STUDIES WITH HUMAN INFLUENZA VIRUS CULTIVATED IN ARTIFICIAL MEDIUM

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A previous report (1) has recorded the successful cultivation of a strain of human influenza virus (P.R.8) (2) in an artificial medium composed of Tyrode's solution and minced chick embryo. The virus cultivated under these conditions was found to retain its capacity to infect mice and ferrets, and it was maintained at about the same concentraton as in the lungs of infected mice from which it had been derived.

Since the preliminary report the P.R.8 strain of human influenza virus has been continuously transferred at 2 day intervals until it is now in the 70th generation. In addition, under similar conditions, a 2nd strain of human influenza virus (Philadelphia) (3) has been cultivated through 45 successive transfers in the artificial medium. Still more recently the virus of swine influenza (4), obtained from the lungs of infected mice,[1] has also been transferred to tissue culture. Burnet (5) and Smith (6) have reported the successful cultivation of strains of human influenza virus upon the chorioallantoic membranes of developing eggs. The latter has also confirmed our results in cultivating the virus in a fluid tissue medium.

Certain observations have been made regarding the conditions affecting multiplication and the time of survival of the virus. Furthermore, studies have been made of the immunological properties of culture virus as compared with the same strain of virus maintained solely by passage through susceptible animals. The present paper comprises primarily the results of these latter investigations.

Methods and Materials

Culture Medium.—The medium employed is that devised by Li and Rivers (7) based upon the earlier procedure of Maitland and Maitland (8). Chick em-

[1] Through the courtesy of Dr. Shope.

bryos after 10 to 14 days' incubation are removed aseptically from the egg, the eyes are taken out, and the remainder of the embryo is finely minced in 2 to 4 cc. of Tyrode's solution, depending upon the size of the embryo. After the preliminary period, embryos of 12 to 13 days were uniformly used. To 4.5 cc. of Tyrode's solution in a Rivers flask or in a 50 cc. Erlenmeyer flask, are added 4 drops (approximately 0.25 cc.) of the suspension of embryonic tissue. To the medium is then added 0.5 cc. of the virus-containing material. The flasks are stoppered with firm plugs of cotton bound in gauze. After 2 days' incubation at 37°C., transfers of 0.5 cc. of the culture are made to flasks containing 4.5 cc. of freshly prepared medium. For routine purposes the transfers have subsequently been made at 2 day intervals. In this manner, the P.R.8 strain has been actively maintained in culture for over 4 months and the Philadelphia strain for a somewhat shorter period. A control culture not containing virus has also been transferred as routine.

Method of Titration.—The presence of active virus in the culture fluid has been demonstrated by the instillation of the material into the nostrils of white mice lightly anesthetized with ether. The active agent induces in these animals gross pulmonary consolidation which varies in extent with the concentration of the virus. Mice receiving 0.05 cc. of culture of the usual titer die or are moribund in 4 to 6 days with complete involvement of the lungs. All mice surviving on the 6th day are sacrificed and their lungs examined for gross lesions. With cultures of lower titer only slight lesions may be produced in this time. For purposes of titration the limit of infectiousness has been taken to be the highest dilution of culture which produces visible areas of involvement in the lungs of inoculated mice. It is realized, however, that this arbitrary limit may not be entirely accurate, for it has been possible to demonstrate, by passage to normal mice, the presence of virus in the lungs of mice in which at the end of 6 days visible lesions were not observed. Nevertheless, for practical purposes, the end-point as measured by the above method has been generally employed.

Multiplication and Survival of Virus under Different Cultural Conditions

Under the standard conditions adopted, the greatest concentration of virus in the artificial medium is usually attained in 36 to 48 hours. In 72 hours a decrease in the amount of virus has begun, and after 5 to 6 days it is difficult to demonstrate active virus in the culture fluid. Nevertheless, in cultures removed from the incubator after 48 hours' incubation and placed in the refrigerator at +4°C., the virus has been found to retain practically full infectiousness for as long as 23 days. Furthermore, cultures made with the same medium in ordinary test tubes plugged with a tight rubber stopper and incubated at 37°C. have been found to contain active virus as long as 18 days. At this

time lesions were not observed in mice inoculated with the material, but when these cultures were transplanted to fresh medium in tightly stoppered test tubes and transferred at 4 day intervals, the virus regained its full potency and was satisfactorily maintained by this procedure. However, if vaseline seals are placed over the culture medium so as to approach anaerobic conditions, the virus does not multiply, and the culture fluid after 48 hours is not infectious for mice.

The effect of variations in the amount of tissue used in the culture medium has not been fully investigated. As previously recognized, this factor plays a definite rôle in the problem of virus cultivation. A very small number of living cells apparently does not support multiplication of the virus, while too great a quantity of tissue is also detrimental. In the present study embryos of 12 to 13 days have appeared to be the most satisfactory, possibly because the embryo contains at this time a greater amount of serum which may serve to protect the virus.

Maintenance of Virulence of Human Influenza Virus in Tissue Culture

The virulence of the culture virus, or the retention of its capacity to produce pulmonary lesions in susceptible mice, has been measured at frequent intervals. In most instances, mice receiving the undiluted culture fluid succumb in 6 days or less and exhibit extensive involvement of the lungs. Titrations of the virus concentration of the standard P.R.8 cultures made at different times are presented in Table I.

It can be seen that a comparatively constant titer of between 1:1000 and 1:10,000 has been maintained.

Ferrets, as well as mice, have been successfully inoculated with virus of the 6th, 37th, and 54th transfers of the P.R.8 strain. In each case the ferret responded with fever, and in those instances in which autopsies were done involvement of the lungs of the ferret was observed. Furthermore, the serum of ferrets recovering from infection with the culture virus was found to contain a high concentration of antibodies effective against the regular mouse passage virus and the animals were found to be actively immune to reinfection when tested with ferret passage virus.

Immunization Experiments with Culture Virus

It has been reported previously that mice inoculated subcutaneously or intraperitoneally with human influenza virus (9, 10), although showing no evidence of experimental disease, develop an immunity

TABLE I

Titrations of P.R. 8 Culture Virus at Intervals during the Course of Cultivation

Transfer	Mouse No.	Dilution of virus					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
6th	1	*++++	±	+	0	0	
	2	*++++	++	+	±	0	
	3	++++	+++	+	±	0	
10th	1	++++	++	0	0	0	
	2	+++	++++	±	0	0	
	3	+++	++	+	0	0	
19th	1	++	+++	0			
	2	+++	±	±			
	3	+++	+	+			
31st	1	*++++	+++	+±	±		
	2	++++	+++	+	±		
39th	1	*++++	+++	++±	0		
	2	+++±	+++	++	++		
42nd	1	*++++	++	++	+		
	2	+++	++	+	±		
55th	1	*++++	*++++	+++	+	0	0
	2	*++++	++++	+++	++	0	0

0 = no gross pulmonary involvement.
± to ++++ = progressive degrees of pulmonary involvement.
* = mouse died.

which is effective against the virus inoculated intranasally. It was of interest, therefore, to determine whether the virus propagating in tissue culture outside the animal body was still capable of exerting this effect.

Experiment 1.—To each of 15 mice was administered subcutaneously 0.3 cc. of P.R.8 virus of the 3rd culture transfer. 9 days and 21 days later, 0.3 cc. portions of the fluid of the 7th and 13th generations respectively were given intraperitoneally. 14 mice of the same stock were kept as controls, receiving no inoculations. 8 days after the last injection both vaccinated and control mice were given 0.03 cc. of a 10 per cent suspension of P.R.8 mouse passage virus intranasally. By the 10th day 11 of the 14 control mice had died with extensive pulmonary involvement; the 3 surviving control mice were killed and marked pulmonary lesions were exhibited. The vaccinated mice had appeared perfectly well, and in the 5 killed on the 10th day no pulmonary lesions were observed.

Experiment 2.—Three groups of 10 mice each were used. One group received, at 10 day intervals, 0.2 cc., 0.3 cc., 0.3 cc., of the 40th, 45th and 49th generations, respectively, of the P.R.8 culture virus subcutaneously; those of the second group received equal amounts of the same active virus cultures intraperitoneally at the same intervals. Animals of the third, or control, group, were given subcutaneously 0.2 cc. of culture medium which contained no virus, and at 10 day intervals thereafter two doses of 0.3 cc. each of similar material were given intraperitoneally. 10 days after the last vaccination all mice were given 0.03 cc. of 10 per cent suspension of P.R.8 mouse passage virus intranasally. By the 10th day after infection, 9 of the 10 control animals had died with typical pulmonary involvement; 2 of the mice which were vaccinated subcutaneously had died, although the others of this group appeared perfectly well; all of the mice which had been vaccinated by the intraperitoneal route remained well throughout the period of observation.

The results cited, and other experiments of a similar nature, have shown conclusively that mice vaccinated subcutaneously or intraperitoneally with human influenza virus transferred through many generations in tissue culture develop a staunch immunity against intranasal infection with large doses of the same strain of virus maintained entirely by serial passages in mice.

Experiments relating to the efficacy of the culture virus in the immunization of ferrets have shown that, following subcutaneous inoculation of the artificially cultivated agent, the animals develop an active resistance which distinctly modifies the disease produced by intranasal instillation of regular ferret passage virus.

Studies in progress regarding the vaccination of human individuals (11) have revealed that, following the subcutaneous inoculation of virus culture fluid, the human subject responds with a development of antibodies capable of neutralizing the virus as demonstrated by mouse protection tests.

Studies of the Immunological Characteristics of Human Influenza Virus Grown in Artificial Medium

The evidence heretofore obtained, through titration in white mice, of the concentration of active agent in culture fluid has indicated that the virus multiplying in tissue culture reaches a concentration and maintains a virulence closely resembling that of the virus in the lungs of infected mice. However, when the capacity of certain sera to neutralize the culture virus was compared with the capacity of these sera to neutralize the same strain of mouse passage virus, distinct

TABLE II

Comparison of Neutralizing Capacity of the Same Sera Tested against Culture Virus of 4th and 15th Transfers

Serum	Culture virus (P.R. 8)					
	15th transfer (Original culture)			4th transfer (New culture)		
	Mouse No.			Mouse No.		
	1	2	3	1	2	3
Normal ferret 1-05	+	++	++	++++	+++	+++
Immune " 1-14	0	0	0	0	0	0
Normal rabbit 1-33	++++	+	+	++++	+++	++++
Immune " 1-29	0	0	0	0	0	0
" swine 14-44	0	0	0	++	++	+
Human—H.F. (acute influenza)	0	0	0	+	0	0
" H.F. (convalescent influenza)	0	0	0	0	0	0
Human—T.F. (normal)	0	0	0	—	—	—

differences were noted. In general, the neutralizing effect of the serum was enhanced when tested against culture virus, so that sera which exhibited little or no protective capacity against mouse passage strain might protect mice completely against the cultivated virus.

In the earlier phases of this study it appeared that the ease with which the virus grown in artificial medium could be neutralized by a given serum was somewhat related to the length of time it had been removed from animal passages. Tests were made simultaneously with the same sera against one culture in its 15th transfer and a culture of the same strain which had been transferred through mice

after culture and was then returned to artificial cultivation for 4 generations. The results are presented in Table II.

It is readily observed that normal ferret and normal rabbit serum exerted a stronger neutralizing effect against the older culture virus than against the more recent culture. Furthermore, the serum of swine convalescent from swine influenza virus infection, and of two human beings, H.F. (acute) and T.F. completely protected against the older culture virus.

When the virus from the 15th generation in artificial medium was passed through mice for 3 transfers and comparative tests were again

TABLE III

Capacity of Various Sera to Neutralize Culture Virus (P.R. 8) before and after Passage through Mice

Serum	Culture virus (21st transfer)			Culture virus (15th transfer) after 3 serial mouse passages		
	Mouse No.			Mouse No.		
	1	2	3	1	2	3
H. F. (acute)	0	0	0	+++	++	++
H. F. (convalescent)	0	0	0	0	0	0
S. S. "	0	0	0	±	0	0
T. F. (normal)	0	+	±	+++	++	++
Normal rabbit 1-29	++	++	++	+++	++++	++++
Immune (P.R.8) rabbit 1-29	0	0	0	0	0	0
Normal swine	++++	++	++	*++++	+++	*++++
Swine, immune to swine influenza	±	0	+	++	++	+++

done, it was found that after animal passages the virus had regained its original characteristics and that neutralization of the virus by these sera was no longer effected (Table III).

Recently, the conditions of cultivation have been somewhat more constant than earlier in the study, and a comparative test was made between the same strain of cultivated virus in its 54th generation and a new culture which was only 5 transfers (10 days) removed from animal passage. In this instance, the older culture virus which had exhibited little fluctuation in its potency for a considerable time, was less easily neutralized than previously and was no more susceptible to the action of serum than the younger culture (Table IV).

These results suggest that the variations observed have been due entirely to alterations of a quantitative, not of a qualitative nature in the virus. Certainly no change in the immunological characteristics has been observed which could be interpreted as due to an alteration in the antigenic constitution of the virus. On the other hand, since the titer of the culture virus, as measured by the customary method in susceptible mice, has remained at a level closely parallel to that of mouse passage virus, the differences observed might be attributed to a qualitative change in the virulence of individual virus particles, so that more would be required to produce a lesion equal in severity to that

TABLE IV

Comparison of Neutralizing Capacity of Same Sera Tested against Culture Virus of 54th Transfer and a New Culture in the 5th Transfer

Serum	Culture virus (P.R. 8)					
	54th transfer (Original culture)			5th transfer (New culture)		
	Mouse No.			Mouse No.		
	1	2	3	1	2	3
Normal rabbit 1-29....	*++++	+++	+++	*++++	*++++	+
" swine 16-52....	*++++	*++++	++++	*++++	*++++	+++
Swine 16-52, immune to swine influenza......	*++++	+++	+++	++++	++	+++
Human R. D..........	++±	++	+	+	++	++
" D. T..........	+++	+++	+++	+±	++	++
" T. F..........	++	++	++	++	++	++
" W. M.........	0	0	+	0	0	0

produced by a smaller number of particles of virus maintained by animal passage. This possibility is heightened by the impression gained that the lesions in the mice infected with culture virus, although of equal extent, are less intense than those obtained with passage of the virus from animal to animal.

SUMMARY

The *in vitro* cultivation of strains of human influenza virus has been successfully conducted through a prolonged series of successive transfers. The cultivated virus has retained the antigenic and immunological properties which characterized the animal passage virus from

which it was derived. The culture virus is still virulent for mice and ferrets; it is capable of inducing an active state of immunity in animals vaccinated subcutaneously or intraperitoneally; it elicits specific neutralizing antibodies in the serum of infected or vaccinated animals.

The virus has been successfully cultivated to date only in the presence of oxygen; when conditions of reduced oxygenation are imposed by the use of vaseline seal, with or without the addition of cystein, multiplication of the virus is not supported. On the other hand, it has been possible to cultivate the virus in the medium of Li and Rivers in ordinary test tubes. This affords a greatly simplified procedure, since the interval between transfers may be prolonged.

The results of neutralization tests with various sera and the culture virus are presented and discussed.

BIBLIOGRAPHY

1. Francis, T., Jr., and Magill, T. P., *Science,* 1935, **82,** 353.
2. Francis, T., Jr., *Science,* 1934, **80,** 457.
3. Francis, T., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1935, **32,** 1172.
4. Shope, R. E., *J. Exp. Med.*, 1931, **54,** 573.
5. Burnet, F. M., *Med. J. Australia,* 1935, **2,** 687.
6. Smith, W., *Brit. J. Exp. Path.*, 1935, **16,** 508.
7. Li, C. P., and Rivers, T. M., *J. Exp. Med.*, 1930, **52,** 465.
8. Maitland, H. B., and Maitland, M. C., *Lancet,* 1928, **2,** 596.
9. Francis, T., Jr., and Magill, T. P., *J. Exp. Med.*, 1935, **62,** 505.
10. Smith, W., Andrewes, C. H., and Laidlaw, P. P., *Brit. J. Exp. Path.*, 1935, **16,** 291.
11. Francis, T., Jr., and Magill, T. P., *Proc. Soc. Exp. Biol. and Med.*, 1936, **33,** 604.