FURTHER OBSERVATIONS ON THE CULTIVATION OF VACCINE VIRUS FOR JENNERIAN PROPHYLAXIS IN MAN

By THOMAS M. RIVERS, M.D., and S. M. WARD

(From the Hospital of The Rockefeller Institute for Medical Research)

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In 1913, Steinhardt, Israeli, and Lambert (1) demonstrated that vaccine virus is capable of multiplication in the presence of bits of viable tissue embedded in plasma. Although this work has been confirmed by a number of investigators, cultures of vaccine virus made by means of the cover-slip technique have proved of no value in the preparation of an active agent for Jennerian prophylaxis. In 1927, Carrel and Rivers (2) devised a method for the cultivation of vaccine virus in which bits of viable chick embryo tissue embedded in plasma in flasks were used. In 1928, Maitland and Maitland (3) showed that they were able to grow vaccine virus in a medium consisting of minced hen kidney suspended in a mixture of hen serum and Tyrode's solution. In 1929, Rivers, Haagen, and Muckenfuss (4) demonstrated that cells remain viable for at least 5 days in a medium made according to Maitland's directions. However, if the cells are killed by repeated freezing and thawing, the medium no longer supports the multiplication of virus.

In a further search for a simple and safe method of cultivating vaccine virus for human use, we devised a highly satisfactory medium which consists of bits of minced chick embryo tissue (0.1 gm.) suspended in Tyrode's solution (4–5 cc.) in "collar flasks." In 1930, the results obtained with cultures of a neurovaccine virus were reported (5). In 1931, we described (6) our work with a dermal strain of vaccine virus and reported that the culture virus had been successfully used for the vaccination of 3 children. In 1932, Herzberg (7) reported that he had been able to vaccinate human beings with virus cultivated in the manner described by us. Since our last report in 1931 we have continued our observations, and it seems appropriate at this time to

record certain facts that have accumulated in regard to the culture virus and its use for Jennerian prophylaxis.

One of the objects of our work on the cultivation of vaccine virus has been to obtain an active agent, free from bacteria and contaminating viruses, that will protect human beings against smallpox with the least amount of inconvenience and discomfort to the individuals vaccinated. Consequently, all cultures of virus used in this type of work have been carefully tested for the presence of bacteria and other undesirable agents.

EXPERIMENTAL

As we have been able to show (5, 6) that vaccine virus will multiply in the presence of bits of minced chick embryo tissue (0.1 gm.) and Tyrode's solution (4–5 cc.), we decided to determine what would happen to the active agent when it was cultivated in such a medium over a long period of time. This was accomplished by making transfers in series from old cultures to flasks of fresh medium at intervals of 4 or 5 days and then testing the activity of the virus in the different culture passages by means of dermal and intradermal titrations in rabbits and vaccination of human beings.

Effect of in Vitro Cultivation on the Activity of Vaccine Virus in the Rabbit

Cultures of vaccine virus were initiated, March 9, 1931, and the titer in rabbits of the active agent in the 1st set of cultures was 10^{-6}. The titer of the virus in the 19th set of cultures was 10^{-6}, in the 30th 10^{-4}, in the 60th 10^{-2}, in the 80th 10^{-2}, in the 86th 10^{-1}. From the 88th set of cultures to the 99th only the undiluted virus produced a lesion when injected intradermally. From the record portrayed in Chart 1 it can be seen that the titer of the virus in the cultures maintained a high level, around 10^{-6}, for 19 generations and then gradually fell until only the undiluted cultures in the 88th generation produced a lesion in rabbits.

With the drop in the titer of the virus in the rabbit came a change in the character of the lesions induced by the active agent. The virus in the first 15 generations upon intradermal inoculation produced large edematous lesions with hemorrhagic and necrotic centers. With

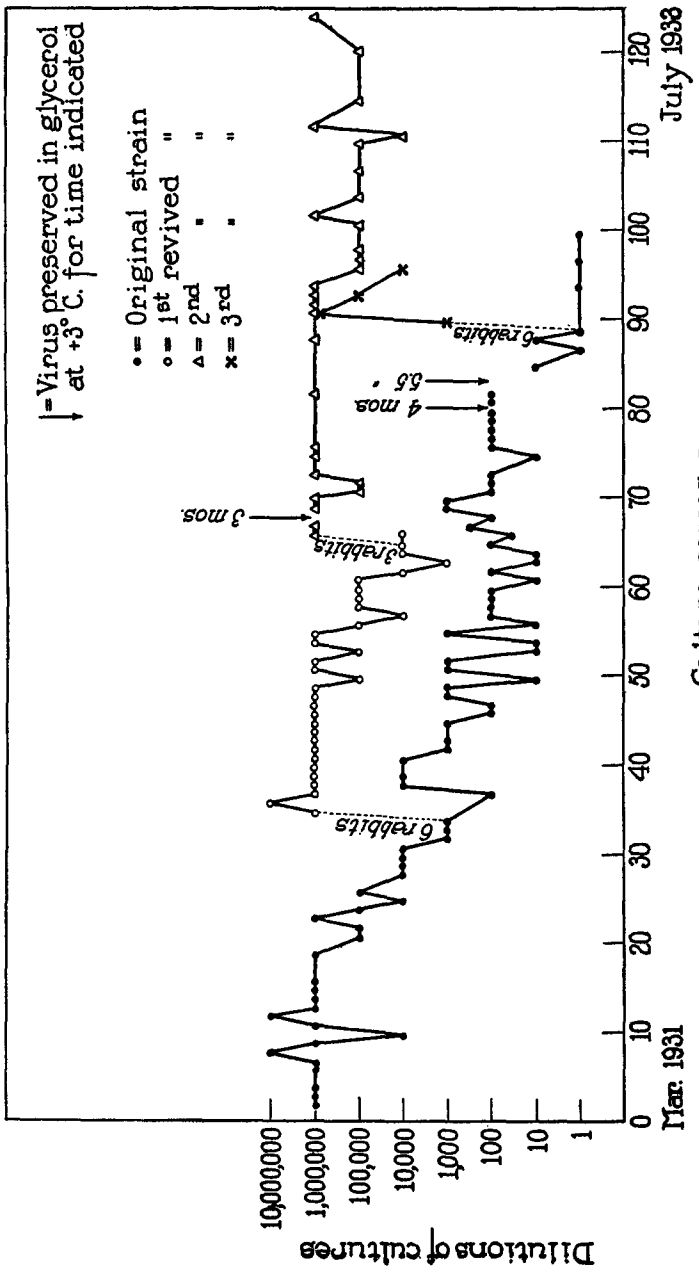

CHART 1. Graphic portrayal of the intradermal titer in rabbits of vaccine virus from different culture passages of the original, 1st revived, 2nd revived, and 3rd revived strains.

material from the 15th to the 70th generations nodular lesions without hemorrhage and necrosis were excited. When the virus was spread on the scarified skin, however, typical vaccinal vesicles appeared. After the 73rd generation, only small flat red areas 1 cm. in diameter were seen at the points of intradermal inoculation of the virus. The erythema faded rapidly and was followed by a superficial scaling of the skin. Upon dermal inoculation of the virus, only a slight amount of redness and scaling was produced which was almost indistinguishable from that caused by scarification alone. In spite of the fact that extremely mild and evanescent lesions were produced in rabbits with material from these cultures, vaccine virus was present in them as will be seen when the reaction induced by them in human beings is discussed.

Revival of the Activity of Culture Virus by Means of Testicular Passages in Rabbits

Inasmuch as vaccine virus for human use is usually tested in rabbits before distribution, we have attempted to obtain a culture strain that retains its pathogenicity both for man and rabbits. Consequently, when the titer of the culture virus for the rabbit began to fall, we passed the virus through several rabbits by means of testicular inoculations. With the passaged virus new cultures were initiated. This procedure has been resorted to three times and the results are detailed below and portrayed in Chart 1.

1 cc. of the pooled 34th generation cultures was injected into each testicle of a rabbit. After 4 days the testicles were removed aseptically and an emulsion was prepared. 1 cc. of this emulsion was then injected into each testicle of another rabbit. This procedure was repeated until the virus had been passed through 6 rabbits. All of the animals had fever. The titer of the virus gradually increased and the intradermal lesions again assumed a hemorrhagic and necrotic character. With testicular virus from the 6th rabbit new cultures were initiated and have been designated as the 1st revived strain. The titer of the first 14 generations of this strain was 10^{-6}, the intradermal lesions were edematous, hemorrhagic, and necrotic. Upon further passage of the virus in cultures the titer gradually fell (Chart 1) and the character of the lesions changed from hemorrhagic and necrotic to nodular.

1 cc. of the pooled cultures of the 31st generation of the 1st revived strain was injected into each testicle of a rabbit. After 4 days the testicles were removed and

an emulsion was made. 1 cc. of this emulsion was injected into each testicle of another rabbit. This procedure was repeated until the virus had been passed through 3 rabbits. All of the animals had fever and typical vaccinal reactions in the testicles. Testicular virus from the 3rd rabbit was used to start a new set of cultures that has been designated as the 2nd revived strain. The titer of this strain has remained around 10^{-6} for 60 culture generations. Although the titer has not fallen to any great extent (Chart 1), the intradermal lesions have become less hemorrhagic and more nodular.

Repeated passages of vaccine virus in the culture medium used in this work appear to decrease the titer of the active agents for rabbits and to mitigate the severity of the lesions produced. We have been able in the manner described above to revive the culture strains by testicular passages in rabbits, and the 2nd revived strain seems to be fairly stable. It may be necessary, however, to repeat the procedure of revival several times more before a completely stable culture virus is obtained.

The original culture strain (Chart 1) was carried for more than 2 years without being revived by passage through rabbits. The 88th culture passage produced little or no reaction in the skin of a rabbit yet produced typical vaccinal reactions in the skin of 2 children. It seemed of interest to find out if this culture could be revived by passages through rabbits.

1 cc. of the pooled cultures of the 88th passage (original strain) was injected into each testicle of a rabbit. The culture virus was also rubbed into the scarified skin and injected intradermally. Only a slight papule appeared at the point of intradermal inoculation and no definite vaccinal lesions were seen in the area of scarification during the 7 day period of observation. The animal had no fever. Nevertheless, the testicles were removed on the 4th day after inoculation and appeared practically normal. An emulsion of the testicles was made and 1 cc. of the emulsion was injected into each testicle of another rabbit. Dermal and intradermal inoculations were also made. The testicles were removed on the 4th day and seemed slightly injected. At the point of intradermal inoculation a lesion, 1 x 1 cm. in diameter, developed and 2 discrete pocks were seen in the area of dermal inoculation. The testicular virus was passed to a 3rd rabbit that developed no fever. The testicles in this animal had the appearance of being affected by a mild vaccinia. At the site of intradermal inoculation a lesion, 6 x 5 cm. in diameter, developed, and 2 discrete pocks were observed in the scarified skin. The 4th rabbit had fever, inflamed testicles, confluent vaccinal eruption at the site of scarification, and an intradermal titer of the virus of 10^{-3}. The 5th rabbit had fever, inflamed testicles, and an intradermal titer of the virus of 10^{-5}. The 6th

rabbit had fever and the testicles were inflamed. From an emulsion of the testicles of the 6th rabbit, cultures were initiated and have been designated as the 3rd revived strain. The titer of the 1st culture was 10^{-3}, of the 2nd 10^{-6}, of the 4th 10^{-5}, and of the 7th 10^{-4} (Chart 1).

From the facts presented above it appears that the 88th culture passage of the original strain of culture virus caused little or no reaction in rabbits. Upon repeated testicular passages in rabbits, however, the virus gradually regained its pathogenicity for that host.

TABLE I

Summary of Results of Vaccinations in Man with the Original Culture Strain of Virus

No. of patients	Culture passage	Preservation of culture		Results	Revaccination with New York City virus
		Time	Manner		
1	5	1 mo.	Glycerol at +3°C.	+	
	10	2 days	" " +3° "	+	
12	11	11 "	" " +3° "	12+	
1	11	2 wks.	" " +3° "	+	−
1	11	3 "	" " +3° "	+	
1	14	6 mos.	Glycerol at −10°C.	−	+
1	14	19 "	" " −10° "	+	−
1	42	1 hr.	Glycerol at +3°C.	+	−
1	42	1 wk.	" " +3° "	+	
1	42	2 wks.	" " +3° "	+	
1	82	1 mo.	" " +3° "	+	−
1	82	39 days	" " +3° "	+	
1	86	1 hr.	Without glycerol at +3°C.	+	−
1	88	1 "	" " " +3° "	+	
1	88	1 "	" " " +3° "	+	−

Jennerian Prophylaxis in Man by Means of Culture Virus

Before discussing the effect that *in vitro* cultivation has on the activity of vaccine virus in man it seems advisable to present in tabular form the results obtained in individuals vaccinated with culture virus. With the original strain, 25 people have been inoculated (Table I). Of these, only 1 failed to develop a typical vaccinal lesion. Six of the individuals who reacted to the culture virus were revaccinated with the New York City Board of Health virus and were found to be refractory. With the 2nd revived strain (Table II), 77 people have

TABLE II

Summary of Results of Vaccinations in Man with the 2nd Revived Culture Strain of Virus

No. of patients	Culture passage	Preservation of culture		Results	Revaccination with New York City virus
		Time	Manner		
1	27_2	1 mo.	Glycerol at +3°C.	+	
1	33_2	2 wks.	Dessiccated	+	
1	36_2	3 "	"	+	
1	42_2	4 days	Glycerol at +3°C.	+	
1	42_2	1 wk.	" " +3° "	+	
1	42_2	11 days	" " +3° "	+	−
1	42_2	1 mo.	" " +3° "	+	−
4	42_2	1 "	" " +3° "	2+	
				2−	
5	43_2	2 wks.	" " +3° "	5+	
1	44_2	6 days	Without glycerol at +3°C.	+	
8	44_2	3 wks.	Glycerol at +3°C.	6+	
				2−	
11	47_2	2 mos.	" " +3° "	8+	
				3−	
1	42_2	1 mo.	" " +3° "	−	
	49_2	4 days	" " +3° "	+	
1	49_2	4 "	" " +3° "	−	
	51_2	1 hr.	Without glycerol at +3°C.	+	
1	49_2	4 days	Glycerol at +3°C.	−	
	51_2	1 hr.	Without glycerol at +3°C.	−	+
2	50_2	1 day	Glycerol at +3°C.	+	
				1−	
9	51_2	4 days	" " +3° "	9+	
8	54_2	3 "	" " +3° "	7+	
				1−	
19	56_2	3 hrs.	" " +3° "	15+	
				4−	

TABLE III

Summary of Results of Vaccinations in Man with the 3rd Revived Culture Strain of Virus

No. of patients	Culture passage	Preservation of culture		Results
		Time	Manner	
16	5_3	4 hrs.	Glycerol at +3°C.	12+
				4−

been vaccinated, 64 of whom developed typical vaccinal lesions. Of those who had reactions, 2 were revaccinated with the New York City Board of Health virus and were found to be refractory. With the 3rd revived strain (Table III), 16 individuals have been inoculated and in 12 of them typical vaccinal reactions occurred. Three children who had been vaccinated with the New York City Board of Health virus were found to be refractory to the culture virus. In summary, 118 individuals have been inoculated with the culture virus and in 100 of them the inoculation was followed by a typical vaccinal pustule. Individuals vaccinated with the culture virus were refractory to a standard dermal strain of calf lymph and *vice versa.* All of the inoculations represent primary vaccinations in infants and children. Approximately one-third of the patients were in the Hospital of The Rockefeller Institute for complete observation during the course of the vaccination. The other two-thirds were vaccinated by us in Dr. Schloss' prophylactic clinic at the Cornell Medical Center and were seen only once after inoculation.

Effect of in Vitro Cultivation on the Activity of Vaccine Virus in Man

With virus from the 5th, 10th, and 11th culture passages of the original strain 15 children were vaccinated. A positive result was obtained in each individual. The reactions were similar to those caused by the New York City Board of Health virus. Consequently, no more children were vaccinated with the virus until it had been passed through 42 successive sets of cultures. Then virus from the 42nd, 82nd, 86th, and 88th sets of cultures were tested in man (Table I). In addition to this, virus from cultures of the 2nd and 3rd revived strains (Tables II and III) were employed for the vaccination of a large number of children. Virus from these cultures did not average as high a percentage of positive reactions as that usually obtained with the New York City Board of Health virus. The reactions, however, were milder than those caused by the Board of Health virus; the children had no fever and were in no way upset. In every respect the results obtained with the culture virus were highly satisfactory.

During the course of the observations it was found that with the later generations of culture virus an area of skin larger than that

usually advised had to be scarified in order to insure a positive reaction. Furthermore, it soon became evident that fresh culture virus (Tables I, II, and III) can be used with complete safety. Such is not the case with green calf lymph. Moreover, in view of our experience with the culture virus, we suggest that it be dispensed in cork-stoppered vials containing enough material for 10 vaccinations instead of in capillary tubes containing only sufficient virus for 1 inoculation. This suggestion is made because the culture virus contains a very small amount of particulate matter and it is believed that sooner or later the virus is adsorbed on these particles which in turn tend to adhere to the sides of the capillary tubes. Under these conditions difficulty is encountered in expressing the virus from the tubes and a low percentage of positive reactions is likely to be obtained.

Vaccination by Means of Intradermal Injection of Culture Virus

The results of intradermal vaccinations have been reported by a number of workers. The literature has been fully reviewed in a communication by Roberts (8). In spite of the favorable reports concerning the matter, most physicians have hesitated to use this method because of the fact that very few vaccine virus preparations are entirely free from living bacteria. Inasmuch as we had a bacteria-free virus that caused mild reactions upon dermal inoculation, we decided to see what it would do when injected intradermally into man.

C. B. received intradermally 0.1 cc. of a 1–10 dilution of culture 42_2 that had been preserved for 11 days in 50 per cent neutral glycerol at +3°C. The first signs of a reaction were observed 9 days later and consisted of erythema and a slight amount of induration. The erythema spread and the induration increased for a few days and then gradually disappeared. No pustule formed and no scar was left. The child was not sick or upset and had no fever. Upon revaccination with the New York City Board of Health virus the child was found to be refractory.

R. R. received intradermally 0.1 cc. of a 1–10 dilution of culture 42_2 that had been preserved for 1 month in 50 per cent neutral glycerol at +3°C. The first signs of a reaction were seen on the 7th day after inoculation. The course of events was practically the same as that described for C. B. with the exception that a very small vesicle formed where the needle was inserted in the skin. Upon revaccination the child was found to be refractory.

J. O'B. received intradermally 0.1 cc. of a 1–10 dilution of culture 44_2 that had been preserved without glycerol for 6 days at +3°C. The first signs of a reaction were observed on the 4th day following inoculation. The course of events was the

same as that described for R. R. The small vesicle resulted in a minute scar. Upon revaccination the child was found to be refractory.

H. J. received intradermally 0.1 cc. of undiluted fresh culture 88. On the 4th day after inoculation a red papule was seen. The course of events was similar to that of the other children. No pustule formed and no scar was left. Upon revaccination the child proved to be refractory.

From the facts presented above it appears that intradermal vaccination with virus that has been passed through a number of cultures is safe. The virus can be used, undiluted or diluted 1–10, in 0.1 cc. amounts either in the fresh or preserved state.

Effect of Storage on the Titer of Culture Virus

It was essential to determine how well the culture virus withstands storage. Consequently, lots of the same virus were preserved in different ways and later tested in rabbits for potency.

One lot of virus was mixed with an equal amount of 100 per cent neutral glycerol, another lot with an equal amount of 100 per cent neutral glycerol to which heated normal chick embryo tissue had been added, and still another lot with an equal amount of 100 per cent glycerol to which sufficient glucose had been added to make a 2.5 per cent solution. Then these lots of virus were placed in small cork-stoppered vials. Half of each lot of vials was stored at −10°C., the other half at +3°C. From time to time a vial of each lot was removed from storage and the virus was titered intradermally in rabbits.

The results of the work described above are shown in Table IV and indicate that the titer of the culture virus in storage (−10°C. and +3°C.) gradually fell from 10^{-6} but was still 10^{-4} at the end of a year. Furthermore, virus in a vial that had been stored at −10°C. for 19 months produced a typical vaccinal lesion in a child.

Desiccation of the Culture Virus

Vaccine virus does not maintain its activity well in the absence of refrigeration. It has been shown, however, that a number of viruses retain their activity better if they are frozen and then desiccated while in the frozen state. In view of this fact we performed an experiment in which culture virus (36th passage of the 2nd revived strain) was frozen, desiccated, and then stored in sealed tubes at 37°C. Each week for 5 weeks a tube was removed from the incubator, the original

volume in the tube was restored with sterile distilled water, and the resulting virus mixture was titered intradermally in a rabbit. The results are brought together in Table V and show that the dried virus

TABLE IV

Effect of Storage on Titer of Culture Virus (14th Generation of Original Culture Strain)

Time in storage	Stored at −10°C. in cork-stoppered vials			Stored at +3°C. in cork-stoppered vials		
	Equal volume of glycerol added to culture	Equal volume of glycerol added to culture plus extra heated embryo tissue	Equal volume of glycerol added to culture containing 2.5 per cent glucose	Equal volume of glycerol added to culture	Equal volume of glycerol added to culture plus extra heated embryo tissue	Equal volume of glycerol added to culture containing 2.5 per cent glucose
Titer by calculation before storage	500,000		500,000	500,000		500,000
1 day		1,000,000			1,000,000	
1 mo.	100,000	1,000,000	1,000,000	100,000	1,000,000	100,000
3 mos.	1,000,000	1,000,000	1,000,000	100,000	100,000	1,000,000
5 "	100,000	10,000	10,000	10,000	10,000	1,000
1 yr.	10,000	10,000	10,000	10,000	10,000	1,000
19 mos.	E. Ramon +					

TABLE V

Effect of Storage at 37°C. on Desiccated Culture Virus (36th Generation of 2nd Revived Strain)

Time of titration	Intradermal titer in rabbits
Before desiccation	100,000
After desiccation	100,000
After storage for 1 wk. at 37°C	100,000
" " " 2 wks. " 37° "	1,000
" " " 3 " " 37° "	1,000
" " " 4 " " 37° "	1,000
" " " 5 " " 37° "	10

maintained some of its activity for 5 weeks even at 37°C. In addition to this fact we have shown (Table II) that desiccated culture virus restored to its original volume with 25 per cent glycerol produces typical vaccinal lesions in human beings.

TABLE VI

Initiation of New Cultures from Preserved Cultures

Virus	Time and temperature of preservation	Titer when stored	Amount of dilution in starting cultures	Titer of new series of cultures				
				1st	2nd	3rd	4th	5th
5th generation original strain in glycerol	1 mo. at +3°C.	500,000	1:400	1,100,000	1,000,000	1,000,000		
5th generation original strain in glycerol	3 mos. at +3°C.	500,000	1:400	1,000,000	100,000	100,000		
5th generation original strain in glycerol	10 mos. at +3°C.	500,000	1:400	1,000,000	1,000,000			
8th generation original strain in glycerol	22 mos. at +3°C.	5,000,000	1:400	10,000	10,000		100,000	100,000
43rd generation original strain in glycerol	3 mos. at +3°C.	500	1:400	1	10	1,000	10,000	1,000
6th generation original strain without glycerol	22 mos. at −10°C.	500,000	1:20	100,000	1,000,000		100,000	
33rd generation 2nd revived strain desiccated	5 days at +3°C.	10,000	1:100	100,000	100,000			

Initiation of New Cultures from Preserved Cultures

If the cultivation of vaccine virus is to become a practical procedure, it is essential to know whether new cultures can be initiated with virus from preserved cultures. Therefore, numerous attempts have been made to ascertain the facility with which new cultures can be successfully seeded with virus from cultures that have been frozen and desiccated or from cultures preserved with or without glycerol. We have experienced no difficulty in obtaining fresh cultures in this manner. For convenience a few of the results are shown in Table VI.

DISCUSSION

From the results of the work presented in this paper it is obvious that we have had no difficulty in cultivating a dermal strain of vaccine virus for a period of over 2 years in a medium consisting of bits of viable chick embryo tissue (0.1 gm.) suspended in Tyrode's solution (4–5 cc.) in flasks. This medium was chosen because it is the least likely of all media containing living cells to be contaminated with an unknown or an undesirable virus. It is also evident that culture virus has been successfully employed by us for Jennerian prophylaxis in man. It is hoped that our observations will tempt workers in vaccine virus laboratories to try to adapt this or a similar method of preparation of the active agent for general use. In view of the purity of the virus prepared in this manner and since it causes such mild, yet effective reactions in man, it seems possible that much of the objection to vaccination might be overcome and that the rare but occasional postvaccinal encephalitis might be rendered even more rare, or avoided wholly, by its use instead of that of calf lymph.

Attention should be focussed on the fact that repeated cultivation of vaccine virus in the medium used by us gradually reduced the titer of the active agent for the rabbit and also led to an alteration in the type of lesions produced by the virus in that host. Indeed, the virus that had been cultivated for 2 years in the manner described induced little or no reaction in rabbits. Material from these cultures, however, gave rise to typical vaccinal pustules in man. This observation appears to us to be of importance and among other things seems to indicate that a virus of a desired character for human use can be produced by culture methods. In view of the findings presented at

this time, it is believed that the change in the activity of the virus for the rabbit was not due entirely—and perhaps not at all—to a gradual diminution in the amount of virus in successive sets of cultures, but to some alteration in the character of the virus itself.

SUMMARY

A dermal strain of vaccine virus has been passed through 99 successive culture passages. This procedure led to a diminution in the pathogenicity of the active agent for the rabbit. By repeated testicular passages in rabbits, however, the virus regained its pathogenicity for that host. New cultures were initiated with the revived virus. A culture strain of virus that has been twice revived in this manner has remained fairly stable for the rabbit through 60 culture passages and it produces mild, yet effective vaccinal reactions in man.

Virus in early cultures was not attenuated for man, but later cultures of the original strain and cultures of the 2nd and 3rd revived strains produced mild reactions without fever and discomfort to the patients. Intradermal vaccinations with the culture virus are safe and satisfactory.

With the culture virus 118 infants and children have been inoculated and in 100 of them a positive reaction occurred. The culture virus produced a refractory state to a standard dermal strain of calf lymph and *vice versa*.

Culture virus stored in 50 per cent neutral glycerol at −10°C. or at +3°C. maintained a considerable amount of its activity for at least 1 year. Desiccated culture virus sealed in tubes maintained some of its activity when stored at 37°C. for 5 weeks.

Fresh cultures can be initiated without difficulty from desiccated virus or from virus that has been stored with or without glycerol.

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