A Genetic Approach to Variation in Influenza Viruses

2. Variation in the Strain NWS on Allantoic Passage

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SUMMARY: When the strain NWS of influenza virus, which is adapted to multiplication in mouse brain, is submitted to allantoic passage in the chick embryo, it maintains its original character if passed at limiting infective dilutions. When passage is made with a low dilution of the same virus preparation the strain changes toward a more 'normal' character. These phenomena are regarded as providing formal proof that the changes are mutational in origin.

In the course of work on the O-D transformation of influenza virus A (Burnet & Bull, 1943; Burnet & Stone, 1945; Burnet, Stone, Isaacs & Edney, 1949) we have repeatedly found that the O form can be maintained on serial passage only if limit or near-limit infective dilutions are used. Unfortunately no record exists of confirmation of this finding from any other laboratory. We have consistently held that the phenomenon provides a formal proof that variation from O to D phase represents a mutation equivalent to a gene-mutation in higher forms, with selective overgrowth of the mutant, and is not a direct effect of the new environment on the virus population as a whole. The O-D change in influenza virus is admittedly difficult to study, entailing the inoculation by the amniotic route of large numbers of eggs and the detailed analysis of the haemagglutinating activities of each fluid and lung emulsion. It seems therefore desirable to present essentially similar evidence that a well-defined variant type of virus established in another host behaves similarly on passage in the allantoic cavity.

The argument is simply that if a strain in phase A on repeated passage by ordinary methods eventually gives rise to a strain showing different behaviour, phase B, one of two mechanisms may have been concerned in the change. The first alternative is that by repeated reproduction in a new intracellular environment the quality of the virus particle is progressively modified till it takes on the character of phase B. By modification we mean a process involving all or the great majority of the individuals in the continuously changing populations concerned, the change being a direct result of the new environment. A process of this type is postulated by Hinshelwood (1946) for the rapid development by certain bacterial strains of the capacity to ferment a sugar which they cannot initially attack. The second possibility is that amongst the mutants constantly being produced by the original strain there are some better fitted than the original to multiply in the new environment. It is these mutants (or further mutants derived from the primary ones) which eventually give to allantoic fluid virus the new B phase characteristics. A distinction between the two alternatives is possible when, as is usually the case, mutation is a relatively infrequent occurrence. In a number of instances the frequency with which a recognizable mutation occurs is of the order of once in every 10^5 or 10^6 life

cycles of the organism concerned. If the conditions for viruses are broadly similar it follows that if one infective virus particle is introduced into the allantoic cavity a mutant is highly unlikely to arise until the original form has multiplied so extensively that there is no possibility of the mutant overgrowing it within the usual 44 hr. before the fluid is harvested. The great majority of virus particles in this fluid will therefore be of the original type. When dilution of 10^{-8} is used for the next passage no more than one to five infective particles will be transferred, all being of the original type; and if this process is continued, the same situation holds at every passage. On the other hand, when passage is made at low dilution so that the inoculum contains in addition to the original form a proportion of mutants capable of multiplying more rapidly, the fluids obtained will eventually be dominated by those mutants which grow most effectively under the experimental conditions.

If it is found that with repeated passage at limiting dilution (10^{-7} or 10^{-8}) a virus retains its original character, but with the same number of passages at a dilution of 10^{-2} gives rise to a recognizably different type, the change must be ascribed to mutation. The virus population obtained after the completion of the limiting dilution series is composed of virus particles which on the average are separated from the original virus particles used to initiate both series by a greater number of generations than those of the low dilution series. The limiting dilution virus has thus had *more* opportunity to be influenced by the intracellular environment than has the other series. A result of this type therefore rules out the possibility of the change being due to direct environmental modification.

The object of this paper is to show that such a situation can be demonstrated by passage of the neuro-WS strain of influenza A (Stuart-Harris, 1939) in the allantoic cavity of the chick embryo.

MATERIALS AND METHODS

The strain NWS (Stuart-Harris, 1939) has been described in the previous paper (Burnet, 1951) as well as the methods by which it can be characterized *in vitro* and *in vivo*. The passage series were commenced with virus two egg-passages removed from the dried mouse-brain material received from Prof. Stuart-Harris through Dr Alick Isaacs. Eggs at the 11th day of incubation were inoculated with 0.05 ml. of virus serially diluted in cold nutrient broth and incubated at 35° . As a matter of convenience two passages each week were made with 44 hr. incubation and one with 68 hr. The proportion of eggs showing haemagglutination by allantoic fluid was determined and selected positive fluids were used for detailed tests and for sub-inoculation. The high dilution passages were made at 10^{-7} and 10^{-8} dilutions, and a fluid from the highest positive dilution used for each passage. In the later passages only the 10^{-8} dilution was used. The 13th failed to cause infection in any of the eight eggs inoculated at 10^{-8} and this had to be repeated at 10^{-7} . This is the reason that there is one less passage in the high dilution series than in the low dilution series. In the latter, four eggs were routinely inoculated with a 10^{-2} dilution of

the previous passage fluid. At various stages tests were made for heat stability of haemagglutinin, capacity to develop indicator state and to produce encephalitis in mice according to the techniques described in the previous paper.

RESULTS

Representative fluids from the 3rd, 6th, 10th and final (13th or 14th) passages were tested with the results shown in Tables 1 and 2. After fourteen passages at 10^{-2} dilution the last fluid of this series was tested at high dilution and the

Table 1. *Characters of high and low dilution passage series of NWS virus*

		Allantoic fluid							
		Haemagglutinin titres							
Passage			Inhibitor titre* of						Intracerebral pathogenicity for mice†
	At	Indicator	heated		Original		Heated at		
No.	dilution	titre	fluid				54° for		
							30 min.		
3rd	10 ⁻⁸	60	48	80	600	700	< 2	< 2	.
	10 ⁻²	60	48	48	600	600	< 2	320	.
6th	10 ⁻⁸	100	80	80	600	280	10	8	4, 5, 6, 8
	10 ⁻²	1,500	28	24	350	500	50	12	5, 5, 6, 6
10th	10 ⁻⁸	100	80	40	300	220	< 2	< 2	.
	10 ⁻²	10,000	12	8	250	400	< 2	< 2	.

* Inhibitor titre against H-LEE of fluid heated at 62° for 30 min.

† Pathogenicity indicated by days to death of four mice inoculated intracerebrally with fluid diluted 10^{-2} .

two positive fluids 53,594 and 53,596 obtained with a dilution of 10^{-8} from eight inoculated embryos were tested in detail against four fluids of the 14th passage of the high-dilution series. The fluids from the low-dilution series thus represent clones derived from two particles of types present in high concentration in the final fluid.

The points to be noted are that the high-dilution series have retained all the characteristics of NWS. In the low-dilution series there are two types of fluid.

Table 2. *Characters of re-isolated high and low dilution passage strains of NWS virus*

(The original haemagglutinin titres were in the range 200-800.)						
Fluid	Haemagglutinin heated at 54° for 30 min.	Indicator titre	Inhibitor titre of heated fluid*	Intracerebral pathogenicity for mice†		
				10^{-4}	10^{-5}	10^{-6}
53,579 H	<2	80	24	5, 6, 6, 9	5, 6, 6, 6	5, 6, 7, 9
53,580 H	<2	40	28			
53,581 H	<2	80	64			
53,583 H	<2	40	32			
53,594 L	<2	20,000	2	6, 6, 6, 7	6, 6, 7, 7	6, 6, 7, 7
53,596 L	120	7,000	<2			

H = high-dilution passage, L = low-dilution passage.

* Inhibitor titre against H-LEE of fluid heated at 62° for 30 min.

† Intracerebral pathogenicity indicated by days to death of four mice inoculated intracerebrally with fluid diluted 10^{-2} .

Both differ from the original in being convertible into the indicator state and in having demonstrable enzymic activity. One fluid is resistant to heating at 54°, the other not. Both high- and low-dilution passage series have retained full virulence for mice by the intracerebral route. Evidence for the existence of other variants was sought by heating fluids to the temperature just capable of completely inactivating the normal form (54° for 30 min.). After three passages two fluids of each series were heated and then tested for infectivity undiluted and at a dilution of 10⁻². Positive fluids were studied in the usual manner.

Table 3. *Characters of fluids obtained from embryos inoculated with heated virus*
(Virus heated at 54° for 30 min.)

Series	Egg no.	Allantoic fluid				
		Infectivity of heated virus		Haemagglutinin heated at 54° for 30 min.	Indicator titre	Inhibitor titre of heated fluid*
		Undiluted	10 ⁻²			
H	52,031	+++	+--	<2 <2	100, 80	64, 40
	52,032	---	---	.	.	.
L	52,039	---	---	.	.	.
	52,042	+++	+++	100	200	20

H = high-dilution passage; L = low-dilution passage.

* Inhibitor titre against H-LEE of fluid heated at 62° for 30 min.

It will be seen that fluid 52,042 contained much virus resistant to heating at 54°. The fluids tested were not from a limiting dilution so a further passage at high dilution was made. Positive fluids gave essentially the same characters as the first passage fluid shown in the table, but there was less residual inhibitor in the heated allantoic fluid. Both first- and second-passage fluids were inoculated intracerebrally in mice and produced no symptoms. It appears therefore that in embryo 52,042 a relatively heat-resistant and non-encephalitogenic variant had been able to multiply to a relatively high titre. On the other hand, the small proportion of virus that survived heating in fluid 52,031 of the high-dilution series gave fluids with the standard character of the original type.

DISCUSSION

These results show just as definitely as the experiments on the O-D phase variation, that influenza viruses are highly labile, changing in character with passage in a new environment, but equally that such change does not occur if transfers are consistently made at the highest infective dilution. As in the earlier case this provides a formal proof that the change in character on passage at low dilutions results not from 'adaptation' but as a result of the selective survival of mutant forms which eventually outgrow the original type.

We would suggest that just as any critical work on bacterial metabolism is always preceded by a replating of the strain concerned and choice of a typical colony, any study of virus properties should be carried out by using only clones of virus derived from an immediately preceding passage at the limiting infective dilution. The rationale is simply that mutation is a relatively rare

occurrence and the product of multiplication of a single infecting particle in one host is, except under most unusual circumstances, bound to contain only a small minority of mutants. These are eliminated when passage is made at high dilution. When passage is carried on at low dilution there is a gradual accumulation of mutants in the stock the proportion of which will be determined essentially by their relative speed of multiplication in the environment. It seems obvious that an inoculum containing only one virus genotype will produce more uniform populations in the infective fluids harvested than an inoculum containing a variety of mutants. Chick embryos are by no means identical, one may well favour mutant *x* while another favours mutant *y*. A mixed inoculum may therefore give very different results in two different embryos (cf. eggs 52039 and 52042 above), but if a high dilution of a pure clone is used the only likely difference will be in regard to the actual concentration of virus attained by the time of harvesting.

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