A Genetic Approach to Variation in Influenza Viruses

3. Recombination of Characters in Influenza Virus Strains Used in Mixed Infections

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SUMMARY: When the non-encephalitogenic strain of influenza virus A, WSM, was mixed with the influenza strain NWS in suitable proportion no encephalitis followed intracerebral injection of the mixture in mice. When the amount of WSM relative to NWS was decreased partial interference resulted and from four mice so inoculated, virus strains referred to as WS-NM were isolated, which appear to be recombinants. The NM strains were encephalitogenic for mice, though in varying degree, their haemagglutinin was almost as resistant to heat inactivation as that of WSM and they had at least one character (position in the receptor gradient) in which they differed from both the original strains.

In the first two papers of this series (Burnet, 1951 *a, b*) the characteristics of the strain NWS were described and the suitability of the WS variants for genetic studies was indicated. At various times we have made unsuccessful attempts to induce recombination of characters between influenza virus strains. Even on the assumption that recombination of characters of influenza viruses occurs freely whenever double infection of a single cell occurs, there are obvious technical difficulties in demonstrating its occurrence. In studies of this sort on bacterial viruses it is relatively simple to arrange that the relevant characters of *all* the progeny resulting from double infection of a single bacterium can be examined. With animal viruses one cannot work with single host cells, and the most that can be hoped is to produce a double infection and to obtain in the population of virus units resulting a proportion of recombinants amongst a majority of one or both the original forms. The existence of recombinants can then only be demonstrated if conditions can be so arranged as to favour their selective proliferation. Our object has been to concentrate on recombinants possessing the capacity to multiply in the mouse brain, so making possible the use of a highly selective environment. The difficulty has been of course to prevent the overgrowth of the original NWS necessarily present as one of the 'parent' viruses. This difficulty was overcome in a quite unforeseen fashion by using serial dilutions of the second 'parent' with a constant dose of NWS. With a suitable non-neurotropic strain, e.g. WSM, the higher concentrations will prevent multiplication of the neuro-virus by 'interference' and at the level where interference is just being overcome it is possible to isolate sharply distinct variants, which are most readily interpreted as recombinants.

MATERIALS AND METHODS

The virus strains NWS and WSM were described by Burnet (1951 *a*). They were used in the form of allantoic fluids from chick embryos inoculated at 11 days with 0.05 ml. of 10^4 dilution of a glycerolated seed virus stored at -10° .

Injections of 0.03 ml. into the right cerebral hemisphere were made in 4-5-week-old mice under light anaesthesia. Deaths were regarded as specific when the mouse had shown nervous signs, excitability or partial paralysis, on the day of or days before death and experiments were terminated after 12 or 14 days, deaths being very unusual after the 9th day. When indicated, brains of mice dead spontaneously or killed when moribund were removed aseptically, ground with alundum and each brain emulsified in 2 ml. saline containing 200 units, penicillin/ml. After being lightly spun the supernatant fluids were tested for their virus content by inoculation into the allantoic cavity of 11-day chick embryos. The eggs were tested for haemagglutinin after 44 hr. incubation at 35° and active fluids were studied in detail.

The principles of pure-clone study described by Burnet (1951*b*) were applied throughout, the practice being to work with limiting infective dilutions (L.I.D.) and to accept a fluid as representing a pure line if (*a*) it was derived from a L.I.D. and showed the same characters as other L.I.D. fluids from the same seed, and (*b*) on being tested by titration to the L.I.D., the L.I.D. fluids resembled each other and the parent fluid in all relevant characters. Serological work was confined to anti-haemagglutinin titrations with ferret sera from animals immunized by intranasal inoculation of active WSM or NWS virus. The animals were exsanguinated 14 days after inoculation.

Interference phenomena in mice inoculated with mixed NWS and WSM virus

When a fresh allantoic fluid of NWS with a haemagglutinin titre of *c.* 320 is inoculated intracerebrally in mice it produces fatal infections in a dilution 2×10^{-5} . Fluids of WSM usually of rather higher haemagglutinin titre produce no symptoms either undiluted or diluted 10^{-2} . When a mixture of the two viruses is used the result depends on their relative proportions. When WSM is in considerable excess signs fail to appear, and if such mice are killed at the time signs are appearing in mice receiving only NWS (i.e. 5-7 days after inoculation) no virus is isolated from the brains.

In Table 1 are shown the results of two experiments in which all the 'recombinant' strains subsequently discussed were isolated. In the first, two sets of mice were inoculated with serial dilutions of NWS; in one set the dilutions were mixed with an equal volume of diluent (10 % horse serum saline) in the other with an equal volume of dilution 10^{-1} of WSM. In the second experiment a standard amount of NWS (diluted 10^{-3}) was used with serial dilutions of WSM. These results, shown in Table 1, are characteristic of five other experiments with the same two viruses. When NWS diluted 10^{-3} was mixed with other viruses interference to an approximately equal extent was shown by influenza virus strains WSM, WSE, PR8, MEL, BEL, less-marked interference was shown by SW and none with mumps virus strain END or Newcastle disease virus.

Isolation of viruses from mice showing partial interference. When brains removed from mice dying from uncomplicated NWS infection are prepared as described and the supernatant fluids titrated, the end-point of infectivity is about 10^{-4} (0.05 ml. inoculum). With WSM alone virus can be isolated from mice killed on the 2nd or 3rd day but it is absent on the 6th day. Mice in which

Table 1. *Interference by WSM with NWS infection*

(The figures are the day of death of each of four mice, and the figures in brackets the day following that on which a sick mouse was killed for culture, i.e. the day on which death would probably have occurred spontaneously.)

	NWS dilutions					
(a)	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	
+ Saline	4, 5, 6, 6	5, 6, 6, 7	5, 6, 7, 7	X, 6, 6, 6	S, S, S, S	
+ WSM (10 ⁻¹)	5, 5, 5, 7	5, 9, 9, (8)	X, (7), S, S	S, S, S, S	S, S, S, S	
	WSM dilutions					
(b)	10 ⁰	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	Nil*
+ NWS (10 ⁻³)	S, S, S, S	S, S, S, S	5, 6, (8), (8)	5, 6, 6, 10	6, 6, 6, 10	6, 6, 6, 6

X = early non-specific death; S = survived for 12 days.

* Control no WSM dilution added.

mixed infection has produced no signs about this time have shown no virus. Even when paralysis or death results after infection with mixtures giving partial interference it is common to isolate no virus from the brain emulsion. This is particularly the case when death or gross symptoms are delayed beyond the 7th day. It is more usual to isolate virus only from the undiluted brain emulsion. From the two experiments shown in Table 1 a special study was made of the groups on the borderline of interference, NWS 10⁻³ + WSM 10⁻¹ in A, NWS 10⁻³ + WSM 10⁻² in B. Three of the mice in A and all four in B were investigated in detail with the results shown in Table 2. In this table, N signifies virus of the same character as NWS. This was the only type isolated (i.e. presumably present in largest amount) from mice inoculated with NWS only. N' differed from N principally in being convertible to the indicator state, having more enzymic action and being slightly more heat resistant. NM strains were characterized primarily by the heat resistance of their haemagglutinin. The fluids still showed haemagglutinating power after being heated to 62° for 30 min. Their other characters are described in the following section.

Table 2. *Isolation of virus from mice at zone of partial interference*

(The day of death or the day on which it was killed (K) is shown after each mouse. The dilutions are those of supernatant fluid from a 10% suspension of mouse brain in saline.)

Virus mixture in original inoculum	Mouse	Virus isolated at dilutions			
		10 ⁰	10 ⁻¹	10 ⁻²	10 ⁻³
NWS 10 ⁻³ + WSM 10 ⁻¹	69,738 K7	NM, N', N', N	.	---	.
	69,739 +9	NM, N', +	.	+, -	.
	69,740 +9	N', N', N'	.	---	.
NWS 10 ⁻³ + WSM 10 ⁻²	70,450 +5	++-	NM, N, +	NM, NM --	--
	70,451 +6	N, ---	++--	---	---
	70,452 K7	NM, N, +-	++--	---	.
	70,453 K7	N, --	N, --	---	.

NM, N', N = virus present of the type indicated; + = virus present but not further investigated, usually because of low titre; - = no virus present.

Isolation and characterization of NM strains

The striking features of the NM strains were their isolation from mouse brain and hence by implication their encephalitogenic capacity, and the heat resistance of their haemagglutinin. These appeared to correspond to qualities specific for the two original strains and immediately raised the question as to whether a recombination of the qualities of the two viruses had occurred. Obviously the first requirement was to ensure that fluids showing these qualities did not contain a mixture of virus types. The only way in which certain characteristics of an infective fluid can be shown to be the manifestation of a single clone of virus is to obtain secondary clones by subinoculation at limiting dilutions. If all the positive fluids obtained have the same qualities as each other and as the parent fluid, the likelihood of single clones being concerned becomes very high. In the present instance it is necessary to show that repeated re-isolation at limit dilution from infective fluids or mouse-brain emulsions regularly gives fluids which are infective for mice by intracerebral inoculation, and which show haemagglutination after being heated to 62° for 30 min. The evidence in this respect for the first NM strain isolated is as follows. The first positive fluid 51,557 from mouse-brain 69,738 showed haemagglutination on the 3rd and not on the 2nd day of incubation. It therefore may well have been infected with only a single particle. Mouse-brain 70,138 infected from 10⁻² dilution of this fluid was titrated in chick embryos giving egg fluid 52,901 at the limit infective dilution of 10⁻³. This fluid titrated in embryos gave infective fluids including 53,243 at 10⁻⁷ and not at 10⁻⁸; it killed mice on intracerebral injection at 10⁻⁵. Table 3 shows the relevant characteristics of the three fluids described above, together with a parallel series derived from mouse-brain 70,139 a duplicate of 70,138.

Table 3. *Characters of fluids containing NM type of virus*

Fluid	Haemagglutinin			
	Original	After heating at 62° for 30 min.	Encephalitis	
			Dilution	*
51,557	200	130	10 ⁻²	(6), (6), 7, 7
52,901	320	140	10 ⁻⁵	8, 8, 8, 8
53,243	420	240	10 ⁻²	6, 6, 6, 8
52,910	240	120	$\begin{cases} 10^{-4} \\ 10^{-5} \end{cases}$	$\begin{cases} 9, 9, 9, 11 \\ 7, 8, 9, S \end{cases}$
53,251	180	100	10 ⁻²	5, 6, 8, S

* Note as at foot of Table 1.

The same general procedure has been followed in isolating the other strains. Four distinct isolations have been made from mice receiving mixtures of NWS and WSM, those concerned (all shown in Table 1) having the numbers I 69,738, II 69,739, III 70,450, IV 70,452. Strain I is that shown in Table 3.

Strains I and IV were highly pathogenic for mice by the intracerebral route but II and III much less so, giving a proportion of survivors from test inoculations of allantoic fluids diluted 10⁻¹ or 10⁻². Of sixteen mice inoculated intra-

cerebrally with one or other of these dilutions of fluids derived by limiting-dilution passage from strain II, four died at 6, 6, 7, 8 days, four were killed for culture when sick at 6, 7, 8, 8 days, and eight survived. All such fluids contained haemagglutinin which resisted heating for 30 min. at 62°. The other three NM strains, all of which had undergone two or more limit-dilution transfers to the allantoic cavity, were tested in parallel for the main characters that can be determined by *in vitro* tests.

Position in the receptor gradient. The position in the receptor gradient was determined by the use of fowl cells treated with graded dilutions of receptor-destroying enzyme (RDE; Burnet, 1951*a*). Table 4 shows the results with three NM strains and the other significant viruses, as well as the results of tests made on fowl cells treated with each of the WS types and stabilized with anti-serum. These cells were treated in the proportion of 0.1 ml. of packed fowl cells to 5 ml. of undiluted allantoic fluid virus for 4 hr. at 37°. It will be seen that NM strains are considerably further along the gradient series than any of the other WS types. They produce full agglutination of cells rendered inagglutinable by the homologous virus through the action of NWS or WSM. In this character they are by no means intermediate between NWS and WSM and it is obvious that here no question of the NM strains being mixtures can arise.

Table 4. *Position of NM strains in receptor gradient*

Strain	Cells treated with RDE dilutions						Cells treated with		
	1/50	1/100	1/200	1/400	1/800	1/1600	WSM	NWS	NM-WS
WSM	—	—	—	+ ±	++	++	—	+	—
NWS 54,530	—	—	—	±	++	++	—	—	—
NM 54,208 I	—	+	++	++	++	++	++	++	—
NM 54,222 III	—	+	++	++	++	++	.	.	.
NM 54,238 IV	—	±	++	++	++	++	.	.	.
NDV	—	—	—	±	+ ±	++	.	.	.
MEL	—	—	—	±	++	++	.	.	.
WSE	—	—	±	++	++	++	.	.	.
LEE	±	++	++	++	++	++	.	.	.

++ = complete agglutination; ± ±, +, ± = degrees of partial agglutination; — = no agglutination of cells treated as shown and tested with five agglutinating doses of virus.

Serological character. There are minor serological differences between NWS and WSM in the sense that a ferret anti-serum against NWS has a considerably higher titre against the homologous strain than against WSM. A WSM anti-serum on the other hand neutralizes both strains to approximately the same extent. Comparative tests of three NM strains, NWS and WSM against ferret sera 304 NWS and 305 WSM are shown in Table 5. The NM strains are inactivated a little less readily than WSM and are relatively insensitive to the action of anti-NWS ferret serum. A similar relative insusceptibility was evident with two immune fowl sera (anti-NWS).

Tests for neutralization *in ovo* (allantoic method) were limited to the experiment whose results are shown in Table 6. This confirms the fact that serologically the NM strains are closer to WSM than to NWS.

Table 5. *Neutralization of viruses (anti-haemagglutinin) by ferret anti-sera*

(Figures give anti-haemagglutinin titres, i.e. the dilution of serum which mixed with five agglutinating doses of virus allows partial (+) agglutination of fowl red cells.)

Strains		F 304 (NWS)	F 305 (WSM)
WSM	54,322	250	1000
NWS	54,314	1000	2400
NM I	54,208	150	1000
NM III	54,222	100	600
NM IV	54,238	150	800

Table 6. *Neutralization of NM strain III (54,222) in ovo by ferret anti-sera*

(Mixtures contained $10^{6.5}$ 50 % infective doses of virus.)

V 1/5 plus	F 304 (NWS)	F 305 (WSM)
S/10	++++	++++
S/3	++++	+ + - -
S undiluted	++++	- - - -

V = virus; S = serum.

+ = infection as indicated by haemagglutination; - = no infection.

In mice that have been immunized with NWS by combined intranasal and intraperitoneal inoculation of formalin-killed virus, intracerebral inoculation of NM virus produces no signs. It is noticeable (Table 7) that strain III is less virulent than the other two, in this respect resembling strain II which was not included in this experiment.

Table 7. *Resistance of mice immunized with NWS against intracerebral challenge with NM strains*

Strain	Dilution	Immune mice*	Control mice of same age
NWS 54,026	10^{-2}	S, S, S, S	6, 6, 7, 7
NM I 54,197	10^{-2}	S, S, S	8, 8, 8, 8
NM III 54,222	10^{-2}	S, S, S, S	8, 12, S, S
NM IV 54,238	10^{-2}	S, S, S	6, 7, 7

* Note as at foot of Table 1.

Enzymic activities. The NM strains can readily be converted into indicators of the inhibitory effect of ovomucin on haemagglutination. They show about the same degree of activity in destroying ovomucin inhibitor as WSM; i.e. much more than NWS. In limited experiments no qualitative differences from other WS strains could be observed either as indicator or enzyme. The NM strains resemble WSM and NWS, and differ from WSE in being capable of stabilizing red cells treated with excess of virus at 37° without the necessity for subsequent treatment with immune serum.

Heat resistance. The comparative heat resistance of the various types which have been mentioned is shown in Table 8. The fluids were all freshly harvested stocks and were heated, undiluted, for 30 min. at the temperatures shown. The close correspondence of the heat resistance of NM to WSM is obvious.

Table 8. *Heat resistance of haemagglutinins of WS subtypes*

(Figures give haemagglutinin titre after fluids had been heated for 30 min. at the temperature shown.)

Temperature of heating (° C.)	Virus type			
	NWS	N'-WS	NM III	WSM
Unheated	320	480	480	640
54	< 2	100	320	640
56	.	< 2	320	600
60	.	.	280	450
64	.	.	120	360
67	.	.	< 10	< 10

DISCUSSION

The evidence shows that from mixed infections with the strains NWS and WSM a new type can be isolated with some regularity in which the thermostable haemagglutinin of WSM and the encephalitogenic quality of NWS are both manifested. The capacity to produce encephalitis is more marked in some strains than in others, but two *in vitro* characters are shown by all, the heat resistance of the haemagglutinin already mentioned and a quality shown by neither 'parent', in the form of a sharply changed position in the receptor gradient. Before the appearance of these strains can be ascribed to recombination it must be established that the fluids whose characters have been tested do in fact consist of pure clones of virus, or more correctly that they do not represent mixtures of the two original strains. We consider that a simple examination of the qualities shown in Tables 4, 5 and 7 will show that no hypothesis of mixture is tenable. A large number of other fluids obtained at various stages in the process of isolation, mouse passage and re-isolation from limit dilutions was similarly tested with the same *in vitro* findings. This uniformity of character rather than any formal proof from the fact that each strain had undergone two or more passages at limiting infective dilution, is the most important indication that a new virus type is being dealt with.

The NM strains are unlike any strain in previous use in this laboratory and the possibility of casual contamination can be excluded. Since they resemble both WSM and NWS serologically and fail to infect mice that have been immunized with NWS, the possibility can be eliminated that some unsuspected neurotropic virus derived from the mice is being activated by the intracerebral injections.

In the circumstances the only possibilities that can be considered are that the NM strains are derived by mutation from one or other strain, or that the new strain is the result of some type of fusion of genetic qualities from both the original types.

All the virus forms with which we have been concerned in this paper are descendants of the first strain of human influenza virus isolated by Andrewes, Laidlaw and Smith. In one sense therefore NM must be regarded as a mutant irrespective of the process by which it was produced, and it may be formally impossible to exclude its origin by direct mutation. We have definitely adopted

the working hypothesis that recombination of characters has in fact occurred, but it would be unjustifiable to speculate further as to the mechanism involved until a completely unequivocal example of recombination can be provided.

Further work has shown that similar recombinant strains can be obtained from mice inoculated with NWS and influenza virus A of different serological subtype. In these strains encephalitogenic activity is combined with the serological character of the non-neurotropic original strain. A more complete discussion of the problems presented by these phenomena will therefore be deferred until these strains are described.

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