STUDIES ON THE MECHANISM OF ADAPTATION OF INFLUENZA VIRUS TO MICE

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Viruses are often adapted to new hosts, but as yet little is known of the mechanisms involved in the process. It is commonly assumed, and in many cases may be true, that on initial passage in a new host a virus grows poorly and few of the numerous particles inoculated survive. The survivors are presumably variants, which differ from the rest in having a greater capacity for multiplication in the new host. By serial passage these variants are selected and fostered, and in the beginning they may have no detectable effect, thus giving rise to the term blind passage. Complete adaptation can be assumed to be due to the emergence of further variants and their selection, until a strain capable of achieving a high titer is obtained. These latter stages may be accompanied by the appearance of pathological lesions or by inability of the host to survive. If such is the case it is not necessary to assume that the occurrence of lesions is correlated with any other change in the virus except its capacity to multiply in host cells.

The adaptation of influenza virus to the lung of the mouse, reported in this paper, may be a special case, but in any event it is an interesting one and necessitates some other interpretation of the adaptive process.

It has been the usual experience that a fresh human strain of influenza virus can be readily adapted to mice after a few ferret passages. Five to twenty mouse passages enhance the virulence to such a degree that lung suspensions will kill in dilutions of 10^{-2} to 10^{-6}. The same strains will cause lung lesions in dilutions about two logs beyond the lethal end point. In the absence of any means of detecting the presence of influenza virus other than by lethal or lung consolidating effect, it has not been possible to tell what amounts of virus there might be in the mouse lung during the early passages, and it has seemed possible that the increased pathogenicity of the virus with passage might be correlated with increased capacity to multiply in the lung.

With the development of the *in ovo* method of titrating influenza virus (1), a very delicate means of virus measurement became available which enables the investigator to follow the course of virus growth in the mouse lung during a period of adaptation and thus to correlate ability of the virus to multiply with changes in mouse virulence. The method is especially suitable, since all the evidence points to the fact that the *in ovo* titer of the virus is quite independent of the degree of mouse adaptation.

A second aspect of the study reported here was the examination of virus strains for change in antigenic pattern in the course of adaptation to the mouse. Practically all the descriptions of antigenic differences among influenza A and influenza B virus strains consist of comparisons of strains which have been repeatedly passed in mice before they have been tested. It has never been certain whether or not many of the differences found were present in the original organisms or were produced by passage in animals. The work on strain adaptation described below offered the possibility of examining such variation.

Methods

Virus Strains.—The strains used in the present study were Ala. 41, Kil. 41, N.Y. 43, and Sinai 45. The first three are strains of influenza A and the last is a strain of influenza B. Ala. 41 and Kil. 41 were isolated from patients in Alabama during the influenza A epidemic of 1940–41, N.Y. 43 was obtained in New York City during the epidemic of December, 1943, and strain Sinai 45 was isolated from the lung of a patient who died in Mount Sinai Hospital, New York City, of acute hemorrhagic tracheobronchitis during the epidemic of influenza B in November, 1945.[1] All these strains were initially isolated by inoculation of garglings or lung suspension into the amniotic sac of the developing chick embryo (2).

Mouse Passages.—The adaptation of the strains to mice was carried out by the usual methods. Suspensions were inoculated intranasally in 0.05 cc. amounts into six mice, which were sacrificed in 3 or 4 days. The lungs of the mice were ground in 10 per cent normal horse serum broth and passed in series at a 10 per cent concentration. As virulence increased, inocula of greater dilution were used. Titration of passage material in mice and *in ovo* was usually done immediately after preparation but sometimes only after storage at −72°C. With passage of strain N.Y. 43, mouse lungs were perfused with buffer before removal. Hemagglutinin titrations were done on perfused lung suspensions, using a pattern technique like Salk's (3). For control purposes similar titrations were done simultaneously on normal mouse lung.

In Ovo Titrations.—The titration of mouse lung suspensions in the allantoic sac of developing chick embryos was carried out by methods already described (1). The virus was diluted in tenfold steps, and each dilution in the amount of 0.1 cc. per egg was inoculated into six eggs. After incubation for 48 hours the eggs were placed in a refrigerator overnight. The allantoic fluids were then tested individually for agglutinins, and the 50 per cent egg infective titer was calculated in the usual manner (4).

Titrations in Mice.—For these titrations the virus was diluted in tenfold steps and each dilution in 0.05 cc. amounts was inoculated intranasally into six mice. All mice dying in the first 10 days were examined for the presence of specific lung lesions; the remainder were sacrificed at the end of 10 days and the extent of their lesions was recorded. The 50 per cent mortality end points and the 50 per cent lesion end points were calculated (5), the latter being based on the presence of one-plus or greater lung lesions.

Cross-Tests for Strain Differences.—The mouse passage strains to be tested were grown in the allantoic sac of chick embryos, a considerable amount of virus being prepared. Ferrets were inoculated with a 10^{-3} dilution of this material, and the serum obtained 2 weeks later was used for cross-tests. Agglutinin inhibition titrations were performed with the aid of a densitometer (6), and all cross-tests were made at the same time (7). The results, as heretofore, have been expressed graphically as a ratio of the homologous titer to the heterologous titer with any given serum.

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[1] This specimen was obtained from the pathology service of Mount Sinai Hospital through the courtesy of Dr. Klemperer.

EXPERIMENTAL

Correlation of Virus Multiplication with Enhancement of Virulence in the Mouse Lung

Influenza A strains which have been isolated in chick embryos can be established directly in mice without any difficulty. This has been true of the strains of 1940–41 and 1943–44. The first strain selected for study of adaptation was Ala. 41, which was isolated by amniotic inoculation and subsequently went through four allantoic passages. The allantoic fluid of the fifth egg passage was titrated *in ovo* and had an infectious titer of $10^{-7.5}$. This material was

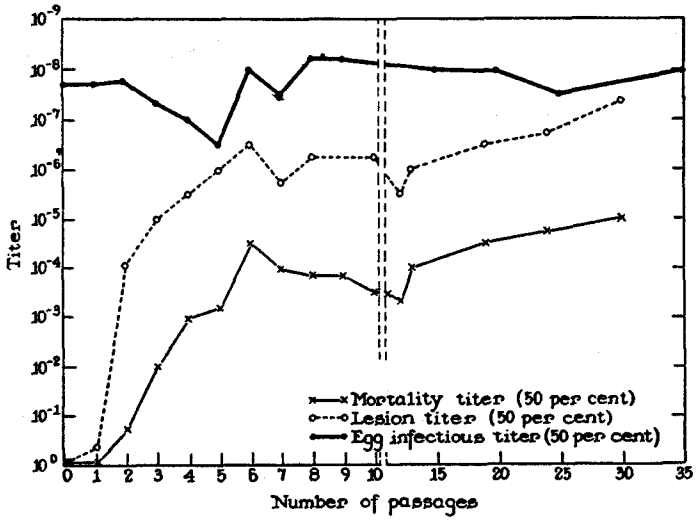

FIG. 1. The adaptation of influenza A strain Ala. 41 to mice. Curves of titer of egg infectivity, mouse lesion titer, and mouse mortality titer of a strain of influenza A virus in different stages of the adaptation to the lungs of mice by serial passage. Zero passage indicates titrations of the allantoic fluid starting material, passage one indicates titrations on lungs of the first mouse passage on the 3rd day, etc.

inoculated intranasally into six mice. After 3 days the mice were killed, and the lungs were ground and passed in 10 per cent concentration to six more mice, and so on in series. Each mouse lung preparation and the original allantoic fluid were titered both *in ovo* and in mice, with results shown graphically in Fig. 1. In this chart zero passage refers to the titration of the allantoic fluid starting material, passage one indicates titration results of the first mouse passage, etc.

Although the initial inoculum was large in terms of egg infectious particles it did not cause any deaths in mice, and only negligible lung lesions. It was very surprising therefore to find that these first passage lungs, most of which appeared perfectly normal, contained very large amounts of virus. In terms

of egg infectivity, mouse lungs of the first passage contained as much virus as did those of subsequent passages when the mouse virulence increased so much that dilutions of 10^{-4} and 10^{-5} of mouse lung were able to kill. This level was achieved by the sixth passage, and virulence was maintained without further increase through many subsequent generations. The lesion end point was fairly consistently two dilutions higher than the mortality end point. While there were minor irregularities in the *in ovo* titrations, in general the egg infective titer remained between 10^{-7} and 10^{-8} throughout the whole period of adaptation and showed no marked tendency to increase with passage.

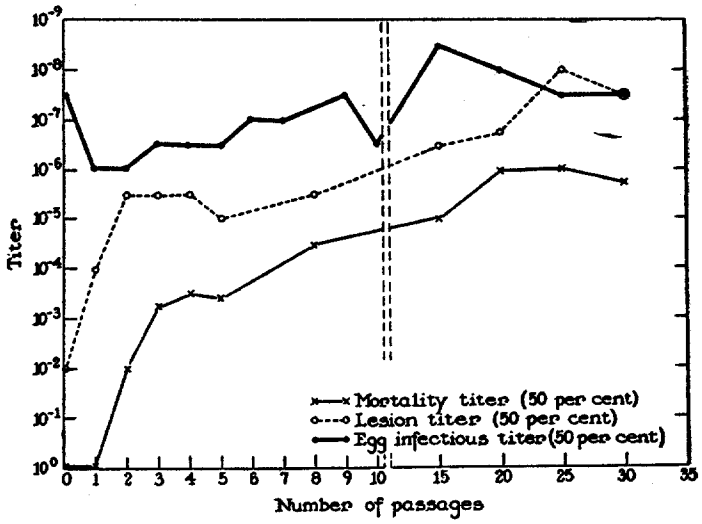

FIG. 2. The adaptation of influenza strain Kil. 41 to mice. The curves are similar to those shown in Fig. 1. The virus being tested is from the same epidemic and in its egg-isolated form was similar antigenically to the strain Ala. 41, of Fig. 1.

Fig. 2 shows the results of a similar effort with the closely related strain Kil. 41 from the same epidemic. With this strain there was some tendency for the allantoic titers to increase between the tenth and the thirty-fifth mouse passage, but no change took place during the early stages when the mortality and lesion titers were rapidly rising. As in the case of strain Ala. 41, there was a high titer of virus in the initial lung passage with very little evidence of gross lesions. It can also be noted that with neither of these strains was the rise in mortality with passage sharply stepwise; it was steady for about five passages before levelling off at a lower rate of increase.

The results of another passage series with strain N. Y. 43 (type A 1943) are shown in Table I. There was no marked difference from the previous results. While the mortality titer rose from less than 10^{0} to $10^{-5.2}$ in four passages, the

in ovo titer remained nearly constant for thirty passages. In this series an attempt was made to correlate the hemagglutinin titer in the lungs with the *in ovo* titer, and all lungs were perfused before removal from the mouse to eliminate murine red cells from the suspension. The hemagglutinin tests were done by the pattern method in order to increase the sensitivity, and suspensions were tested with both guinea pig and chicken red cells. The results were not entirely consistent; but two trends may be noted: (1) The chicken cell agglutinin titer did not increase with passage, while the guinea pig titer increased markedly; and (2) the guinea pig/chicken cell titer ratio of the starting virus was 1, but during passage was usually greater than 1 and increased to 128 on the twenty-eighth passage. It might be inferred from this that the virus

TABLE I

Adaptation of Strain N.Y. 43 to Mice by Serial Passage

Passage No.	*In ovo* titration	Titration in mice		Hemagglutinin titer	
		Mortality	Lesion	Chicken cells	Guinea pig cells
0	$10^{-8.2}$	10^{0}	$10^{-2.0}$	1:320	1:320
1	$10^{-7.4}$	$10^{-3.5}$	$10^{-5.0}$	1:10	1:40
2	$10^{-6.7}$	$10^{-5.0}$	$10^{-5.0}$	1:10	1:40
3	$10^{-6.2}$	$10^{-4.0}$	$10^{-5.0}$	1:10	1:160
4	$10^{-6.8}$	$10^{-5.2}$	$10^{-6.0}$	1:10	1:10
5	$10^{-6.6}$			1:10	1:20
10	$10^{-7.0}$			1:10	1:10
15	$10^{-6.5}$			1:10	1:160
20	$10^{-7.5}$			1:10	1:40
25	$10^{-7.0}$			1:10	1:20
28	$10^{-6.5}$			1:10	1:1280
30	$10^{-7.0}$			1:20	1:640

(originally D form) was becoming with passage more like the O form of Burnet and Bull (8), although this seems at variance with the contention of these authors that the latter multiplies poorly in mice compared with the D form.

A passage series was also started with the type B strain Sinai 45. This strain, in common with other examples from this epidemic (9), did not adapt quickly to the allantoic sac, and even after egg adaptation it could not be established in mice. Virus persisted detectably for only one or two passages and then disappeared. After passing the egg-isolated strain through three ferrets it became possible to infect mice regularly in passage series. In the ferret the virus was obtainable only from the turbinates. In the initial mouse passage no lesions were present (*i.e.* 10 days after inoculation); after fifteen passages, only small and scattered lesions were seen and there were no deaths among the animals. Allantoic titrations of passage lungs have shown a max-

imum titer of 10^{-5}, but the results have not been consistent, presumably because of the poor allantoic adaptation of the strain. This passage series is being continued.

The Development of Strain Differences with Mouse Passage

Magill and Francis (10, 11) and Smith and Andrewes (12) demonstrated with a large number of examples that influenza A strains, even from patients in the same epidemic, often showed marked antigenic differences one from the other. Acceptance of these differences for human virus necessitates the assumption that influenza virus undergoes continual radical changes in antigenic pattern with human to human passage or that epidemics arise commonly from multiple foci and are due to heterogeneous agents of the same type. There is another interpretation of these observations however which seems equally likely, namely that the strain variations found did not exist in the original human strains but were the result of the adaptation of these strains to mice. This does not seem like a remote possibility, since mouse adaptation induces marked behavior changes in strains, and it is by no means unlikely that these are accompanied by antigenic pattern changes as well.[2]

With the advent of the agglutination test, another method became available for making strain comparisons, which has been shown to be adequate for detecting minor antigenic differences. A reexamination of the strain difference problem with this *in vitro* method completely confirmed the findings of earlier workers when the same (mouse-adapted) strains were compared (7). However, when a large number of strains from the 1940–41 epidemic of influenza A were cross-tested it was found that viruses isolated directly in chick embryos did not differ from one another at all, while two strains isolated in ferrets and mice differed from each other and from all the egg-isolated examples. These findings also suggested that mouse adaptation might be at the bottom of the strain difference problem, and further data from two later epidemics (9) has reenforced this view. To furnish a direct test of this possibility, two strains (Ala. 41 and Kil. 41) identical in their egg-isolated form have been studied.

Each strain was started from throat washings inoculated into ferrets. Six ferret passages were necessary before they could be carried in mice. Virus could be obtained only from ferret turbinates. It was maintained in mice by passage at 4-day intervals. After thirty mouse passages of Ala. 41 (Ala. M) and twelve of Kil. 41 (Kil. M), antisera were prepared against these strains in ferrets, and they were cross-tested with their non-mouse–adapted counterparts (Ala. E and Kil. E). The results of this cross-test are shown graphically

[2] Magill and Francis (11) discussed this possibility and tested for antigenic changes in PR8 virus between the thirtieth and the two hundred eighty-fifth mouse passage, but found none. The necessity of using a mouse-virulent virus for cross-testing did not permit the examination of unadapted virus of early passages.

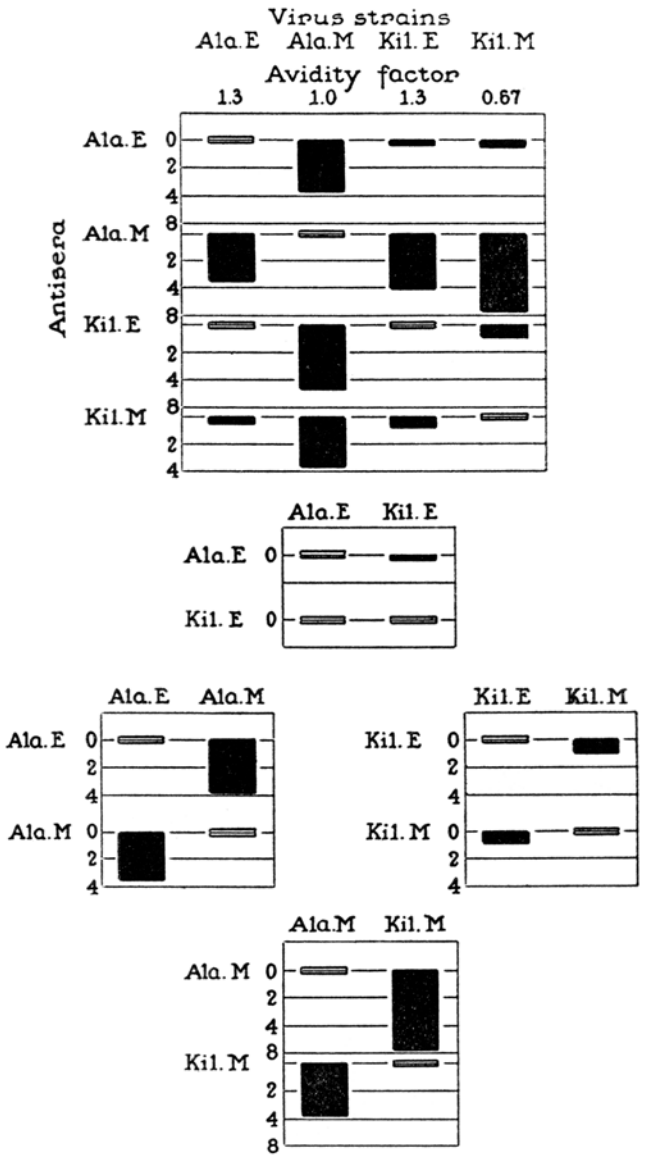

FIG. 3. Antigenic comparison between two influenza strains isolated in chick embryos and their mouse-adapted counterparts; results of cross-tests between the various strains and their ferret antisera by agglutination inhibition. The homologous value is by definition zero; the black bars indicate how much less the titer was with heterologous than with homologous strains and their height is an indication of the degree of antigenic shift.

An empiric correction for the avidity factor is given at the top of the figure. This factor renders the differences found more nearly equal on both sides of a cross-test and is probably made necessary by varying states of aggregation in different virus suspensions. The complete test given in the upper figure is broken down below to facilitate specific comparisons.

in Fig. 3. Small corrections have been applied to the results to make them more reciprocal. The rationale of these has been previously described (7), but the essential results can be demonstrated without this factor. As may be seen from Fig. 3 the sera prepared from strains Ala. E and Ala. M inhibit the agglutination of the heterologous strains only one-fourth as well as that of the homologous strains. This result is highly significant of a true strain difference as measured by this method. The mouse strain Kil. M had had only twelve passages in mice and differed from strain Kil. E by less than twofold, just on the borderline of significance. Both strains in the egg-adapted form (Ala. E and Kil. E) are identical within the limits of this test, but the mouse-passage derivatives of these two strains differ from each other to a greater degree than either one differs from its egg-passage antecedent. Examination of these strains at other stages of mouse adaptation gave confirmatory results.

Thus the *in vitro* type of cross-test showed that two strains of influenza virus shifted in antigenic pattern with mouse passage. The strains in their egg-isolated form were identical, and with mouse passage they not only deviated from their original pattern but deviated from each other.

DISCUSSION

The results of experiments with mouse lungs which had been inoculated with egg-adapted influenza A virus make it clear that a full-blown influenza infection with maximum virus multiplication may occur in the mouse without any gross evidence of a pathological process. In this case the enhancement of virus virulence with passage is not due to an increasing ability of the agent to grow in the lung. Rather, the egg-adapted type of virus seems to be gradually replaced by an entity possessing the same growth potentialities but new and different pathogenic qualities. The pathogenic virus, in order to predominate, must have a more rapid growth rate than its innocuous predecessors. Preliminary experiments have indicated that both forms reach the same maximum titer in the lung, but the pathogenic strain reaches the maximum a little faster. The way in which the mortality titer rises, slowly and steadily for four or five passages, is also consistent with the gradual predominance of a pathogen which grows at a slightly faster rate. The regularity with which mouse virulence is attained with egg-passage strains suggests that the lethal strain may be produced with a fairly high frequency and consistency. The sharp initial rise in virulence levels off at 10^{-4} to 10^{-5}, and subsequent increases, possibly due to other variants, are less predictable.

Just how comparable results of serial passage of influenza B virus may be cannot be stated at present since in forty passages of this virus no appreciable enhancement of virulence has occurred, and the relatively poor adaptation to the allantois makes the significance of the titrations less certain.

While the behavior of red cell agglutinins in passage mouse lungs was notable

for its extreme variability, there was nevertheless a tendency toward the development of guinea pig cell agglutinins and not chicken cell agglutinins. This finding and those of Burnet and Bull (8) on freshly isolated human strains suggest that mammalian cells may have some characteristics in common in respect to agglutinability by certain forms of influenza virus. Maintenance of the virus in man (or in the mouse) fosters the development of the mammalian type of virus agglutinin, while with chick embryo growth the avian agglutinin is very rapidly developed *without*, however, any loss of affinity for mammalian cells. This can be described as only a tendency, since there are numerous examples of laboratory strains which have been carried in mammals only but which possess high chicken cell agglutinins.

The change in antigenic pattern on passage of a strain through mice is of interest mainly in that it removes a good deal of the foundation on which the knowledge of antigenic differences began. We now have evidence that with mouse passage two similar strains will not only change from their original pattern but will deviate from each other. This source of error has in no sense been eliminated from the original, and much of the later, work on this subject. Further evidence will be given in the succeeding paper on the homogeneity of strains in various epidemics, and the implications of these findings will be developed in the light of more complete evidence. As has been stressed earlier the occurrence of antigenic differences with mouse passage is by no means unexpected, and the fact that the shift in antigenic pattern does not follow a definite direction but diverges in different series is also in line with what might be expected from the selection of chance variants. The degree of antigenic difference produced was of the same order of magnitude as that found by Magill and Francis (11) but is definitely less than that described by Smith and Andrewes (12). Why the English workers found such large variations is not clear; antigenic discrepancies of similar magnitude have never been found in the United States. It should be emphasized that the description of antigenic shifts by animal passage does not rule out the occurrence of differences in human strains but merely requires added proof of such differences, which at the present time is not sufficient where strains from a single epidemic are concerned.

SUMMARY

1. When strains of influenza A virus which have been isolated in chick embryos are introduced into the mouse lung, the virus multiplies readily and achieves initially a titer which is as high as is ever obtained, even after repeated passage. The high initial titer of virus may be unaccompanied by any lethal or visible pathogenic effects; but with four or five mouse passages the agent becomes lethal in high titer and causes extensive pulmonary consolidation, though its capacity to multiply in the lung has not increased. In one

example the adaptation to mouse lung was accompanied by increasing capacity to agglutinate guinea pig red cells without a corresponding increase in agglutinating power for chicken cells. Influenza B virus, in preliminary tests, did not behave in a similar fashion.

2. The adaptation of influenza A virus to mice is accompanied by changes in antigenic pattern, as detected by cross-tests with the agglutination inhibition method. Two strains, initially similar, with passage, changed in pattern along divergent paths so that they became not only unlike the parent strains but unlike each other. This finding has important implications for the interpretation of the strain difference problem in human influenza.

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