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Problems of Influenza Virus Vaccine Standardization *

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The lack of reliable laboratory methods of determining the antigenicity of inactivated influenza virus vaccines prompted a reinvestigation of the reproducibility of the tests used for measuring the antigenic content of influenza vaccines, namely, the CCA and mouse potency tests.

The data obtained in the second part of the mouse potency test, i.e., the neutralization test performed either in mice or in embryonated eggs, statistically demonstrated protective differences between 2 vaccines differing in antigenic mass by as little as 2-fold. However, the dependence upon a single egg or mouse neutralization test to provide the correct vaccine/reference ratio assumed more than "biological" variation would allow. Further, the test was long and tedious and it would be impracticable to perform the number of tests needed to obtain statistically significant results. Thus, the extreme variability observed between individual mouse potency tests and the impracticability of performing this test in statistically sufficient numbers precluded its use for measuring antigenic content of inactivated influenza vaccines.

The simpler CCA test, on the other hand, did provide the reproducibility required for the correct determination of the vaccine/reference ratio once a stable CCA reference vaccine was prepared. This test was easily reproducible and results obtained were sufficient to allow a meaningful and reliable conclusion to be drawn with respect to vaccine potency.

The problems of measuring the relative content of several components in multivalent vaccine preparations and of finding a test which positively correlates with vaccine potency in man, however, remain unsolved.

HISTORICAL PERSPECTIVE

Several years ago when we began an intensive investigation of the efficacy of inactivated influenza vaccines, it became quite obvious that reliable laboratory methods for determining the antigenicity of these vaccines were lacking. Since it was unrealistic and virtually impossible to test each vaccine lot in man prior to release for general use, a test (or tests) was needed which could be performed easily in the laboratory and which positively correlated with what the vaccines did in man. Thus, the search began for such a test. Initially, we reinvestigated the reliability of the tests being utilized at that time for measuring antigenic content of influenza vaccines—namely, the CCA (chicken-cell agglutination) and the mouse potency tests.

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The CCA test

At that time the CCA test was used only as a guide in preparing virus concentrates and for pooling these concentrates in the preparation of final vaccine. It was not used to measure the antigenic content of the final vaccine because of one serious drawback—the test could measure only total CCA content and could not be used to determine the relative content of several components in a multivalent vaccine preparation. It would have been useful for monovalent vaccines if the test were not so difficult to reproduce between laboratories.

In April 1968 the Division of Biologics Standards (DBS) sponsored an Influenza Virus Vaccine Workshop. Representatives from the United States manufacturers licensed to prepare influenza vaccine and DBS scientists met to determine why the CCA test was irreproducible between laboratories. During the Workshop, we discovered that a critical factor, which was noted by Hirst & Pickles (1942) in their

initial description of the CCA test, was seemingly overlooked when Miller & Stanley (1944) modified the test so that it could be performed in the easily available Klett-Somerson colorimeter. The critical factor was that, at the time of reading, the tube was to be positioned so that the centre of the colorimeter light beam and sensing window should be in a plane 1 cm above the bottom of the tube. When one realizes that the CCA test is a dynamic test that measures a specific concentration of chicken red blood cells settling for a definite length of time, then the position of the light beam becomes very important—a fact well appreciated by Hirst & Pickles (1942). When the colorimeters were properly adjusted, there was little difficulty in reproducing the CCA test between laboratories, and what had been an unreliable test became an extremely reliable and the most reproducible test for measuring influenza virus haemagglutinin content.

Because the CCA test made use of a colorimeter, another indispensable requirement for standardization of the test was a stable CCA reference vaccine. Since our experience with some earlier reference preparations taught us that the stability of the preparations may vary on storage, we attempted to decrease the variables by using zonal-purified antigens to prepare the NIH CCA reference vaccine. This was done initially when we prepared the CCA-10 reference preparation and subsequently when the 69C and 69M references were made. The CCA-10 reference was used as the primary reference vaccine in the preparation and final testing of the monovalent A2 (Hong Kong variant) vaccine during the 1968–69 season. This achieved a high degree of success.

The problems of measuring the relative content of several components in a multivalent preparation and of finding a test which positively correlates with vaccine potency in man remained unsolved.

The mouse potency test

The mouse potency test (Eddy, 1967) involves inoculating mice with serial dilutions of influenza vaccine, bleeding the mice 14 days later, performing a neutralization test with the undiluted mouse serum and a known quantity of challenge virus, and finally determining what vaccine dilution was sufficient to elicit enough antibody to protect 50% of the mice in the neutralization test. Essentially, the mouse potency test is an antigen extinction test. A major problem is that a mouse-adapted challenge virus is used to check antibodies developed to an egg-adapted virus. The changes which can occur in a strain of influenza virus after adaptation to mice are well known (Hirst, 1947; Sugg, 1949). In addition, there appeared to be no relationship between the results of mouse potency tests and the results of immunization studies in man. Although this test was the only method being used to determine the antigenicity of influenza vaccine, it was surprising that there were absolutely no data on its reproducibility. It was obvious that, if a test could not be reproduced to within a small margin of error, it would have little value and would not be a test which should be evaluated with human experience.

The scientific portion of this report presents some of our data on the reproducibility of the CCA test. In addition, we are describing the results of our studies evaluating the second part of the mouse potency test, i.e., the neutralization test. These latter results not only were quite revealing but also prompted us to philosophize on our scientific naïveté.

MATERIALS AND METHODS

Vaccines

In the animal potency tests, vaccine X was the undiluted NIH 67CP (civilian polyvalent) reference vaccine.[1] This vaccine was prepared by the conventional Sharples process. Vaccine Y was made by diluting vaccine X 1 : 2 with 0.01 M phosphate-buffered saline (PBS), pH 7.2, so that the Y/X ratio of the actual antigenic content was 0.50.

Chicken-cell agglutination (CCA) test

The CCA test was performed according to the methods of Miller & Stanley (1944) as described in a National Institutes of Health (NIH) memorandum.[2]

Haemagglutination (HA) test

The HA test was performed in the microtitre system (Sever, 1962) (1) using as reagents 0.01M phosphate-buffered saline (PBS), pH 7.2, and 0.5% cockerel red blood cell (RBC) suspension with PBS as the diluent, and (2) according to the following procedure: antigen was serially diluted 2-fold in 0.025 ml PBS using microdiluters; after adding a second drop (0.025 ml) of PBS diluent to each well, 0.05 ml of 0.5% cockerel RBC suspension was

[1] NIH 67CP reference vaccine formulation: A/PR/8/34, 100 CCA; A1/Ann Arbor/1/57, 100 CCA; A2/Japan/170/62, 100 CCA; A2/Taiwan/1/64, 100 CCA; B/Mass./3/66, 200 CCA.

[2] *Titration of chicken red cell agglutination (CCA) value* (NIH memorandum of 16 September 1946, available from the Division of Biologics Standards, National Institutes of Health, Bethesda, Md. 20014, USA).

added; after being sealed with tape and shaken for thorough mixing, plates were incubated at room temperature (23°–25°C) until cells had settled. The HA end-point is the highest antigen dilution showing equal to or greater than 50% haemagglutination.

Haemadsorption (HAd)

Haemadsorption tests were performed as initially described by Vogel & Shelokov (1957).

Animal potency test

Swiss white mice, CFW strain, weighing 10 g–14 g were immunized in the usual manner for the influenza mouse potency test except that 3-fold, instead of serial 5-fold, dilutions of vaccine were used. Groups of 500–1000 mice were immunized with serial 3-fold dilutions (from 1 : 5 to 1 : 32 805) of each vaccine, and the sera obtained from these immunized mice 14 days after inoculation were pooled for each vaccine dilution (National Institutes of Health, 1947). Prior to storing, serum was filtered through a set-up consisting of an AP-25 Millipore prefilter disc and a 1.2 μ Millipore membrane. Serum was stored at −20°C or below and heated (at 56°C for 30 minutes) prior to use. Control serum reagents prepared from unimmunized mice were treated similarly. Replicate tests were performed using aliquots of these mouse sera which had been frozen and thawed only once, and using different sets of serum–virus mixtures.

Serological studies

Mouse serum was analysed for the presence of antibody by the following tests: mouse neutralization (MoNT),[1] egg neutralization (EgNT),[1] and tissue culture neutralization (TCNT).[1] The challenge virus used in the egg neutralization test was a strain having had all its passages in eggs; for the tissue culture neutralization test the challenge virus was the "egg" strain passaged 3 additional times in primary rhesus monkey kidney cell cultures; and for the mouse neutralization test the challenge virus was a "mouse-adapted" strain. Three strains of influenza virus (A2/Japan/170/62, A2/Taiwan/1/64, and B/Mass/3/66) were used in each of the above neutralization tests.

Calculations and statistical analyses

All infectivity end-points and 50% neutralization end-points were determined by the Kärber method (Lennette, 1964) and expressed as decimal exponent (dex) values (Haldane, 1960). Mean, variance, standard deviation (SD), and standard error (SE) of each column of results of the replicate neutralization tests were determined from antilog values. Although these statistics were derived from antilog values, subsequent analysis on the data was performed from dex values, except for the Y/X ratios.

[1] A detailed description of the methods employed in the Respirovirus Unit, Division of Biologics Standards, is available on request.

RESULTS

Analysis of reproducibility of CCA test

Table 1 shows the results obtained during a 2-week period of measuring the CCA content of the CCA-9 and CCA-10 reference vaccines. The former

TABLE 1

REPRODUCIBILITY OF CCA TESTS PERFORMED ON THE CCA-9 AND CCA-10 REFERENCE VACCINES

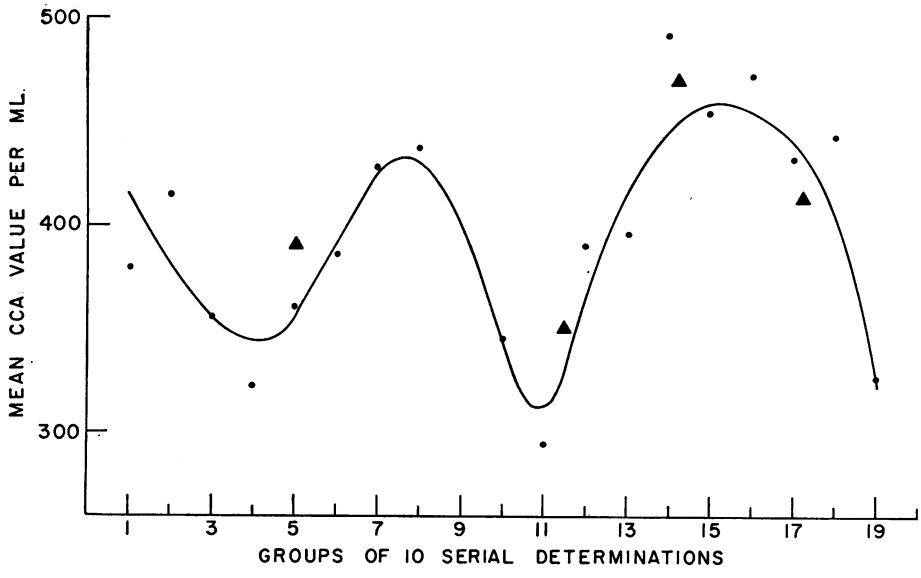
Test No.	Date of test, 1968	CCA values[a]/ml		
		CCA-10	CCA-9	CCA-9/ CCA-10
1	27 Sept.	344	193	0.56
2	30 Sept.	403	158	0.39
3	1 Oct.	383	228	0.60
4	1 Oct.	356	193	0.54
5	1 Oct.	410	205	0.50
6	2 Oct.	402	364	0.91
7	2 Oct.	383	364	0.95
8	2 Oct.	453	380	0.84
9	2 Oct.	436	372	0.85
10	4 Oct.	402	380	0.95
11	5 Oct.	445	428	0.96
12	7 Oct.	373	295	0.79
13	8 Oct.	373	302	0.81
14	10 Oct.	399	307	0.77
15	12 Oct.	402	292	0.73
Mean		397.6[b]	297.4[b]	0.74
Standard deviation		30.8	84.5	0.18
t-test[c]		−0.3	+1.3	+1.49

[a] Actual (uncorrected) values.

[b] Assigned values for the CCA-10 and CCA-9 reference vaccines were 400 and 269 respectively.

[c] In performing the t-test, the population mean (μ_0) for CCA-10 was assumed to be 400; for CCA-9, 269; and for CCA-9/CCA-10, 0.67. The sample means for CCA-10, CCA-9, and CCA-9/CCA-10 are indicated above. The null hypothesis tested (H_0) was: μ_0 = sample mean; the alternative hypothesis (H_1) was $\mu_0 \neq$ sample mean. The level of significance was 5%, with 14 degrees of freedom (DF = $n-1$).

ARITHMETIC MEANS OF CCA VALUES OBTAINED DURING 1-YEAR PERIOD [a]



[a] The CCA values obtained for the CCA-10 reference vaccine over a period of 1 year were divided into 19 groups of 10 serial determinations. Arithmetic means calculated for each group of 10 are plotted as dots and the best-fitting curve was drawn. The "x" plots are arithmetic means of determinations obtained during a 3-month period, and the actual values of these are from left to right 383, 379, 456, and 422. The 2nd and 3rd figures differ significantly from the theoretical mean of 400.

vaccine was prepared from Sharples concentrates of A/PR/8/34, and the latter from zonal-purified concentrates of A2/Japan/170/62. It had been our experience (and that of the United States manufacturers) that the CCA value of CCA-9 varied considerably from test to test; this is borne out by the data presented in Table 1. The CCA value of CCA-10 proved to be much more constant. The data in Table 1 were statistically analysed. The critical region for the t-test was $t < -2.145$ and $t > +2.145$. As indicated, the t values obtained for CCA-10, CCA-9 and CCA-9/CCA-10 are outside the critical region. Therefore it is highly probable that the population means for CCA-10, CCA-9 and CCA-9/CCA-10 were 400, 269 and 0.67 respectively.

One of the problems of the CCA test which has yet to be resolved is the variability experienced over long periods of testing. The results of 192 CCA tests performed on the CCA-10 reference vaccine over a period of 1 year in our laboratory were statistically analysed. The CCA values were divided into 19 groups of 10 serial determinations, and arithmetic means calculated for each group are plotted in the accompanying figure. When an interval of 3 months is considered, the second and third arithmetic means were significantly different (1% level of significance) from the theoretical mean value of 400 (see footnote to the figure). There was, however, no significant difference with the first and fourth values. The arithmetic mean for all 192 CCA determinations was 399. When statistically analysed (t-test) at the 1% level against the theoretical mean of 400, the difference was not significant. This is strong evidence that the theoretical (assigned) value of 400 for the CCA-10 reference vaccine was accurate over the long term. The error which might be introduced from these variable results is considerably lessened by a provision of the test which states that, if the correction factor [1] for a test is less than 0.5 or greater than 2.0, the test is considered invalid and must be repeated. Ordinarily, when a lower or higher than usual value is obtained for the reference vaccine, correspondingly lower and higher values are obtained for other vaccines in the test. This indicates that some factor(s) other than the vaccines contributed to this variability. However, the use of a standard reference vaccine in each test should correct for these other variables.

[1] Correction factor is obtained by dividing the observed value by the assigned value of the reference vaccine.

TABLE 2
LOG ED_{50}(Y) − LOG ED_{50}(X) [a]

Test	Strain			
	A2/Japan/170/62	A2/Taiwan/1/64	B/Mass./3/66	Sum of test
EgNT	4.07 10	5.22 10	6.82 11	16.11 31
MoNT	6.61 11	7.55 15	5.49 10	19.65 36
TCNT	12.68 10	7.21 10	10.32 10	30.21 30
Sum of strain	23.36 31	19.98 35	22.63 31	Total : 65.97 97

[a] First entry in each cell is sum of individual readings; second entry is number of readings.

Analysis of the mouse, egg, and tissue culture neutralization tests for determining influenza virus vaccine potency

The results of replicate mouse, egg, and tissue culture neutralization tests are given in the Appendix Table, part of which includes a partial analysis of each column of results.

Three statistical analyses were performed with the information contained in the Appendix Table. First, an analysis of the ratio ED_{50} (Y)/ED_{50} (X) was made (Tables 2 and 3). A highly significant difference exists between the means of the Y/X ratios (Table 3, line 2), with the tissue culture neutralization test yielding the higher mean. There is no significant difference between the egg and mouse neutralization test means. The challenge virus strains showed no significant differences (Table 3, line 1).

Secondly, an analysis of the observed variation within each vaccine for each test was made of the variances within each "cell" of the differences between the common logarithms of the 2 ED_{50}s for each replication (Tables 4 and 5). Although there was a suggestion that the egg test was more variable, no significant difference in variability was established among the vaccines or among the tests.

Thirdly, to test the significance of the differences of the means of each cell from the theoretical mean of 0.50, a *t*-test was performed on the mean of the 9 cells. Only 2—A2/Japan/170/62 (TCNT), B/Mass./3/66 (TCNT)—were significantly different.

TABLE 3
ANALYSIS OF VARIANCE OF TABLE 2

Line	Source	Sum of squares	Degrees of freedom	Mean square	Error line	F	P
1	Strain	0.554	2	0.277	4	1.321	NS
2	Test	4.5333	2	2.267	4	10.812	S (at 1 %)
3	Strain × Test	0.121	4	0.03025	4	1	NS
4	Error	17.612	84	0.20967			
5	Total	22.584	92				

TABLE 4
LOG (VARIANCE X 10^5)DEX(X) – DEX(Y)

Test	Strain			
	A2/Japan/170/62	A2/Taiwan/1/64	B/Mass./3/66	Sum of test
EgNT	3.5784	4.1518	4.0511	11.7813
MoNT	3.6520	3.7769	3.3278	10.7566
TCNT	3.8062	3.8436	3.1072	10.7570
Total	11.0366	11.7723	10.4861	33.2949 97

Also, the mean of the TCNT was significantly different from 0.50 for all strains tested.

DISCUSSION

In our hands the CCA test is both simple and reliable, although not completely free of variation. It may be hoped that additional research might reveal the causes of the variation we encounter between tests.

The immunological test for potency—the mouse potency test—leaves much to be desired. The extreme variability between tests precludes its usefulness for measuring the antigenic content of inactivated influenza vaccines. The test is long and tedious and it would be impracticable to perform the number of tests needed to obtain statistically significant results. On the other hand, the CCA test can be easily repeated to obtain sufficient results to make our conclusions on potency meaningful and reliable.

Investigators have assumed that neutralization tests performed in cell cultures were " more reproducible " than tests performed in animals and eggs. The results of these experiments, however, reveal a different picture. Although the mouse and egg neutralization tests used 10 mice and 10 eggs respectively at each point while the tissue culture test used only 2 cell culture tubes, statistically this is not considered sufficient to explain the consistently higher Y/X ratios obtained in the tissue culture tests.

The actual Y/X ratio of 0.50 and the serial 3-fold vaccine dilutions used to immunize the mice were circumstances calculated to provide a severe test upon the reproducibility of the neutralization tests employed. Thus, if a particular test is unable to show that the antigen content of vaccine is half that of another, it would not be a reliable test for ensuring consistency in influenza vaccine potency.

The data obtained in the second part of the mouse potency test, i.e., the neutralization test performed in either the mouse or the embryonated egg, statisti-

TABLE 5
ANALYSIS OF VARIANCE OF TABLE 4

Line	Source	Sum of squares	Degrees of freedom	Mean square	Error line	F	P
1	Strain	0.2333	2	0.1167	3	<1	NS
2	Test	0.1999	2	0.1000	3	<1	NS
3	Strain × Test	0.4392	3	0.1464	5	3.195	S
4	Total	0.8724	7				
5	Error			0.0458			

cally demonstrated protective differences between 2 vaccines differing in antigenic mass by as little as 2-fold. However, the dependence upon a single egg or mouse neutralization test to provide the correct vaccine/reference ratio assumed more than "biological" variation would allow. The simpler CCA test, on the other hand, did provide the reproducibility required for the correct determination of the vaccine/reference ratio once a stable CCA reference vaccine was prepared.

Although we were surprised to find such variability in the neutralization tests, we now feel that this is scientific naïveté on our part. We would never consider evaluating the antigenicity of a vaccine in man by immunizing 10 individuals, pooling their sera and performing a neutralization test on the single serum pool. Yet we do this in the laboratory, not only with inactivated influenza vaccine, but also with killed typhus vaccine. To evaluate the antigenicity of a vaccine in the field, we know from experience that large numbers are needed to obtain meaningful results. However, we justify using a meagre number of animals because we believe that a laboratory-reared animal is less variable than man. This is scientifically untenable. Probably the most variable factors in all these living systems are biological factors, composed of such things as genetic make-up, age, sex, weight, etc. The development of inbred animal strains does not completely solve this problem, for the multiplicity of sizes in animals of similar sex and age is well known. The competition for acquired characteristics begins as soon as one suckling animal has to compete with his litter-mate for milk.

An interesting method for measuring antigenicity of influenza virus is the serum-blocking technique described by Hebeka et al. (1968). Whether this could be used to measure the relative content of each of several components in multivalent vaccine preparations has yet to be determined. We are working on the development of an immunological CCA test to accomplish this. Our results are too preliminary for us to be able to know whether this test is useful.

Obviously, more research is needed on solving the problem of how to determine the potency of inactivated antigens. This is basic to the problems we encounter in the standardization of influenza vaccines. We shall continue our search for better methods to evaluate influenza vaccines until we are satisfied that we have or have not an effective means of immunizing against the ever-recurring influenza.

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APPENDIX TABLE

RESULTS AND PARTIAL ANALYSIS OF THE MOUSE, EGG AND TISSUE CULTURE NEUTRALIZATION TESTS FOR DETERMINING RELATIVE POTENCY OF INFLUENZA VIRUS VACCINES

TESTS WITH A2/Japan/170/62 VIRUS

Test No.	Challenge virus dose: antilog (dex)[a]	ED_{50}[b] of vaccines		Y/X ratio[e]
		Vaccine X:[c] antilog (dex)[a]	Vaccine Y:[d] antilog (dex)[a]	
		Mouse neutralization test (MoNT)		
1	631 (2.8)	50 (1.698)	40 (1.602)	0.80
2	631 (2.8)	56 (1.746)	13 (1.122)	0.23
3	1 000 (3.0)	29 (1.458)	17 (1.218)	0.59
4	251 (2.4)	40 (1.602)	29 (1.458)	0.73
5	200 (2.3)	45 (1.650)	26 (1.410)	0.58
6	398 (2.6)	87 (1.938)	29 (1.458)	0.33
7	631 (2.8)	50 (1.698)	50 (1.698)	1.00
8	159 (2.2)	78 (1.890)	32 (1.506)	0.41
9	100 (2.0)	135 (2.130)	87 (1.938)	0.64
10	159 (2.2)	70 (1.842)	70 (1.842)	1.00
11	126 (2.1)	234 (2.370)	70 (1.842)	0.30
Mean[e]	390	79	42	0.60
Variance[e]	86 235	3 469	582	0.07
SD[e,f]	294	59	24	0.27
SE[e,g]	89	18	7	0.08
		Egg neutralization test (EgNT)		
1	317 (2.5)	70 (1.842)	26 (1.410)	0.37
2	317 (2.5)	78 (1.890)	50 (1.698)	0.64
3	317 (2.5)	78 (1.890)	62 (1.794)	0.80
4	500 (2.7)	108 (2.034)	26 (1.410)	0.25
5	794 (2.9)	168 (2.226)	50 (1.698)	0.30
6	200 (2.3)	108 (2.034)	36 (1.554)	0.33
7	500 (2.7)	168 (2.226)	32 (1.506)	0.19
8	794 (2.9)	97 (1.986)	26 (1.410)	0.27
9	317 (2.5)	78 (1.890)	36 (1.554)	0.46
10	794 (2.9)	70 (1.842)	32 (1.506)	0.46
Mean[e]	485	102	38	0.41
Variance[e]	53 446	1 398	153	0.04
SD[e,f]	231	37	12	0.19
SE[e,g]	73	12	3.9	0.06
		Tissue-culture neutralization test (TCNT)		
1	200 (2.3)	2 090 (3.32)	1 200 (3.08)	0.57
2	316 (2.5)	1 200 (3.08)	398 (2.60)	0.33
3	316 (2.5)	692 (2.84)	398 (2.60)	0.58
4	100 (2.0)	1 200 (3.08)	2 090 (3.32)	1.74
5	316 (2.5)	1 200 (3.08)	2 090 (3.32)	1.74
6	316 (2.5)	692 (2.84)	2 090 (3.32)	3.02
7	1 000 (3.0)	1 200 (3.08)	1 200 (3.08)	1.00
8	316 (2.5)	692 (2.84)	692 (2.84)	1.00
9	316 (2.5)	692 (2.84)	692 (2.84)	1.00
10	100 (2.0)	692 (2.84)	1 200 (3.08)	1.70
Mean[e]	30	1 035	1 097	1.27
Variance[e]	63 642	201 130	582 127	0.64
SD[e,f]	252	449	763	0.80
SE[e,g]	80	142	241	0.25

APPENDIX TABLE (*continued*)

TESTS WITH A2/Taiwan/1/64 VIRUS

Test No.	Challenge virus dose: antilog (dex)[a]	ED_{50}[b] of vaccines		Y/X ratio[e]
		Vaccine X:[c] antilog (dex)[a]	Vaccine Y:[d] antilog (dex)[a]	
Mouse neutralization test (MoNT)				
1	501 (2.7)	365 (2.562)	87 (1.938)	0.24
2	79 (1.9)	327 (2.514)	210 (2.322)	0.64
3	126 (2.1)	791 (2.898)	365 (2.562)	0.46
4	251 (2.4)	508 (2.706)	78 (1.890)	0.15
5	158 (2.2)	407 (2.610)	455 (2.658)	1.12
6	158 (2.2)	368 (2.754)	708 (2.850)	1.25
7	126 (2.1)	327 (2.514)	188 (2.274)	0.58
8	126 (2.1)	634 (2.802)	234 (2.370)	0.37
9	79 (1.9)	568 (2.754)	234 (2.370)	0.41
10	100 (2.0)	883 (2.946)	210 (2.322)	0.24
11	251 (2.4)	708 (2.850)	234 (2.370)	0.33
12	100 (2.0)	455 (2.658)	262 (2.418)	0.58
13	100 (2.0)	210 (2.322)	108 (2.034)	0.51
14	80 (1.9)	292 (2.466)	108 (2.034)	0.37
15	316 (2.5)	327 (2.514)	97 (1.986)	0.30
Mean[e]	170	491	239	0.50
Variance[e]	13 533	39 059	27 921	0.10
SD[e,f]	116	198	167	0.31
SE[e,g]	30	51	43	0.08
Egg neutralization test (EgNT)				
1	200 (2.3)	408 (2.610)	508 (2.706)	1.25
2	316 (2.5)	708 (2.850)	622 (1.794)	0.88
3	316 (2.5)	1 215 (3.090)	708 (2.850)	0.58
4	200 (2.3)	708 (2.850)	708 (2.850)	1.00
5	251 (2.4)	708 (2.850)	508 (2.706)	0.72
6	316 (2.5)	407 (2.610)	78 (1.890)	0.19
7	316 (2.5)	568 (2.754)	78 (1.890)	0.14
8	631 (2.8)	508 (2.706)	70 (1.842)	0.14
9	631 (2.8)	455 (2.658)	108 (2.034)	0.24
10	316 (2.5)	708 (2.850)	56 (1.746)	0.08
Mean[e]	349	639	344	0.52
Variance[e]	24 278	57 118	83 477	0.18
SD[e,f]	156	239	289	0.42
SE[e,g]	49	76	91	0.13
Tissue-culture neutralization test (TCNT)				
1	100 (2.0)	2 090 (3.32)	2 090 (3.32)	1.00
2	1 000 (3.0)	1 200 (3.08)	1 200 (3.08)	1.00
3	1 000 (3.0)	2 090 (3.32)	1 200 (3.08)	0.57
4	32 (1.5)	2 090 (3.32)	692 (2.84)	0.33
5	32 (1.5)	2 090 (3.32)	692 (2.84)	0.33
6	100 (2.0)	2 090 (3.32)	692 (2.84)	0.33
7	32 (1.5)	1 200 (3.08)	2 090 (3.32)	1.74
8	316 (2.5)	692 (2.84)	229 (2.36)	0.33
9	1 000 (3.0)	692 (2.84)	398 (2.60)	0.58
10	316 (2.5)	692 (2.84)	692 (2.84)	1.00
Mean[e]	393	1 493	998	0.72
Variance[e]	186 652	430 949	421 382	0.21
SD[e,f]	432	656	649	0.46
SE[e,g]	137	208	205	0.15

APPENDIX TABLE (*concluded*)

TESTS WITH B/Mass/3/66 VIRUS

Test No.	Challenge virus dose: antilog (dex)[a]	ED_{50}[b] of vaccines		Y/X ratio[e]
		Vaccine X:[c] antilog (dex)[a]	Vaccine Y:[d] antilog (dex)[a]	
Mouse neutralization test (MoNT)				
1	158 (2.2)	70 (1.842)	56 (1.746)	0.80
2	126 (2.1)	234 (2.370)	78 (1.890)	0.33
3	50 (1.7)	708 (2.850)	292 (2.466)	0.41
4	200 (2.3)	262 (2.418)	108 (2.034)	0.41
5	63 (1.8)	987 (2.994)	634 (2.802)	0.64
6	158 (2.2)	262 (2.418)	135 (2.130)	0.52
7	50 (1.7)	234 (2.370)	151 (2.178)	0.65
8	100 (2.0)	78 (1.890)	29 (1.458)	0.37
9	80 (1.9)	70 (1.850)	63 (1.803)	0.90
10	126 (2.1)	108 (2.034)	50 (1.698)	0.46
Mean[e]	111	301	160	0.55
Variance[e]	2 624	93 552	33 524	0.04
SD[e,f]	51	306	183	0.19
SE[e,g]	16	97	58	0.06
Egg neutralization test (EgNT)				
1	200 (2.3)	70 (1.842)	19 (1.266)	0.27
2	316 (2.5)	26 (1.410)	21 (1.314)	0.81
3	316 (2.5)	21 (1.314)	5.5 (0.738)	0.26
4	316 (2.5)	23 (1.362)	13 (1.122)	0.57
5	126 (2.1)	19 (1.266)	50 (1.698)	2.63
6	251 (2.4)	62 (1.794)	26 (1.410)	0.42
7	316 (2.5)	29 (1.458)	5.5 (0.738)	0.19
8	631 (2.8)	18 (1.266)	13 (1.122)	0.72
9	316 (2.5)	28 (1.410)	8.5 (0.930)	0.30
10	398 (2.6)	9.5 (0.978)	4.4 (0.647)	0.46
11	317 (2.5)	32 (1.506)	6 (0.786)	0.19
Mean[e]	319	31	16	0.62
Variance[e]	15 967	346	182	0.49
SD[e,f]	126	19	14	0.70
SE[e,g]	38	6	4	0.21
Tissue-culture neutralization test (TCNT)				
1	63 (1.8)	2 090 (3.32)	3 645 (3.56)	1.74
2	316 (2.5)	2 090 (3.32)	2 090 (3.32)	1.00
3	316 (2.5)	2 090 (3.32)	2 090 (3.32)	1.00
4	100 (2.0)	2 090 (3.32)	2 090 (3.32)	1.00
5	316 (2.5)	2 090 (3.32)	2 090 (3.32)	1.00
6	1 000 (3.0)	1 200 (3.08)	1 200 (3.08)	1.00
7	1 000 (3.0)	1 200 (3.08)	692 (2.84)	0.58
8	100 (2.0)	2 090 (3.32)	2 090 (3.32)	1.00
9	1 000 (3.0)	2 090 (3.32)	2 090 (3.32)	1.00
10	1 000 (3.0)	2 090 (3.32)	2 090 (3.32)	1.00
Mean[e]	521	1 912	2 017	1.03
Variance[e]	178 676	140 818	567 867	0.08
SD[e,f]	423	375	754	0.28
SE[e,g]	134	119	238	0.09

[a] Dex = decimal exponents; see Haldane (1960).
[b] 50 % effective dose, i.e., that dilution of vaccine that elicited sufficient antibody to protect 50 % of the mice, eggs or tissue cultures in the respective neutralization tests.
[c] Vaccine X is NIH 67CP.
[d] Vaccine Y is NIH 67CP diluted 1 : 2 with 0.01 M phosphate-buffered saline.
[e] Determined from antilog values.
[f] Standard deviation.
[g] Standard error.