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A Proposed Potency Test for Smallpox Vaccine. I. Evaluation of Chick Embryo LD_{50} Method. (23305)

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The official potency requirement in force today, at least in the United States, for release of smallpox vaccine is based on a modification of the method of Calmette and Guérin(1,2), suggested by Force and Leake (3). This method has been most valuable over the years, but those engaged in the production and distribution of smallpox vaccine are familiar with the irregularities to which the rabbit test is subject and are aware of the lack of quantitative criteria for interpretation and recording of results. In fact, only relative values of the potency of a vaccine under test can be estimated, since a control vaccine of acceptable potency always has to be titrated in parallel on the same animal. The desirability, therefore, of a test method less susceptible to variation and capable of measuring the absolute titer of smallpox vaccine is quite evident. A titration method for vaccinia virus based on the number of pocks formed on the chorioallantoic membrane of embryonated hen eggs was first described by Burnet(4). Although this technic was found most effective for titration of vaccinia virus preparations of relative purity, it has not generally proved very satisfactory with the comparatively coarse smallpox vaccine suspensions. Furthermore, even with purified vaccinia virus, difficulties have been encountered with this method unless applied under specified optimal conditions(5).

Observations in this laboratory over the years(6) have indicated that embryos inoculated on the chorioallantoic membrane with dilutions of smallpox vaccine eventually die from the infection, and that death is invariably associated with the presence of pocks on that membrane. Moreover, there seems to be a linear relation between log-dilution of vaccine and reciprocal embryo survival time, similar to that reported for the Psittacosis-LGV group of viruses(7,8).

The present communication reports results of repeated titrations of the same smallpox vaccine preparation by the chick embryo LD_{50} method over a period of two years.

Materials and methods. Virus strain. The virus used in this study originated from human vaccination scabs obtained from the New York City Board of Health in 1909. It has since been maintained in this laboratory by irregular, alternating passages through calves, rabbits and humans, and has been used continuously for commercial production of calf lymph vaccine by the standard procedure. *Smallpox vaccine.* All titrations to be reported here were carried out with the same *glycerinated control vaccine virus* prepared in accordance with the minimum requirements set forth by the National Institutes of Health, Bethesda, Md., and meeting the requirement

of potency on humans and rabbits(9). It consisted of a 20% calf lymph suspension in 45-50% neutral glycerine. At the onset of this study, the vaccine was distributed in 1.0 ml amounts in sealed glass ampoules and stored at a constant −60°C. Individual ampoules were thawed out at the time of use. *Chick embryo and inoculation methods.* The chick embryos employed were of the Leghorn breed. They were routinely received from the same supplier on the day of inoculation, when 11 to 12 days of age. Regardless of the route of injection, a uniform inoculum volume of 0.25 ml was used per embryo. *Allantoic cavity (AC) inoculation.* The margin of the air sac was located by candling, and a small hole was drilled through the shell at a point ¼″ above the margin and as far as possible from the embryo. Inoculation was done by inserting a ¾″-23 gauge needle full length through the hole at a slight angle toward the shell and with the egg standing upright on its small end. *Yolk sac (YS) inoculation.* Provided the air sac was in its proper position, a small hole was drilled at the highest point of the large end of the shell. A 1″-21 gauge needle, inserted full length vertically through the hole as the egg stood upright, was used for inoculation. *Chorioallantoic membrane (CAM) inoculation.* In a departure from the classical method of Beveridge and Burnet (10), routine CAM inoculations were carried out on large numbers of embryos as follows: The embryo was held against the candler in a horizontal position with an appropriate area of the CAM uppermost, and two 1/16″ holes were made with an electric hand drill, one at the center of the natural air sac and the other over the area to be inoculated. Care was always exercised not to injure the underlying shell-membrane (SM). At first, a sterile hypodermic needle was used to pierce the SM over the air sac and then, bevel up, to nick it very lightly over the CAM. Experience soon indicated that the CAM separated from the SM and collapsed just by gravitation, expelling the air from the natural air sac. Only occasionally was it found necessary to apply a slight suction over the air sac while nicking the SM over the CAM. Following inoculation by any of these 3 routes, the shell holes were sealed with collodion and the embryos incubated at 36°-37°C. Care was taken to place the CAM inoculated embryos in a horizontal position with the collodion seal uppermost. *Vaccine titration and interpretation of results.* The virus was diluted routinely in 10-fold steps in beef heart infusion broth containing 500 units of penicillin and 100 μg of streptomycin per ml. Inoculated eggs were candled every day at the same time, and dead embryos were removed and examined carefully. Those dead 24 hours after inoculation were excluded from calculation, as were those who died after 48 hours without showing evidence of infection. Similarly, embryos inoculated on the CAM, whose death on any day could not be associated with virus multiplication by the presence of pocks, were considered to have died from other causes and were also excluded. Calculation of LD_{50} titers was done by the Reed and Muench formula(11) at the end of various incubation periods.

Results. Comparative titration of smallpox vaccine by CAM, AC and YS routes. It has been indicated(6) that, whereas a $10^{-5.0}$ dilution of the control vaccine virus caused death of the embryos in 96 to 120 hours when inoculated on the CAM, a $10^{-3.0}$ dilution of the same vaccine was required by the AC route to kill the embryo within the same interval. Variation in susceptibility of embryos depending upon route of inoculation was repeatedly observed in subsequent experiments as illustrated by the following typical simultaneous titration: the control vaccine virus was diluted from $10^{-3.0}$ to $10^{-9.0}$, and each dilution was inoculated into 6 embryos by either the CAM, AC, or YS routes. The time of death at each dilution and the LD_{50} endpoint for each route of inoculation on the 7th day are given in Table I. It is readily seen that the LD_{50} titer is at least 2 logs higher in the CAM group than in the groups inoculated by either the AC or YS routes. Because of the greater susceptibility of embryos injected on the CAM, this route was employed exclusively for all subsequent titrations.

Repeated titrations of smallpox vaccine by the CAM route. Between Sept. 1949 and

TABLE I. Comparative Chick Embryo Titration of Smallpox Vaccine by Various Routes of Inoculation.

Route of inoc.	Virus dilution	Death pattern: No. dead/No. inoc. (hours) 24	48	72	96	120	144	168	Accumulation Dead	Alive	LD_{50} titer
CAM	10^{-3}	0/6	3/6	6/6					31	0	
	-4		0/6	5/6	6/6				25	0	
	-5		"	3/6	"				19	0	
	-6	1/5*		0/5	5/5				13	0	
	-7				0/6	4/6	4/6	5/6	8	1	$10^{-7.8}$
	-8	" *					0/5	2/5	3	4	
	-9				"	1/6†	0/5	1/5	1	8	
AC	10^{-3}	0/6	2/6*	0/4	0/4	4/4			14	0	
	-4				0/6	2/6	2/6	5/6	10	1	
	-5		1/6*				0/5	4/5	5	2	$10^{-5.4}$
	-6						0/6	1/6	1	7	
	-7		1/5*					0/5	0	12	
	-8							0/6	0	18	
	-9							"	0	24	
YS	10^{-3}			1/6	1/6	6/6			17	0	
	-4				0/6	3/6	3/6	6/6	11	0	
	-5						0/6	3/6	5	3	$10^{-6.3}$
	-6		1/6*				0/5	2/5	2	6	
	-7							0/6	0	12	
	-8							"	0	18	
	-9		" *					0/5	0	23	

* Traumatic deaths: excluded from calculation.
† No pocks on CAM: " " " .

Sept. 1951, the same control vaccine virus was titrated on the CAM 28 times using dilutions ranging from $10^{-6.0}$ to $10^{-9.0}$ and 6 embryos per dilution. In an effort to determine the optimal interval, LD_{50} titers were calculated at the end of 5, 6, 7, and 8 days incubation. Geometric means of the 28 titrations at these time intervals, and the standard deviation of log titers are shown in Table II. Although the majority of deaths occurred by the 5th day, the number of embryos dying was significant until the 7th day, after which relatively few died. The standard deviation of log titers was lowest on either the 6th or 7th day. An incubation period of 7 days was, therefore, selected for termination of titrations, and the frequency distribution of LD_{50} titers from the 28 titrations, given in Table III, shows that titers lower than $10^{-7.2}$ or higher than $10^{-8.0}$ were only infrequently obtained.

TABLE II. Geometric Means of Chick Embryo LD_{50} Titers of Control Vaccine Virus Incubated for 5, 6, 7 and 8 Days Post-Infection.

Days incubation	No. of tests	Mean LD_{50} titers	Stand. dev. of log titers
5	27	$10^{-6.6}$	.43
6	28	$10^{-7.3}$	.34
7	28	$10^{-7.5}$	.33
8	25	$10^{-7.6}$	.36

These titrations, made at regular intervals over a period of 2 years, afforded a unique opportunity to determine whether seasonal changes had any effect on susceptibility of the embryo to vaccinia virus. The 7-day data were, therefore, reexamined according to the seasonal distribution of the titrations (Table IV). Because of the small number of tests,

TABLE III. Frequency Distribution of Chick Embryo LD_{50} Titers of Smallpox Vaccine on 7th Day of Incubation.

LD_{50} titers	$10^{-6.7}$	$10^{-7.0}$	$10^{-7.2}$	$10^{-7.4}$	$10^{-7.5}$	$10^{-7.6}$	$10^{-7.7}$	$10^{-8.0}$	$10^{-8.4}$	Total	Geometric mean	Stand. dev. of log titer
No. of assays	1	1	4	5	7	4	2	3	1	28	$10^{-7.5}$	.33

TABLE IV. Seasonal Variation of Mean Chick-Embryo LD_{50} Titers of Smallpox Vaccine on 7th Day of Incubation.

Season	No. of titrations	Mean LD_{50} titers	Stand. dev. of log titers
Spring (Mar. 23-June 15)	9	$10^{-7.4}$	.36
Summer (June 22-Sept. 8)	8	$10^{-7.7}$	.39
Fall & Winter (Sept. 28-Mar. 16)	11	$10^{-7.4}$	.20
Total	28	$10^{-7.5}$	.33

those made in fall and winter were calculated together. It can be seen that seasonal variations of mean titers and of respective standard deviations were not significant.

Discussion. The chick embryo LD_{50} method, using 24 embryos, 6 at each of 4 dilutions, was used in this study. Applying Gaddum's formula(12) to determine standard error of a log LD_{50} calculated by the Reed and Muench method, a theoretical value of 0.30 in logarithmic units was obtained. The 28 titrations gave individual log LD_{50} titers ranging from −6.7 to −8.4 with a standard deviation of 0.33. This is in good agreement with the theoretical value of 0.30, and accordingly, there seems to be no indication that the LD_{50} titer may change appreciably with time. It would thus be legitimate to calculate potency of a sample in relation to long-term average LD_{50} titer of the control vaccine, rather than to titrate each new vaccine lot in parallel with the control vaccine, as is done in the rabbit test. Check titrations of the control vaccine in the chick embryo, at periodic intervals, however, may be advisable.

These results would seem to indicate that production lots of vaccine should have minimum chick embryo LD_{50} titers of $10^{-7.5 \pm 0.33}$ to be of acceptable potency. From the frequency distribution of 7-day titers of the standard vaccine, it seems reasonable to assume that, should a vaccine lot fail to meet the lower limit on first test, one, or at most 2 additional titrations may be required to determine definitely whether it should be retained or rejected. The chance for such an occurrence, however, is of the order of 2 in 28. Since this study was undertaken, many production lots of calf lymph prepared in these laboratories, as well as several vaccines of chick embryo origin, were tested for potency by both the conventional rabbit and the chick embryo LD_{50} method. In not a single instance did a vaccine which passed the rabbit test fail to reach a chick embryo LD_{50} titer as described above. Admittedly, the chick embryo LD_{50} titer would assume a greater degree of significance if it were correlated with human vaccination by simultaneous titration of the same vaccine in humans and in the chick embryo. Such a study is under consideration.

Summary. 1) A glycerinated control smallpox vaccine of calf lymph origin was titrated for a total of 28 times, over a period of 2 years, by the chick embryo LD_{50} method. Calculations of LD_{50} titers were made at the end of 5, 6, 7, and 8 days incubation, and those of the 7th day were found to be optimal. Individual titers on the 7th day ranged from $10^{-6.7}$ to $10^{-8.4}$, giving a geometric average titer of $10^{-7.5}$ for the control vaccine with a standard deviation of 0.33 log unit. No significant seasonal variations of the average LD_{50} titers were noted. 2) The precision of the method and its applicability to the potency testing of smallpox vaccine have been discussed.

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New Antigenic Variant in Far East Influenza Epidemic, 1957. (23306)

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During the late spring of 1957, epidemic influenza characterized by clinically mild illness and by high attack rates occurred throughout the Far East, following a winter of relatively low influenza prevalence throughout most of the world. The viruses responsible for the Far East epidemic were shown(1) in this laboratory to be type A influenza of strikingly different antigenic composition from strains recovered in previous years. This paper summarizes the clinical and epidemiological reports from the Far East outbreak, presents the results of the detailed antigenic analyses of these viruses and the antibody response to them in patients, and gives information concerning the biological properties of these viruses and the antibody status of U.S. military personnel.

Materials and methods. Far East virus strains designated A-Japan-305-57 and A-Japan-307-57 were recovered in embryonated eggs from American military personnel by Drs. S. E. Grossberg and I. Gresser at U.S. Army 406th Medical General Laboratory, Zama, Japan, and were forwarded to this laboratory. The A-Malaya-309-57, 310-57, and 311-57 strains were recovered in Malaya by Dr. J. H. Hale of the University of Singapore. A-Hong Kong-304-57 virus was isolated in this laboratory from throat washings collected by Dr. C. Kraul of the 406th Medical General Laboratory from a patient in the Hong Kong outbreak. The A-Formosa-313-57 strain, recovered at the Taiwan Serum Vaccine Laboratory, was sent by the U.S. Naval Medical Research Unit #2 to the 406th Medical General Laboratory and forwarded to this institution. *Prototype strains.* Strains selected from previous epidemics for purpose of comparison are referred to as prototype strains. The prototype virus designated A-Japan-301-56 was recovered in Dec. 1956 in Japan and was furnished by Dr. M. Kitaoka, of the Nat. Inst. of Health, Tokyo, Japan. Miss Betty Ransom of the U.S. Army Hawaiian Med. Laboratory furnished the A-Hawaii-303-56 strain. The remaining prototype viruses used to prepare antigens or antisera in this laboratory have been described (2,3). *Hemagglutinating antigens* were allantoic fluids from embryonated eggs infected with influenza virus. The antigens prepared from the new Far East isolates were in egg passage 2 to 5 and titered 1:40 to 1:320 in tests with human "O" red blood cells. Drs. Thomas Francis, Jr., Keith Jensen, Bernice Eddy, or Robert M. Chanock furnished hemagglutinating antigens for the A-AA-1-57 (Mich.), A-Denver-2-57 (Colo.), A-AA-4-56 (Mich.), A-Ned-36-56 (Netherlands), A-Sendai-5-56 (Japan), B-GL-1739-54 (Ill.), A-Swine, and type D-Sendai (Japan)(4,5) viruses. The remaining antigens were standard reagents used by this laboratory and the strains employed in their preparation are described above or elsewhere(2,3). The methods used to prepare the antigens have been described(2,3,6). *Animal antisera.* Sera against the new Far East isolates were prepared in chickens by methods described earlier(2,3,6). Each chicken received 20 ml of undiluted infected allantoic fluid on 2 successive days and the animal was bled 9 or 10 days following the first injection. The ferret