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A Study of the Antigenicity of Strains of Vibrio Cholerae

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A STUDY OF THE ANTIGENICITY OF STRAINS OF VIBRIO CHOLERAE

By

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Contents

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Introduction

Public health workers in those countries where cholera is prevalent are convinced of the value of vaccination as a prophylactic measure against the disease. The significance of most statistical investigations concerning cholera vaccination has been open to question because of the unequal numbers of persons in the vaccinated and control groups, the unequal time intervals between vaccination and the outbreak of epidemics, and unequal risks of exposure to cholera of the persons within the two groups. The investigations of Greenwood and Yule (1), whose subjects were members of the Sanitary Corps and other combatant groups, appear to be above these criticisms. These authors offer support for vaccination in the form of statistical data.

* The work reported in this paper was carried out in the Biologics Laboratory of the Michigan Department of Health, Lansing, while the author was on study leave as a Research Fellow under the cultural cooperation program of the United States Department of State.

Additional support for anti-cholera vaccination is offered by immunological studies concerned with the bactericidal and agglutinative properties, and mouse protective antibodies present in the sera of persons who have been vaccinated against the disease (2, 3, 4, 5).

Burrows and his co-workers (6) have pointed out that Vibrio cholerae, unlike the typhoid bacillus, remains within the lumen of the bowel and exterior to the tissues of the body in the human disease; and that artificial immunization against cholera has not been as efficacious as artificial immunization against typhoid. These investigators differentiated Vibrio cholerae into four immunologic types A, AB, AC, ABC according to the distribution of major somatic antigens. They are presently engaged in studies aiming toward the elucidation of the functional mechanisms of immunity in Asiatic cholera.

In general, manufacturers of cholera vaccines endeavor to employ virulent strains of Vibrio cholerae; however, little is known of the relationship between the virulence and antigenicity of this organism. Yu (7) has been the only one to use mice for determining the virulence of saline suspensions of smooth strains of Vibrio cholerae. This author demonstrated that vaccines prepared with highly virulent strains induced better protection than those prepared from strains of low virulence.

Tang et al (8) studied serologically 83 strains of Vibrio cholerae, isolated from the 1942 Kuming epidemic, but made no observations on either virulence or antigenicity.

The National Institute of Health of China isolated 12 strains of Vibrio cholerae from patients dying with Asiatic cholera during the Chungking epidemic of 1945. The present study was undertaken to compare the virulence and antigenicity of these newly isolated strains with strains currently in use for the production of cholera vaccines in the United States and in China, and to determine whether or not a relationship exists between the virulence and antigenicity of the organism. It was also hoped that some recommendations might be forthcoming in regards to the selection of strains of Vibrio cholerae for use in cholera vaccine production.

Experimental Methods and Results

A total of 16 strains of Vibrio cholerae were included in this study. Of these, 2 were obtained from the National Institute of Health of the United States Public Health Service, 10 were recovered from 12 strains isolated during the 1945 Chungking epidemic, while 4 others were chosen at random from 8 strains currently in use in China for cholera vaccine production.

The strains of Vibrio cholerae were received in either the dried or semi-solid state. These were transferred to veal

infusion broth at pH 8.4 and incubated for 18 hours at 37°C., after which transfers were made onto veal infusion agar of similar pH. No organism was propagated on artificial medium beyond the third subculture before use in either lyophiling, animal injection, or vaccine preparation.

Characterization Studies of the Strains of Vibrio cholerae

The various strains of Vibrio cholerae were carefully studied as to their morphological, cultural, biochemical and serological characteristics. These included cellular and colonial morphology, staining properties, motility, Heiberg's sugar reactions, the cholera-red reaction, the Voges-Proskauer test, the Grieg hemolytic test and immunological typing with specific somatic antisera. Monospecific A, B, and C antisera were furnished by Dr. William Burrows of the University of Chicago. Typing was carried out according to the technique recommended by Burrows and associates (6) while the biochemical tests were performed according to the methods outlined in Diagnostic Procedures and Reagents (9).

The results of the characterization studies are summarized in Table I. All the strains studied showed typical characteristics of true, smooth Vibrio cholerae, the only atypical finding was the slight hemolytic activity exhibited by strains 5122 and 5149. Serologically, all 4 immunologic types A, AB, AC and ABC

Table I

Characteristics of Strains of Vibrio cholerae

Number	Source	Appearance of colony	Motility	Staining	Heiberg type	Cholera-red	V-P	Grieg	Typing	Japanese type
NIH41	NIH, USPHS	Smooth	+	Gram-negative	I	+	-	-	AB	Ogawa
NIH35A3	"	"	+	" "	I	+	-	-	AC	Inaba
5122	Chungking epidemic strain	"	+	" "	I	+	-	sl.	A	-
5149	"	"	+	" "	I	+	-	sl.	AB	-
5104	"	"	+	" "	I	+	-	-	A	-
5038	"	"	+	" "	I	+	-	-	AB	-
5284	"	"	+	" "	I	+	-	-	AB	-
5137	"	"	+	" "	I	+	-	-	AB	-
5102	"	"	+	" "	I	+	-	-	AB	-
5044	"	"	+	" "	I	+	-	-	A	-
5053	"	"	+	" "	I	+	-	-	ABC	-
5353	"	"	+	" "	I	+	-	-	ABC	-
35	Chinese vaccine strain	"	+	" "	I	+	-	-	AB	Ogawa
36	"	"	+	" "	I	+	-	-	AB	"
45	"	"	+	" "	I	+	-	-	AB	"
48	"	"	+	" "	I	+	-	-	AB	"

were represented. The majority, 10 out of 16 belonged to type AB. The strains isolated from the 1945 Chungking epidemic included 3 types A, AB, and ABC.

Animal Passage and Virulence Titration of Strains of Vibrio cholerae

After the various characteristics of the strains had been determined, attempts were made to increase the virulence of the organisms through serial passages in mice. The virulence of the strains was titrated by intraperitoneal injections of mucinized suspensions of the organisms into mice. The procedure used for handling the strains for animal injection was as follows:

1. Equal number of male and female white Swiss mice weighing from 10-20 grams were employed. The mice were bred on the Laboratory's own farm.
2. Only the third subcultures of 5-6 hours of age were used for animal injection. The parent state was either a fresh recovery from the dried state or a fresh isolate from the heart's blood of injected and dead mice. The complete sequence of cultures was veal infusion broth 18 hours, veal infusion agar 24 hours and veal infusion agar 5-6 hours.
3. The organisms were suspended in warm buffered physiological saline solutions from veal infusion agar. The suspension

of the organisms was facilitated mechanically by using sterile cotton swabs.

4. This suspension was standardized to an arbitrary reading of 50 on a Coleman Universal Spectrophotometer.
5. Serial ten-fold dilutions of this standardized suspension were prepared in saline and the test doses were made by ten-fold dilutions in 5% mucin from the next lower saline dilution.
6. Groups of six mice each were given 0.5 ml amounts of the test doses ranging from mucin dilutions 10^{-5} to 10^{-8}. Injections were begun with the highest dilution in each series. All injections were finished within an hour from the time the suspensions had first been prepared.
7. Duplicate samples of 1 ml portions of the 10^{-7} saline dilutions were pour-plated in veal infusion agar for colony counting.
8. The 5% mucin solution was prepared according to the method recommended by the Biologic Products Division of the United States Army Medical School (10).

The mice were observed for deaths for a 72 hour period after the injection of the mucinized suspensions of the organisms. The Reed and Muench method (11) was used in determining the 50% end point (LD_{50}) of the injected mice, care being taken to include both zero or

near zero and 100% or near 100% end points in each experiment. This method minimizes the intrusion of statistical weighing into results by test animals failing to give an average response. The calculation is based on the assumption that if one animal survives a low dilution it would certainly have survived at higher dilutions; and if it dies in a higher dilution, it would die in lower dilutions. The method is especially valuable in comparative studies, such as comparing the results of one experiment with another or comparing the data of one laboratory with that of another. A sample protocol of an experiment with NIH35A3 strain is given to illustrate the method of calculation.

Protocol of Virulence Titration
(strain NIH35A3)

Dilution	No. of organisms in 0.5ml dose (pour-plate)	No. of mice	Deaths	Survivals	Accumulated Deaths	Accumulated Survivals	Percent mortality	LD_{50}
10^{-5}	8,200	6	4	2	11	2	85	
10^{-6}	820	6	4	2	87	4	64	
10^{-7}	82	6	2	4	3	8	27	=1/2,390,000
10^{-8}	8	6	1	5	1	13	7	=343 organisms

The 50% end point dilution lies between 10^{-6} and 10^{-7}, and is obtained by use of the fraction:

$$\frac{50\% - 27\% \text{ (mortality next under 50\%)}}{64\% \text{ (mortality next over 50\%)} - 27\% \text{ (mortality next under 50\%)}}$$

which is equal to 23/37. The product of this fraction and

the logarithm of the dilution factor (log 10 = 1) subtracted from the logarithm of the denominator of the dilution next under 50% gives the logarithm of the denominator of the 50% end point dilution.

Log denominator of dilution next under 50% = 7.0000

$$\frac{23/37 \times \log 10}{\text{Log denominator of 50\% end point dilution}} = \frac{.6216}{6.3784}$$

6.3784 is the logarithm of 2,390,000. The 50% end point then is 1/2,390,000 dilution. The 1 ml samples of the 10^{-7} saline solution pour-plated averaged a colony count of 164. Since 0.5 ml amounts were used for animal injections, the 1/2,390,000 dilution contained

$$\frac{82 \times 10^{-7}}{2,390,000} = 343 \text{ organisms.}$$

The logarithm of the denominator of 50% end point dilutions (log. titre) and the corresponding number of organisms in the LD_{50} doses for the various virulence titration experiments are summarized in Table II. Table III segregates the 16 strains of Vibrio cholerae according to their relative virulence into three broad groups. The virulence of an organism as titrated in experimental animals varies with the mean reactivity or sensitivity of the animal population. Thus even with organisms of established virulence, duplicate experiments do not necessarily yield identical

results. The virulence of the 16 strains of Vibrio cholerae was compared in groups of either two or four. When the LD_{50}'s expressed either in terms of dilution or in number of organisms oscillated in both directions during excessive experiments, the virulence of the organisms was taken as fixed if the relation of the LD_{50}'s of the various strains remained more or less constant. No attempt was made to determine the precise order of virulence among the various strains of each group.

Table II

Virulence of Strains of *Vibrio cholerae*

Strain of *Vibrio cholerae*	Log. titre (LD_{50})	No. of organisms in 0.5 ml 10^{-7} dilution (plate count)	No. of organisms LD_{50} dose	
			Single determination	Average
NIH41	6.6600	80	175	114
	7.4014	100	40	
	6.8450	90	120	
	7.0000	112	112	
NIH35A3	6.3784	82	343	212
	7.0000	100	100	
	6.6000	110	279	
	6.8065	80	125	
5122	6.3341	70	319	160
	7.2000	100	62	
	6.9000	80	100	
5149	6.2500	76	422	193
	7.3200	90	43	
	7.0000	115	115	
5038	5.4710	85	2,873	5019
	5.2000	90	5,683	
	5.0820	78	6,500	
5104	7.0000	75	75	137
	6.7000	100	200	
	6.8400	94	136	
5137	6.3180	75	375	826
	5.8000	85	1,350	
	6.0000	82	820	
5284	6.5400	100	287	866
	5.6300	76	1,780	
	6.2000	84	530	
5102	5.1760	82	6,133	8378
	5.0000	90	9,000	
	4.8700	74	10,000	
5044	7.2500	80	44	92
	6.9100	90	111	
	7.0000	120	120	
5053	7.0000	80	80	106
	7.1000	118	94	
	6.8400	100	145	
5353	6.9000	74	93	105
	7.0000	90	90	
	6.7800	80	133	

Table II (continued)

Strain of *Vibrio cholerae*	Log. titre (LD_{50})	No. of organisms in 0.5 ml 10^{-7} dilution (plate count)	No. of organisms LD_{50} dose	
			Single determination	Average
35	6.0000	90	900	432
	6.5300	97	287	
	6.4200	81	308	
36	6.8100	104	161	121
	7.0000	92	92	
	7.0000	110	110	
45	6.1800	80	533	407
	6.3000	74	372	
	6.5000	80	316	
48	6.6800	115	219	141
	6.8450	80	114	
	7.0000	90	90	

Table III

Strains of *Vibrio cholerae* Grouped According to Their Relative Virulence

LD_{50} dose expressed in No. of Organisms		
80-400	400-2000	2000-10,000
NIH41	5137	5038
NIH35A3	5284	5102
5122	35	
5149	45	
5104		
5044		
5053		
5353		
36		
48		

Preparation of Lyophile Cultures and Vaccines

When the virulence of the organisms reached a "steady" level, a generous supply of cultures of each strain dried from the frozen state in sterile normal horse serum was made and maintained. A 24-hour growth of the second subculture of a fresh isolate from the heart's blood of injected and dead mice was used for lyophiling. The normal horse serum employed was recovered from the dried state by adding the appropriate amount of physiological saline. Sterilization was effected by filtration through a Seitz filter. After thoroughly washing the growth off from the veal infusion agar, 0.2 ml amounts of the serum suspension were carefully delivered by capillary pipettes into each of 10-20 lyophile tubes. Freezing was accomplished either in an alcohol-dry-ice bath or in a deep freezer. The frozen cultures were dried for 48-72 hours and sealed under vacuum.

Vaccines were also prepared separately for each strain after the completion of its virulence determinations. The parent state used for vaccine production was an 18-hour growth in veal infusion broth of organisms either freshly recovered from the dried state or freshly isolated from the heart's blood of injected and dead mice. The planting was made by the loop method. Physiological saline containing 0.5 per cent phenol was used to wash off the 24-hour growth from veal infusion agar. The washing off of the organisms was facilitated mechanically by using sterile cotton swabs. The phenolized saline suspensions

were immediately stored at 5°C. Testing for non-viability of the Vibrio cholerae was generally begun on the third day. The organisms were found to have been killed by the 4th to the 7th days. The phenol-killed vaccines were standardized before use to contain 1,600 million organisms per ml. The standardization was carried out as follows: When the organisms were found to have been killed, small lots of different dilutions were prepared from the stock vaccines. The spectrophotometric reading of each of these diluted vaccines was recorded and the number of organisms per ml of the corresponding dilutions was determined by direct count. A white blood cell counting pipette and Petroff-Hauser counting chamber were used for the latter purpose. Figure 1 gives a composite curve with the logarithm of the spectrophotometric readings plotted against the number of organisms in millions per ml. In practice, vaccines were first standardized to read "40" on the spectrophotometer which represented a count of 3,400 million organisms per ml and then diluted 1:2. The direct count method here employed with the vaccines gave higher values than the pour-plate method utilized in the case of viable organisms. The latter yielded an average of 180 organisms per ml in the 10^{-7} saline dilutions. The original undiluted suspension therefore contained 1,800 million organisms per ml. From the calibration curve a suspension with a spectrophometric reading of 50 corresponds to a count of 2,400 million organisms per ml. The ratio

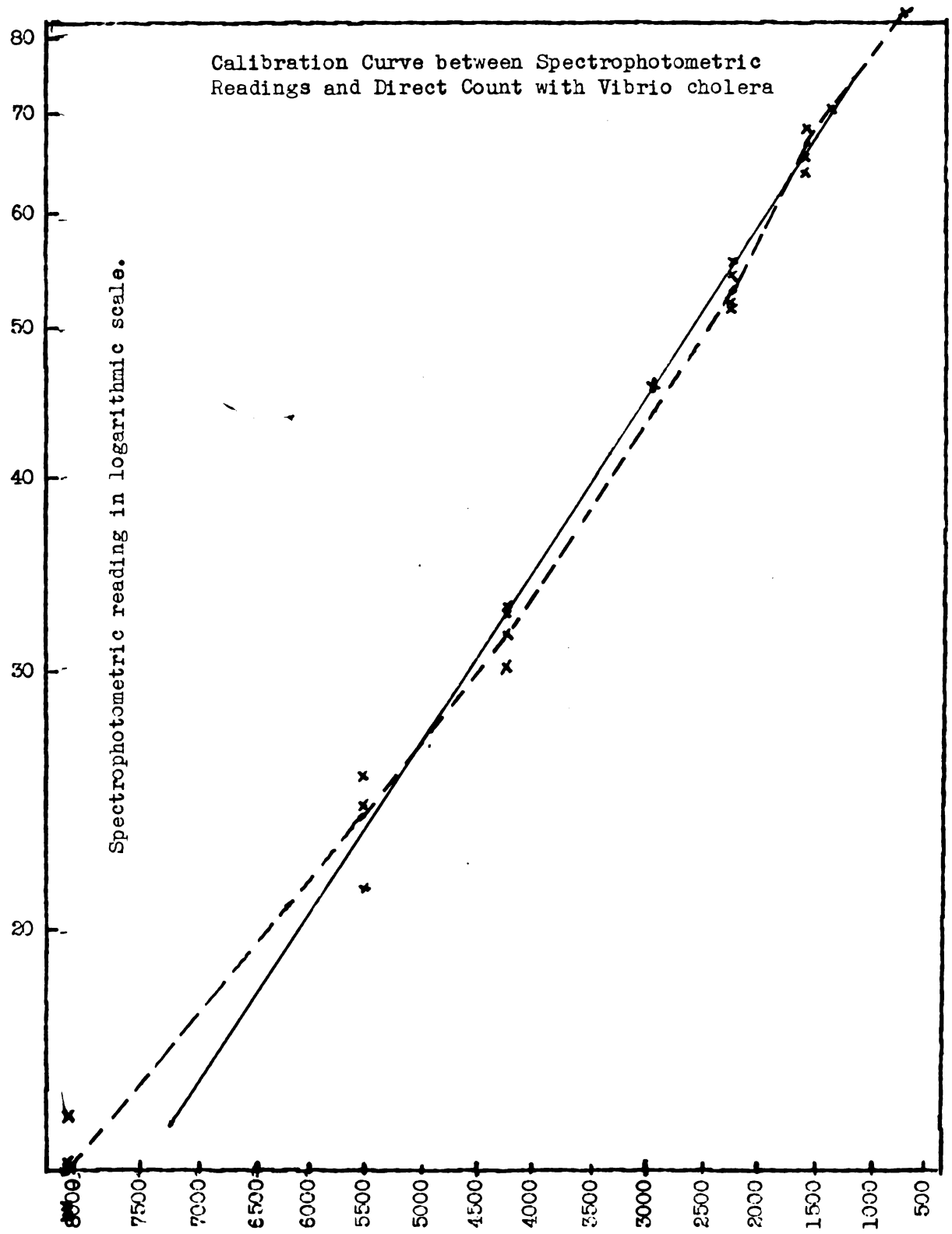
between the direct count and pour-plate count is roughly 3:2.

Comparison of the Antigenicity of Various Strains of Vibrio cholerae

1. Vaccines of Various Strains Tested Against Homologous and Reference Strains.

Three groups of 50 mice each weighing from 10-20 grams were set aside for each experiment. One of these groups was maintained as controls. The other two groups were vaccinated with vaccines prepared from two different strains of Vibrio cholerae. Each group was given two intraperitoneal injections of 0.25 and 0.5 ml respectively of a given vaccine with an interval of one week between the two injections. Two weeks after the last injection, the vaccinated mice in groups of six each were given challenge doses of mucin dilutions 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} of the homologous and the NIH41 strains of Vibrio cholerae. The unvaccinated controls were given test doses of the same strains ranging from 10^{-5} to 10^{-8}. The NIH41 strain, being the most virulent strain, was chosen as the reference strain. The 50% end point dilution was calculated for both the control groups and the vaccinated groups with reference to both the homologous strain and the reference strain in the same way as in virulence titration experiments. The 50% end point dilution of the control group receiving test doses of one strain of Vibrio cholerae divided by the 50% end point dilution of the vaccinated group challenged with the same test strain of organism gives the protective value in M.L.D. of the given vaccine against the test strain of

Figure 1


Calibration Curve between Spectrophotometric Readings and Direct Count with Vibrio cholera
Spectrophotometric reading in logarithmic scale.
80
70
60
50
40
30
20
8000
7500
7000
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
Millions of organisms per ml.

organism. When the LD_{50} of the vaccinated group lies beyond the lowest dilution 10^{-1}, the protection is expressed as $> \frac{\text{control 50\% end pt. dilution}}{10}$. If it lies beyond the highest dilution 10^{-4}, the protection is given as $< \frac{\text{control 50\% end pt.dilution}}{10000}$. When the LD_{50}'s of both vaccinated and control groups lie beyond the ranges under test, the protection values is of course undeterminable. The antigenicity of each given vaccine was tested twice on different days. The protective values of vaccines prepared from various strains of Vibrio cholerae are given in Table IV.

ii. Vaccine of Reference Strain Tested Against All Strains

In addition to challenging mice vaccinated with vaccines prepared from the various strains with organisms of the homologous and reference strains, other groups of mice were given vaccine of the reference strain and challenged separately with organisms of all the various strains of Vibrio cholerae. Normal unvaccinated controls received appropriate LD_{50} test doses of the same strains. The results of these experiments are summarized in Tables V a and b.

Table IV

Protective Values of Vaccines of Various Strains Against the Homologous and Reference Strains

Serial No. of Expts.	Vaccines	Type of Org.	Challenged with Homologous Strain			Challenged with Reference Strain		
			Log titre vaccinated	Log titre controls	M. L. D.'s protected	Log titre vaccinated	Log titre controls	M. L. D.'s protected
1	NIH41	AB	2.5700	7.0000	26,920	2.5700	7.0000	26,920
2	"		2.2530	6.3170	11,590	2.2530	6.3170	11,590
1	NIH35A3	AC	1.5000	6.5000	100,000	2.5000	7.0000	31,600
2	"		2.0000	6.6000	31,500	2.5000	6.3170	6,566
2	5122	A	1.6780	6.4700	62,000	2.5000	6.3170	6,566
3	"		2.5000	7.4950	100,000	3.0000	7.0000	10,000
2	5149	AB	1.5400	6.5400	100,000	2.5000	6.3170	6,566
3	"		1.1500	7.1520	1,000,000	2.4100	7.0000	38,900
4	5038	AB	1.5000	4.5000	1,000	3.7200	6.5000	603
5	"		1.4200	4.6250	1,600	> 4.0000	6.3900	< 246
4	5104	A	< 1.0000	7.6250	>4,217,000	4.0000	6.5000	316
5	"		1.5000	7.5000	1,000,000	4.0000	6.3900	246
6	5137	AB	< 1.0000	5.5000	> 31,600	3.8480	6.5900	552
7	"		< 1.0000	5.7160	> 52,000	3.5900	6.7160	1,330
6	5284	AB	1.5000	5.4700	9,330	4.0000	6.5900	390
7	"		< 1.0000	6.1480	>140,600	3.8100	6.7100	810
8	5102	AB	< 1.0000	< 4.0000	?	> 4.0000	6.6300	< 427
9	"		1.5000	4.5000	1,000	3.8000	6.5000	500
8	5044	A	3.5000	7.5000	10,000	3.5000	6.6300	1,350
9	"		2.3700	7.0000	42,700	3.5000	6.5000	1,000
10	*5053	ABC	3.1300	7.5900	28,840	2.8600	6.5130	4,500
11	"		> 4.0000	7.5000	< 3,160	> 4.0000	7.6370	< 4,336
10	*5353	ABC	< 1.0000	7.0910	> 123,000	2.8800	7.5130	4,300
11	"		> 4.0000	7.6370	< 4,336	>4.0000	7.6370	< 4,336
12	*35	AB	2.3700	6.0000	4,284	2.8300	7.6380	64,600
13	"		> 4.0000	7.7500	< 5,624	> 4.0000	7.8500	< 7,080
12	*36	AB	2.8470	7.2430	24,844	3.0000	7.6380	43,450
13	"		> 4.0000	7.6370	< 4,336	> 4.0000	7.8500	< 7,080
14	*45	AB	< 1.0000	6.8480	> 704,700	2.5000	6.3590	7,300
15	"		< 1.0000	< 5.0000	?	> 4.0000	7.0000	< 1,000
14	*48	AB	1.0000	< 5.0000	< 10,000 ?	1.8250	6.3590	34,200
15	"		> 4.0000	> 8.0000	?	> 4.0000	7.0000	< 1,000

*The wide discrepancy between duplicate experiments makes it impossible to interpret the results.

Table Va

Protective Values of Vaccine of Reference Strain Against the Various Strains of Vibrio cholerae

Serial No. of Expts.	Protection in M.L.D.'s against challenges of															
	NIH41	NIH35A3	5122	5149	5038	5104	5137	5284	5102	5044	5053	5353	35	36	45	48
1	26,920	100,000	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	11,590	31,600	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	42,200	-	11,000	382,000	>10,000	?	>100,000	>100,000	-	-	-	-	-	-	-	-
17	-	-	10,000	682,400	-	<1,000	-	-	>10,000	?	<1,000	>286,400	-	-	-	-
18	-	-	-	-	-	-	-	-	-	<1,000	-	-	?	?	>376,000	?
19	-	10,000	-	-	-	<4,200	-	-	-	-	<1,500	-	<3,160	< 1,500	-	<2,100

Table Vb

Protection of Reference Vaccine against Various Strains

Strain	Expt. No.	Log Titer	
		Vaccinated	Controls
NIH41	1	2.5700	7.0000
	2	2.2530	6.3170
	16	2.3748	7.0000
NIH35A3	1	1.5000	6.5000
	2	2.0000	6.5000
	19	3.0000	7.0000
5122	16	2.7840	6.8340
	17	3.0000	7.0000
5149	16	1.4188	7.0000
	17	1.0000	6.8340
5038	16	< 1.0000	5.0000
5104	16	> 4.0000	> 8.0000
	17	> 4.0000	7.0000
	19	> 4.0000	7.6250
5137	16	< 1.0000	6.0000
5284	16	< 1.0000	6.0000
5102	17	< 1.0000	5.0000
5044	17	> 4.0000	> 8.0000
	18	> 4.0000	7.0000
5053	17	> 4.0000	7.0000
	19	> 4.0000	7.1760
5353	17	< 1.0000	6.4288
35	18	> 4.0000	> 8.0000
	19	> 4.0000	7.5000
36	18	> 4.0000	> 8.0000
	19	> 4.0000	7.1760
45	18	< 1.0000	6.5752
48	18	> 4.0000	> 8.0000
	19	> 4.0000	7.3220

Strains NIH41 and NIH35A3 are known to possess good antigenicity (5,12,13,14) and the results here obtained confirm such findings. Strains 5122 and 5149 isolated from the Chungking epidemic showed good protection against both the homologous and reference strains. Of the strains that are currently in use for vaccine production in China and strains 5053 and 5353 isolated from the Chungking epidemic, the great discrepancy between duplicate experiments precluded possible conclusions. Strains with low virulence, 5038 and 5102 yield vaccines of low antigenicity. Vaccines from strains of high virulence generally produced good homologous protection but did not always accord good protection against the reference strain.

The reference strain NIH41 showed good cross protection against strains NIH35A3, 5122, 5149, 5038, 5137, 5284, 5102, 5353 and 45. It did not protect well against strains 5104, 5044, 5053, 35, 36 and 48. Strains 5104 and 5044 belong to type A, strain 5053 belongs to type ABC, but strains 35, 36 and 48 are of the same type AB as the reference strain NIH41. Among the strains well cross protected by the reference vaccine, NIH35A3 is type AC, 5122 is type A, 5353 type ABC, while the others are type AB. The virulence of the various strains does not seem to be related to the degree of protection accorded to them by the reference vaccine.

The Toxicity of the Vaccines

Both the vaccinated and the control groups of mice were weighed at 0, 1 and 3 weeks, i.e., before each vaccination and prior to the final challenge. The number of deaths in these groups occurring during these intervals were also recorded. These were undertaken with the purpose in mind of taking the number of deaths and the average gain or loss in weight of the vaccinated groups as contrasted with the controls as measures of the toxicity of the various vaccines. The National Institute of Health, United States Public Health Service, recommended using the average gain in weight of mice vaccinated with varying amounts of the same pertussis vaccine as an indication of the toxicity of the vaccine (15). The same principle is here applied to identical amounts of different vaccines prepared from various strains of Vibrio cholerae. The relevant data are presented in Table VI.

The toxicity of the various vaccines is difficult to analyze from the data presented. The wide variations among the different control groups speak for non-homogenicity of the existing conditions. The initial average weight of the groups of mice was approximately the same. The temperature of the mouse room fluctuated between 60° - 80°F. owing to a defect in the thermo-regulating system. Ample feed of the same kind was provided for all groups but it had been noticed that some groups of mice ate better than others. The amount

of water supplied might have constituted another uncontrolled factor.

Even the comparison between the vaccinated groups with reference to the control group in a given experiment is not without difficulty. When a greater number of deaths concurs with a lesser gain in weight, or when a fewer number of deaths concurs with a greater gain in weight in one vaccinated group as compared to another, the first vaccine may be reasonably inferred to be either more or less toxic than the second. But when a greater number of mice die leaving a resultant higher increase in weight, or when fewer animals die giving a concurrent lesser gain in weight, the inference is not at all clear.

Vaccines of strains 5038, 5104, 35 and 36 appear to be highly toxic. That these strains happened to fall into two groups where both the tested vaccines yielded high toxicity might have aroused the suspicion of a chance factor. The duplicate checks in both instances, however, did not uphold such an assumption. It is of interest to note that strain 5038 is a comparatively non-virulent organism. Vaccine prepared from the other non-virulent strain 5102 was tested side by side with the vaccine of a virulent strain 5044. There was no appreciable difference in the toxicity of the two vaccines, both being rather mild in character. It would thus appear that the toxicity of a vaccine is not related to the virulence of the organism.

Table VI

The Average Increase in Weight and the Number of Deaths in the Control and Vaccinated Groups of Mice

Serial No. of Expts.	Groups of Mice (50) Vaccinated	Average gain in Wt. (grams) 0-1st wk.		0-3rd wk.		Average gain expressed as percentage of controls 0-1st wk.		0-3rd wk.		No. of Deaths 0-1st wk.		1st-3rd wk.		Total
1	Control	2.6		4.6		100		100		0		0		0
	NIH41		1.9		4.8		73.1		104.3		2		5	7
	NIH35A3		1.8		4.5		62.9		97.8		0		2	2
2	Control	4.7		5.5		100		100		0		0		0
	NIH41		4.3		5.3		91.5		96.4		0		1	1
	NIH35A3		4.4		6.4		93.4		116.4		1		0	1
	5122		4.0		5.4		85.1		98.2		0		0	0
	5149		4.8		5.2		102.2		94.5		0		1	1
3	Control	2.2		8.7		100		100		0		2		2
	5122		2.0		6.9		90.9		79.3		3		8	11
	5149		2.4		7.6		109.1		87.3		0		0	0
4	Control	5.2		9.1		100		100		3		0		3
	5038		5.3		7.8		119.1		85.7		1		4	5
	5104		3.3		4.6		63.5		50.6		0		3	3
5	Control	-		4.9		-		100		-		1		1
	5038		-		2.0		-		40.8		-		9	9
	5104		-		2.9		-		59.6		-		8	8
6	Control	1.7		6.7		100		100		2		3		5
	5137		1.1		6.3		64.7		94.0		4		2	6
	5284		1.7		5.8		100.0		86.6		0		1	1
7	Control	3.2		3.5		100		100		0		3		3
	5137		3.0		5.6		93.8		160.0		4		5	9
	5284		3.5		4.9		109.4		140.0		1		2	3

Table VI (continued)

Serial No. of Expts.	Groups of Mice (50) Vaccinated	Average gain in Wt. (grams) 0-1st wk.		0-3rd wk.		Average gain expressed as percentage of controls 0-1st wk.		0-3rd wk.		No. of Deaths 0-1st wk.		1st-3rd wk.		Total
8	Control	2.9		6.3		100		100		1		1		2
	5102		3.1		5.9		110.4		93.7		2		1	3
	5044		1.9		5.7		65.5		90.5		1		0	1
9	Control	3.0		7.1		100		100		1		0		1
	5102		2.9		6.8		96.6		95.9		1		1	2
	5044		2.2		6.7		73.4		94.4		1		0	1
10	Control	0.9		8.2		100		100		0		4		4
	5053		1.9		4.9		211.0		60.0		2		5	7
	5353		1.8		5.9		200.0		72.0		0		4	4
11	Control	1.2		3.8		100		100		1		7		8
	5053		1.2		5.8		100.0		152.6		2		6	8
	5353		1.9		6.1		158.3		157.8		2		6	8
12	Control	0.4		3.6		100		100		1		1		2
	35		0.2		1.9		50.0		63.3		0		4	4
	36		0		1.6		0		53.3		1		0	0
13	Control	2.7		7.7		100		100		4		5		9
	35		1.0		7.2		37.0		93.5		1		10	11
	36		1.0		3.3		37.0		43.0		2		3	5
14	Control	2.6		2.4		100		100		4		0		4
	45		1.7		2.0		65.4		80.0		0		4	4
	48		1.5		3.1		57.7		120.0		0		6	6
15	Control	2.1		6.2		100		100		3		0		3
	45		2.2		6.2		104.8		100.0		0		0	0
	48		2.5		6.0		119.0		96.8		2		3	5

Discussion

In the selection of mice, it was originally intended to follow the more strict criteria of Griffitts (5) rather than the relatively looser requirements of Ranta and Dolman (13). The former author used equal numbers of female and male mice of the same strain weighing from 10 to 13 grams. Ranta and Dolman employed inbred white mice of either sex of 10 to 25 grams initial weight. Equal sex distribution was effected in the above studies, but it had not been possible to adhere to the strict weight specifications. The non-homogenicity of the experimental conditions as evidenced by the wide discrepancies of deaths and weight increases among the different control groups emphasizes that the subsequent care of the animals is just as important, if not more so than the initial selection.

Mucin enhances the virulence of Vibrio cholerae for mice (12). By the use of the mucin vehicle, the virulence of cultures and the antigenicity of vaccines could be more accurately compared. Different lots of mucin may exhibit different enhancing capacities. While this would not have materially affected the ratio of the 50 per cent end points between the vaccinated and unvaccinated mice in an antigenicity test, it would have made some difference in the comparison of virulence titrations between the various strains. To minimize this factor, the same lot of mucin was used for the whole series of virulence titrations of all strains throughout.

The use of living vaccine has long since been replaced by that of killed vaccines. Killed vaccine may be prepared by using either chloroform, heat, formalin or phenol. Phenol-killed vaccine has been shown by Ranta and Dolman (14) to produce the highest and most enduring agglutinative titre in rabbits. It is also the most commonly used vaccine at the present time. Recently Jennings and Linton (16) recommended a direct cholera vaccine prepared from a fluid medium in which phenyl-mercuric acetate had been used as the killing agent. This method of vaccine preparation has not yet been widely adopted.

The mouse protection test was chosen because the National Institute of Health of the United States has recommended it as a tentative procedure for manufacturers of cholera vaccine (17). Griffitts (5) and Ranta and Dolman (13) also considered it as a satisfactory antigenicity test. As no definite correlation has as yet been found to exist between the level of agglutinin and mouse protective titres, it would appear that the mouse protection test, being a more direct test, should be preferred over the agglutinative test.

The two dose method of vaccination was adopted in immunizing the mice since the work of Ranta and Dolman (13) demonstrated that a single dose vaccination does not confer high enough immunity to be clearly demonstrable. A three dose vaccination procedure was found by the same authors not to induce significantly higher immunity than the two dose.

The fact that the strains of Vibrio cholerae isolated from the Chungking epidemic included three different types of the organism is of interest. Tang et al (8) in the study of strains isolated from Kuming epidemic, and Burrows (18) in typing strains brought back by Dr. Reiman from Chungking, encountered similar findings. These findings denote either that the particular epidemic did not originate from a single source, or that the antigenic structure of the causative organism has undergone changes in the course of the epidemic. In any event, unless good cross protection is demonstrable among the various types of Vibrio cholerae, which at present does not seem to be so demonstrated, it would be best to employ a mixed vaccine consisting of highly antigenic strains of various types for prophylaxis against the disease.

The experiments so far conducted represent a preliminary survey of the problem. The results indicate that a virulent organism is required for the production of a vaccine of good antigenicity but virulence is not the sole criterion. The toxicity of a vaccine does not appear to bear any relationship to the virulence of the organism. While good cross protection exists among some of the strains, it does not exist among all strains. Reviewing the data in retrospect, it is clear that the problem demands more intensive study before definite conclusions can be made. It is particularly disappointing that no observations could be made concerning the strains that are currently in use for vaccine production in China.

The mucin suspensions used in the virulence titration experiments and in the mouse protection tests are virtually mixtures of living and dead organisms. The proportion between the living and the dead is apt to vary in different experiments. Biological assays using living organisms also generally yield less uniform results than those in which dead materials like toxins are employed. The endotoxin of Vibrio cholerae has been prepared and studied by Burrows and his associates and has been shown to exhibit immunologic properties (19). It certainly would seem worthwhile to try to substitute endotoxins of the various strains of Vibrio cholerae for the mucinized suspensions of the organism in the virulence and antigenicity determinations.

Summary

Sixteen strains of Vibrio cholerae were included in the present study. Of these, 2 came from the National Institute of Health of the United States Public Health Service, 10 were recovered from 12 strains newly isolated during the 1945 Chungking epidemic, and 4 were chosen at random from 8 strains currently in use in China for cholera vaccine production.

All the strains showed typical morphological, cultural and biochemical characteristics of true Vibrio cholerae. The only atypical finding was the slight hemolytic activity exhibited by strains 5122 and 5149. Serologically all 4 types A, AB, AC, and ABC were

represented. The majority belonged to type AB. The strains isolated from the Chungking epidemic included types A, AB and ABC.

The relative virulence of the strains of Vibrio cholerae was titrated in mice by intraperitoneal injection of mucinized suspensions of the organisms using the Reed and Muench method for LD_{50} determination. Ten strains of Vibrio cholerae are highly virulent, four strains are moderately so and two strains are comparatively non-virulent. The LD_{50} doses are respectively 80-400, 400-2000 and 2000-10,000 organisms. The third group is composed of strains isolated from the Chungking epidemic. The second group includes two epidemic strains and two vaccine strains from China. The first group includes 6 epidemic strains, 2 vaccine strains from China and 2 NIH strains.

Phenol-killed vaccines were prepared separately from each strain. The vaccines were standardized to contain 1,600 million organisms per ml. A two dose method of vaccination was adopted, administering 0.25 and 0.5 ml amounts of a given vaccine one week apart intraperitoneally into mice. Two weeks after the 2nd injection, the protective values of these vaccines were tested against the homologous strains and a reference strain, the NIH41 strain. The immunizing potencies of the vaccines was calculated by means of the Reed and Muench method. The protective value of the reference vaccine was also tested against the various strains.

The antigenicity of vaccines prepared from strains of low virulence was poor. They protected against 1000 M.L.D.'s of the homologous

strains and 500 M.L.D.'s of the reference strain. The more virulent strains yielded vaccines of good immunizing potency against the homologous organisms, giving a protection in the order of 10,000 to 1,000,000 M.L.D.'s. They did not always protect well against the reference strain. Two of the epidemic strains yield vaccines as good as those of the two NIH strains in that they gave good protection against both homologous and reference strains. Duplicate tests on vaccines of strains 5053 and 5353 isolated from the Chungking epidemic and those of strains 35, 36, 45 and 48 currently in use for vaccine production in China gave such erratic results that no possible interpretation could be made.

The reference vaccine gave good cross protection to nine heterologous strains but did not protect well against the six others. The former group included representatives of all four types A, AB, AC and ABC. The latter group included members of type A, AB and ABC. There was only one type AC organism among the whole 16 strains.

Vaccinated and control groups of mice were weighed at 0, 1 and 3 weeks, i.e., before each vaccination and prior to the final challenge in an attempt to evaluate the toxicity of the vaccines from the average gain or loss of weight of the vaccinated groups as contrasted with the controls. The data obtained is difficult to analyze. Four vaccines showed high toxicity. Two of these were prepared from virulent strains, one from a moderately virulent strain and another

was derived from a comparatively non-virulent strain of Vibrio cholerae. Vaccine of another non-virulent strain was tested side by side with the vaccine of a virulent strain. There was no appreciable difference between the toxicity of the two vaccines, both being mild in character.

The wide variations of the weight gains among the different control groups speak for non-homogenicity of the animal population. Factors which were either known to have existed or were suspected to have been present to contribute to such conditions were reviewed in the text.

It is felt that the problem deserves more intensive study and that definite conclusions can not be made at the present time. The isolation of multiple types of Vibrio cholerae from a single epidemic, however, justifies the recommendation of the use of a mixed vaccine, made and pooled from highly antigenic strains of various types, for prophylaxis against the disease.

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