. It is hoped that the clinical study will determine whether or not there is a correlation between the laboratory assay and untoward reactions in children.

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