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Comparative Susceptibility of Cell Cultures to Vaccinia Virus: Application to the Standardization of Smallpox Vaccine. (23774)

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Although the vaccine employed for immunization of man against smallpox is prepared from bovine or avian tissues, the assay method generally used for determining vaccine potency has employed still another species, the rabbit(1). Because considerable natural resistance and variable sensitivity are encountered in the routine performance of vaccine potency assays in rabbit epithelium, in recent years titration of vaccinia in the embryonated egg has supplemented and in some cases supplanted the rabbit dermal techniques(2,3). Whether based upon pock counts or lethal dose determinations the chorioallantoic method appears simpler, cheaper and more reliable than the rabbit assay(4a,b,c). The growth of human, simian, and bovine cells in tissue culture and the marked cytopathic effects produced by vaccinia virus in all mammalian cells studied offers still another means of testing smallpox vaccine and the attractive possibility that this biologic could be simultaneously assayed in cell systems homologous to the source and to the primate recipient. In addition plaque techniques may permit the use of more quantitative procedures in the manufacture and standardization of this biologic.

The following report is concerned with the susceptibility of several tissue cultures to vaccinia virus derived from bovine embryonic cells, calf lymph or embryonated eggs. The high susceptibility of monkey kidney monolayers to vaccinia virus has been utilized for comparative titrations of this agent by roller tube and plaque methods.

Materials and methods. Vaccinia seed virus was obtained as the National Institutes of Health Calf Lymph Reference Standard, Lot 1b, a lyophilized preparation of dermal pulp. Serial passages of this strain were initiated in various cell lines by inoculating 2 or more cultures of each type. When all the cells in the culture showed cytopathic changes, the supernatant fluids from one type were pooled and 0.1 ml of the pool passed to fresh tubes of the same tissue culture. The remaining pooled fluids from each passage were stored at −20°C until used. Certain of the calf lymph and egg vaccines were generously supplied by Cutter Laboratories, Lederle Laboratories, Merck, Sharp and Dohme, National Drug Co., Parke, Davis and Co., Wyeth Laboratories, Inc., and the New York City Board of Health. *Titrations* in rabbits (weighing 3-4 kg) were performed in two

TABLE I. Multiplication of Vaccinia in Bovine and Simian Cell Cultures.

Source	Test cell	$TCID_{50\%}$/ml at hours 30	48	68	90	115
Bovine lung	Bovine kidney	4.5	5.5	5.5	4.0	3.5
	Monkey "	4.5	4.5	6.5	4.5	7.5
" muscle	Bovine muscle	4.5	6.5	7.5	6.5	
	Monkey kidney	6.0	6.5	7.5	6.5	7.0
" kidney	Bovine "	4.5	5.5	6.5	5.5	3.5
	Monkey "	6.0	7.5	6.5	7.5	6.0
Monkey "	" "	4.5	5.5	5.0	5.5	

fashions; by injecting 0.1 ml of the virus dilutions intradermally and observing for pock formation and by scarification (0.2 ml per scarified area) performed in accordance with the "Minimum Requirements: Smallpox Vaccines"(1) of the National Institutes of Health. Egg titrations were made by pock count on the chorioallantoic membrane of chick embryos as described by Jackson *et al.* (4b). Monkey and rabbit kidney epithelium cultures were prepared from the trypsinized organs by the method of Youngner(5), HeLa cell cultures as described by Scherer *et al.*(6). Serial passage bovine embryonic lung, muscle and kidney cultures were established from a single embryo as reported earlier(7).* Roller tube cultures were used for serial passages and titrations whereas for production of large pools of virus 32 oz. prescription bottles containing 50 ml of medium were employed. Plaque cultures of monkey kidney epithelium used in certain experiments were prepared by a method similar to that of Dulbecco and Vogt(8). Prior to use all cultures were drained of spent medium and washed once with Hanks' salt solution. Bovine and monkey cell cultures were fed Eagle's basal medium(9) containing 0.5% bovine albumin as a protein source. HeLa cells were cultivated in Eagle's medium with 10% horse serum and rabbit kidney cells were grown in either Eagle's medium or 0.5% lactalbumin hydrolysate in Earle's salt solution(10) plus 10% horse serum. Monkey kidney monolayers for plaque titrations were fed Eagle's basal medium (2X concentrated) supplemented with 20% skim milk; a medium suggested by Dr. S. Baron. Virus titrations were performed by inoculating 3-4 tissue cultures per dilution with 0.1 ml of virus diluted in Hanks' salt solution or in the case of plaque counts 0.5 ml of virus into triplicate bottles. Tubes were then fed with 0.9 ml of medium and plaque bottles overlaid with 5.0 ml of 3% Noble's agar plus the medium described above. In experiments in which titers were compared by roller tube and plaque methods (Table IV) the same medium was employed, *i.e.,* Eagle's basal medium with 10% skim milk. A tube was read as positive when at least one area of vaccinal necrosis had developed. In the case of plaque preparations the time of virus contact before adding overlay was found to be of importance. Accordingly a 6-hour interval at 37°C was adopted as standard procedure between inoculation of virus and addition of overlay.

Results. Growth of vaccinia in serial passage bovine embryonic tissue. Calf lymph vaccinia was readily propagated in bovine cell cultures derived from lung, kidney or muscle tissue, in HeLa cells and monkey and rabbit kidney epithelium cultures. In view of Wesslen's demonstration of bovine embryonic skin as a satisfactory source of smallpox vaccine(11), the behavior of vaccinia in bovine cell embryonic cultures was studied in some detail.

Growth curve experiments in bovine lung, muscle, kidney and, for comparison, monkey tissue cultures were performed with calf lymph virus which had a single passage in bovine lung, muscle or kidney. Culture fluids were sampled 30, 48, 68, 90 and 115 hours after infection and titrated in the homologous cell and monkey kidney cultures. The results of this experiment are shown in Table I. It

* A portion of the bovine cell cultures was obtained from Microbiological Assoc., Bethesda, Md.

TABLE II. Comparative Cell Susceptibility to Vaccinia Grown in Bovine and Simian Tissue Culture.

Source	Passage	TCD_{50}/ml in: Bovine Muscle	Bovine Kidney	Monkey kidney	HeLa	Rabbit kidney	Titer in rabbit: Scarif.*	I.D.†	CAM†
Bovine muscle	13	5.5	5.5	5.7	5.5	5.7	1: 1,000	4	5.8
	25	5.0	4.7	5.5	5.6	6.0	1: 100	<4	4.3
" kidney	13	4.5	4.0	4.5	4.5	3.7	1:30,000	"	4.0
	25	4.0	4.5	5.0	4.3	4.3	1: 100	"	2.8
" lung	10	5.5	5.0	5.7		5.5		5	5.8
Monkey kidney	13	5.6		6.0		6.5	1:30,000	5	6.0
	25			5.5					

* 0.2 ml of virus dilution/scarified area. † Per ml.

will be noted that the maximum yield in all tissues was reached approximately 3 days after infection. The yield of virus was comparable for each of the 4 cell lines, ranging from $10^{5.5}$ to $10^{7.5}$ TCD_{50} per ml, and there was no difference in susceptibility between bovine or simian cells.

To obtain tissue culture strains of vaccinia, which might have developed a predilection for the host tissue type, calf lymph virus was further passaged in bovine muscle, kidney and lung and in monkey kidney tissue cultures. The 13th and 25th serial passages in bovine muscle and kidney, in monkey kidney and the 10th passage in bovine lung were then titrated in a variety of cells, the rabbit and CAM as shown in Table II.

Propagation in each of these tissues resulted in decreased but stable infectivity when compared with the original calf lymph which had a titer of $10^{7.5}$ in monkey kidney. The passaged strains titered between $10^{5.7}$ and $10^{6.5}$ TCD_{50} per ml in tissue cultures of bovine, simian or human cells and the chorioallantoic membrane. In these systems no significant decrease occurred between 13 and 25 passages.

In contrast, the findings obtained by rabbit skin titration, using either scarification or intradermal injection, indicate a loss of infectivity for this host since the 25th bovine cell passage caused pocks at only a dilution of 1:100 and failed to elicit any intradermal reaction at 1:10,000. It is to be noted that on the basis of their infectivity for bovine or monkey kidney cells the concentration of virus in these 2 bovine lines had not changed between the 13th and 25th passage.

Comparative infectivity of smallpox vaccines in various systems. A clearer definition of gradation in cellular susceptibility to vaccinia virus was obtained when the infectivity of a group of commercial smallpox vaccines was determined using tissue cultures, rabbits, and embryonated eggs.

A total of 10 vaccines, 9 of calf and one of egg origin and from 7 commercial laboratories were tested. All of these had met the potency requirements of the National Institutes of Health. As can be seen from Table III three ranges of susceptibility were encountered. Tested on monkey kidney these biologics had infectivity titers in the region of 10^8 TCD_{50} per ml with an average of 7.5. HeLa and rabbit kidney cells, the chorioallantoic membrane and bovine cells constituted a second order of susceptibility with, in certain instances, a 2 log difference in titer as compared with that in monkey kidney. It should be noted that in 4 instances different lots of calf lymph titered $10^{8.0}$ or higher in the monkey kidney whereas these same products possessed 0.3 to 2.0 logs less virus on the basis of the rabbit intradermal or CAM titration. This greater susceptibility of monkey kidney tissue culture over that of bovine cells was also found during an experiment designed to determine the yield of vaccinia virus per cell after 27 passages in bovine muscle tissue culture. This strain of virus had a calculated yield of 2.1 infectious units produced per cell when titration was made in bovine kidney as compared with a yield of 20 infectious units per cell when the same material was titrated in monkey kidney.

Titration in the rabbit skin by intradermal

TABLE III. Infective Titration of Smallpox Vaccines in Various Systems.

Vaccine	Log TCD_{50}/ml: Bovine Muscle	Bovine Kidney	Monkey kidney	HeLa	Rabbit kidney	Rabbit scarif. titer†	ID_{50}/ml: Rabbit I.D.	Egg CAM
NIH Ref.		6.7	8.0	7.3	7.5	1:10,000	7.2	7.7
1*	4.5	5.5	4.5		4.5			4.5
1A		7.3	>8.0	6.7	7.5	1:30,000	6.0	7.3
2	6.5	7.3	8.5		7.0	"		6.5
3	6.7	7.5	>8.5			"		6.5
3A		6.5		7.0	7.5			
4	6.0	6.5	>7.0		7.0	"		4.5
5		7.0	>8.0	7.2	7.5	"	7.0	7.2
6		7.5	8.0	7.5	7.5	"	7.0	7.2
7	6.3		7.3	7.0	7.7	"	7.5	7.3
8		7.5	7.7	7.2	7.5			

* 1-7 are calf lymph vaccines; 8 is egg vaccine. † 0.2 ml of virus dilution/scarified area.

inoculation seems comparable to the chorioallantoic membrane but less sensitive a procedure than when performed in monkey kidney cultures. The infectivity levels established by scarification of the rabbit were at the low end of the scale. In fact, Vaccines #2 and #4 gave only a single pock at a dilution of 1:30,000 on the scarified rabbit skin although their titers in a monkey kidney tissue culture were $10^{8.5}$ and greater than $10^{7.0}$ TCD_{50} per ml respectively. Unfortunately there were no smallpox vaccines of substandard potency available with which to compare levels with those illustrated.

Relative sensitivity of monkey kidney tissue culture and the rabbit to vaccinia virus. When it became apparent that monkey kidney epithelium could be readily infected with vaccinia virus to high terminal dilutions a more detailed study was made of this culture involving a series of comparative titrations of calf lymph vaccine in plaques and roller tubes and in the scarified rabbit epithelium.

In agreement with Youngner's(5) earlier observations was the finding that successful plaque counts of vaccinia depended upon adequate attachment time of virus to cells and the medium used in the agar overlay. In a series of experiments with 25th passage monkey kidney-adapted virus the plaque titers were compared after varying periods of virus-cell contact at 37°C and with or without shaking during these intervals. Results of such an experiment, shown in Table VI, indicate that approximately 6 hours is required for complete adsorption of virus. Shaking for this interval at a rate of 60 excursions per minute on a reciprocating type machine did not influence the infectivity end points. Nevertheless the practice of agitating the bottles once during the attachment period was adopted.

Comparison of the media in the overlay for the monkey kidney-vaccinia plaque system provided data which, although not susceptible to tabulation because most of the differences consisted of qualitative changes in the cell sheet rather than infective titer, suggested that the skim milk Eagle's medium formula was the most satisfactory. This was adopted as the standard medium. The overlay media tested were as follows:

A. Medium 199
B. Medium 199 with 20% skim milk
C. Eagle's basal medium
D. Eagle's basal medium with 20% skim milk
E. Eagle's basal medium with 0.25% bovine albumin
F. Youngner's medium(5)

Because the focal necrosis caused by vaccinia virus resembles somewhat the open areas which occur in monkey kidney monolayers microscopic examination of vaccinia plaques proved useful in recording endpoints. Characteristic rolled edges and loose swollen cells at the periphery of the necrotic plaque make differentiation of the specific virus lesion a simple matter. Agreement between roller tube and plaque titrations of vaccinia virus in monkey kidney epithelium is good. The results of one such experiment are shown in Table IV. Three lots of calf lymph virus were simultaneously titrated in cells of the same lot by

TABLE IV. Comparative Assay of Smallpox Vaccines in Monkey Kidney Tissue Culture Using Plaque and Roller Tube Methods.

Vaccine	Conc.	Plaque: Count*	Plaque: Log titer PFU/ml	Roller tube: CPE	Roller tube: Log TCD_{50}/ml
3A	10^{-5}	TNC†	$10^{7.8}$	4/4	$10^{7.6}$
	10^{-6}			"	
	10^{-7}	3		"	
	10^{-8}			1/4	
1A	10^{-5}	TNC	$10^{8.0}$	4/4	$10^{7.6}$
	10^{-6}			"	
	10^{-7}	5		"	
	10^{-8}			1/4	
5	10^{-5}	TNC	$10^{7.0}$	4/4	$10^{7.6}$
	10^{-6}			"	
	10^{-7}	.6		"	
	10^{-8}			0/4	

* Avg counts for triplicate cultures.
† Too numerous to count.

both methods using triplicate plaque bottles and quadruplicate tubes per dilution. The data indicate that a single plaque infectious unit resulted in a positive roller tube and endpoints were the same by either method. Another example of this is shown in Table V discussed below.

It has been long recognized that the titration of calf lymph vaccines on the scarified skin of the rabbit, a procedure based on Calmette and Guerin's work of 55 years ago(12), affords only a rough approximation of absolute infectivity. Although intradermal titration in rabbits, as suggested by Smadel *et al.* (13), is more consistent, the standard potency assay for smallpox vaccines in the U.S. continues to employ scarification. On the arbitrary assumption that titration in monkey kidney cultures yields an index more closely approximating the number of infective particles, it was considered profitable to conduct a series of titrations in tissue cultures and scarified rabbits with lots of fresh calf lymph and egg vaccine to determine the differential of sensitivity of the two methods. Both plaque and roller tube cultures were employed. The detailed results included in Table V illustrate the consistency of the pro-

TABLE V. Comparative Titration of Commercial Smallpox Vaccines in Monkey Kidney Tissue Culture and the Scarified Rabbit.

Vaccine	Source	Monkey kidney tissue culture: Exp. No.	Plaque PFU/ml (Log)	TCD_{50}/ml (Log)	Scarified rabbit*: Rabbit No.	Pocks/dilution 1:1000	1:3000	1:10,000	1:30,000
NIH control 1b	Calf lymph	1	7.3	7.0	1–4 avg	Conf.†	Conf.	Conf.	5
		2	7.7	8.0					
		3	7.8	7.8					
		4	7.6	7.6					
1a	*Idem*	1	8.0	7.6	1	"	"	"	Conf.
3a	"	1	7.8	7.6	1	"	"	"	"
5	"	1	7.0	7.5	1	"	"	"	17
9	"	1	7.6	8.0		Not done			
					1	14	14	3	1
		2	7.7	8.3	2	7	5	1	0
					3	15	Conf.	10	5
					4	2	2	1	2
					1	3	5	3	1
		3	7.5	7.5	2	11	9	7	3
					3	Conf.	Conf.	8	4
					4	8	5	5	3
10	"	1	8.5	8.3		Not done			
					1	Conf.	Conf.	Conf.	2
		2	8.5	8.3	2	"	"	"	Conf.
					3	"	"	"	"
					4	"	"	"	"
					1	"	"	"	"
		3	8.6	8.3	2	"	"	"	5
					3	"	"	"	Conf.
					4	"	"	"	"
11	Egg	1	8.0	8.0		Not done			

* 0.2 ml/site. † Confluent lesions.

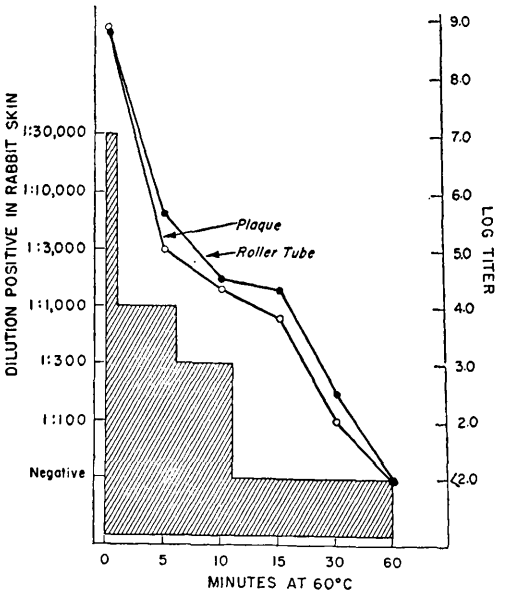

FIG. 1. Thermal inactivation of vaccinia virus as determined in monkey kidney tissue culture and scarified rabbit skin.

cedures as well as the inadequacy of the rabbit method in reflecting the infectiousness of a preparation. For example, Reference Vaccine 1b was retested at 4 intervals over a period of 6 months. By tissue culture this product had a titer of $10^{7.0}$ to $10^{8.0}$ TCD_{50}/ml with an average of $10^{7.6}$ TCD_{50}/ml and 4.3 x 10^7 PFU/ml. However an average of only 5 pocks (several rabbits were negative) was elicited when 0.2 ml of a $10^{-4.5}$ (1:30,000) concentration was tested on the scarified rabbit skin. Still more striking are the results of assays on Vaccines 9 and 10 which had monkey kidney infectious titers of $10^{7.9}$ and $10^{8.3}$ TCD_{50}/ml respectively. In 2 experiments involving 8 rabbits, Lot 9 produced poor lesions at all dilutions tested including 1:1000. Lot 10 was quite regular in its rabbit pathogenicity with an endpoint greater than 1:30,000.

To obtain additional data on the difference of titers measured by these two methods a thermal inactivation experiment was performed on the premise that partially inactivated vaccine would be titratable in monkey kidney cells after it had lost its rabbit infectivity. Such proved to be the case (Fig. 1).

Aliquots of a lot of calf lymph vaccinia were submerged in sealed glass ampoules in a 60°C water bath for the incubation times shown. These were arbitrarily selected after preliminary experiments established a range for complete inactivation. The fractions were then simultaneously titrated in monkey kidney epithelium cultures and by rabbit scarification. When the infectivity of the lymph had decreased to approximately $10^{4.5}$ TCD_{50}/ml, the lowest concentration positive in rabbit skin was $10^{2.5}$. After 15 minutes at 60°C, there were approximately 4 logs of residual virus infectivity as measured in tissue culture but none detectable on rabbits at the highest concentration tested. Thus, although considerable loss of potency can be established by the rabbit scarification method it by no means can be used to determine residual infectivity of vaccinia virus in calf lymph.

Discussion. Highest yields of vaccinia virus were found in first or second bovine or simian tissue culture passages from calf lymph after which titers appeared to stabilize at a lower level. No evidence of species adaptation, as evidenced by enhanced infectivity for homologous or heterologous host cells, has been observed with serial passage of the virus. However, a significant qualitative change in its dermotropism for rabbit skin was noted even after 1 or 2 tissue culture passages. Whereas calf lymph vaccinia produced firm, well demarcated pocks on the scarified rabbit epithelium by the 5th or 6th day, the virus after passage in tissue culture caused a more accelerated but milder pock formation with less induration. Nevertheless, as demonstrated by Wesslen(11) vaccinia grown in bovine skin culture possessing titers of $10^{6.0}$ or greater is a satisfactory source of human smallpox vaccine even when pock formation in rabbits is of the less invasive type.

This observation is in accord with that of Buddingh and Randall(14) who noted a similar alteration of tropism without loss of infectivity in their studies of chick embryo smallpox vaccine. It is of interest that this modified dermotropism should occur even when calf lymph virus is serially propagated in cultures of calf embryonic tissue.

It should be stressed that although calf

TABLE VI. Relationship between Adsorption Time and Plaque Formation by Vaccinia Virus in Monkey Kidney Cells.

Adsorption time (hr)	Exp. No.	Avg plaques/bottle* 10^{-3}	10^{-4}	10^{-5}	10^{-6}
2	1	TNC†		6	
	2		1		0
4	1	"		1.5	
	2		16		0
6	1	"		10	
	2		24		1
8	1	"		3	
	2		27		0

* 0.5 ml virus dilution/bottle.
† Too numerous to count.

lymph vaccinia was most infectious for monkey kidney eipthelium, it did not multiply to any greater level in this cell. We have no evidence that the practice of "lapinizing" or "humanizing" the calf lymph seed used for smallpox vaccine alters the direct infectivity of calf lymph for monkey kidney cells in tissue culture.

These experiments suggest that replicate titration in tissue culture provides a sensitive and reproducible technic for the measurement of the viral content of smallpox vaccines. A sample of the regularity of the data obtainable by this method is shown in Table IV which illustrates a typical vaccine titration. In simplicity it is comparable to the use of the embryonated egg and with plaque procedures, still greater exactitude may be attainable. Although the use of monkey kidney permits the assay in a cell species closer to that of the human recipient, it is premature to state whether such a tissue culture assay of smallpox vaccine accurately measures the quantitative and qualitative factors involved in successful human vaccination. At present it can only be said to constitute a titration system providing a close approximation of infectious units and one which can very readily supplement the standard rabbit assay.

Summary. The infectivity of vaccinia virus in calf lymph for bovine, avian and simian cell cultures has been measured in tissue culture, embryonated eggs and rabbit epithelium. Monkey kidney roller tube or plaque titrations appear most sensitive to this virus and provide a simple and reproducible assay method which should have applications in the potency assay of smallpox vaccines.

We wish to thank Miss Elizabeth Jackson for performing the titrations on chorioallantoic membrane.

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