A STUDY OF THE NEUROTROPIC TENDENCY IN STRAINS OF THE VIRUS OF EPIDEMIC INFLUENZA*

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In our laboratory repeated efforts to adapt the virus of epidemic influenza to the brains of mice had been completely unsuccessful. The virus ordinarily failed after intracerebral inoculation to survive in the brain more than one or two days. Cerrutti (1), however, reported the recovery of virus from the brains of mice infected intranasally. Subsequently, Daddi and Pavà (2), and Cerrutti and di Aichelburg (3) noted that influenza virus survived in the brains of rabbits without producing symptoms. Finally, Stuart-Harris (4) described the development of a variant which produced a fatal neurological infection in the brains of mice after intracerebral inoculation. This variant was derived in the following manner: The W.S. strain maintained by pulmonary infection in mice was transferred to the chorioallantois of the developing chick. After 14 transfers on this tissue, hemorrhagic encephalitis of the embryo was noted and on the 21st transfer mice were inoculated intracerebrally. Serial passages at 2 or 3 day intervals were then made in mice until the 12th passage, after which neurological symptoms and deaths occurred regularly. During this entire period the presence of influenza virus was demonstrated by the occurrence of typical lesions in the lungs of mice receiving the same material intranasally.

These observations indicated that the success of the latter experiments was due to an adaptation of the virus to the nervous tissue of the chick embryo. It was decided, therefore, to attempt a similar adaptation by cultivation of influenza virus in tissue cultures containing embryonic chick brain and either Tyrode's solution or physiological salt solution. Since our earlier unsuccessful attempts had all been made with the PR8 strain while the reported positive results had all been obtained with the W.S. strain, the W.S. strain was used.

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Materials and Methods

Strains of Virus.—The strains of the virus of epidemic influenza employed in this study had all been maintained in chick embryo tissue cultures for several years. The W.S. strain (5) was chosen for the primary investigation since the positive results mentioned above had been obtained with that strain. The PR8 strain which had yielded consistently negative results in our hands was studied simultaneously.

Subsequently other strains were used: Alaska; Henry, isolated in this country; Talmey, from England (6); Melbourne, from Australia (7); two strains of swine influenza virus, S-15 and S-1976, obtained originally from Dr. R. E. Shope.

Methods of Cultivation.—For ordinary purposes the virus was propagated in the customary medium of minced chick embryo (8), although physiological salt solution was usually substituted for Tyrode's solution (regular medium).

For the preparation of cultures in embryonic chick brain the brain of the embryo was removed, minced, and added to physiological salt solution in the absence of all other tissue (brain culture medium).

In some instances combinations of the two were used (mixed culture medium).

Cultures were incubated for 48 hours when transfers to fresh medium were made.

To determine whether virus was surviving in the different media, portions of the cultures at intervals after the 3rd transfer were inoculated intranasally into mice which were then observed for the development of characteristic pulmonary lesions. Neutralization tests with known immune serum were conducted from time to time for serological identification as well.

Attempts to Cultivate Strains of Epidemic Influenza Virus in Brain Culture Medium

In cultures containing only embryonic chick brain the W.S. strain was maintained with ease for as long as 20 transfers while the PR8 strain, in all of five attempts, had disappeared by the 3rd transfer. In two attempts each with Henry, Talmey, and Swine-1976 strains, and three attempts each with Alaska and Swine-15 strains, the virus failed to survive 3 transfers. It appeared at first, therefore, that the capacity to propagate in a medium of chick embryo brain was a characteristic essentially limited to the W.S. strain. The Melbourne strain, however, appeared to occupy an intermediate position between these extremes. In eight of twelve experiments, the virus survived from 3 to 6 transfers in brain or mixed culture medium but as will subsequently be seen differed from the W.S. in its adaptability to mice.

The results of these experiments, summarized in Table I, indicate the sharp differences in ability of different strains to propagate in cultures of brain of the embryonic chick.

Attempts to Obtain a Neurotropic Strain of Epidemic Influenza Virus by Intracerebral Passage in Mice

1. Preceded by Cultivation in Brain Culture Medium.—When it was observed that the W.S. strain of virus multiplied satisfactorily in brain tissue culture, fluid from the 7th transfer was introduced by direct inocula-

TABLE I

Attempts to Cultivate Strains of Influenza Virus in Brain Tissue Culture

Strain	Number of attempts	Results and comments
W.S.	1	Positive to 20th transfer. Discontinued (Experiment 1)
	1	Positive to 10th transfer. Discontinued
Melbourne	4	Negative by 3rd transfer
	3	Positive to 3rd transfer; negative by 5th
	1	Positive to 5th transfer in mixed culture medium (Experiment 10)
	1	Positive to 5th transfer in mixed culture medium and 3 transfers in brain culture medium (Experiment 11)
	1	Positive to 6th transfer in mixed culture medium and 6 transfers in brain culture medium (Experiment 12)
	1	Positive to 6th transfer (Experiment 13)
	1	Positive to 6th and 10th transfer; negative by 14th (Experiment 14)
	1	Positive to 3rd transfer (Experiment 15)
PR8	5	Negative by 3rd transfer
Swine-1976	2	" " " "
Swine-15	3	" " " "
Alaska	3	" " " "
Henry	2	" " " "
Talmey	2	" " " "

The number of an experiment corresponds with the number given in Table II.

tion into the brains of three mice of 10 to 12 gm. weight. At 2 or 3 day intervals the brains were removed, ground in physiological salt solution to form a 20 per cent suspension, and serial intracerebral passages were carried out in the same manner. The mice were closely examined for any signs of nervous disturbance. From time to time in the course of intracerebral passages the suspension of brain was also given to mice by the intranasal route to test for the survival of influenza virus. It was found that the W.S. strain was maintained indefinitely by passage in the brains

of mice. On the 11th passage definite neurological signs in the form of hyperesthesia and tremor were observed and one mouse died in convulsions. All mice of the 12th passage presented marked signs on the 3rd and 4th days and were sacrificed. Two of the mice of the 13th passage were killed when quite sick on the 3rd day, two others were found dead on the 6th day. Since that time the virus has been uniformly pathogenic by the intracerebral route, producing a consistent clinical picture and a fatal infection 4 to 5 days after inoculation with 5 per cent brain suspension (Experiment 1, Table II). This strain (WS-7) has been carried through 67 serial passages. The usual course of the disease is briefly as follows: On about the 3rd day the mice appear huddled, their fur is ruffled, and they are hypersensitive to external stimuli. A generalized tremor is present. On the 4th to 6th day the animal dies in tetanic convulsions. Paralyses are not seen. Pathologically, the picture conforms to that described by Stuart-Harris. Hyperemia and lymphocytic meningitis with little or no involvement of the brain parenchyma are the essential features.

2. Without Preceding Transfer in Brain Culture Medium.—In view of the fact that the W.S. strain multiplied in cultures of brain tissue and became pathogenic in the brains of mice, it was of interest to ascertain whether its neurotropic properties could be developed without previous cultivation in embryonic brain. Accordingly, the W.S. strain, maintained through 194 transfers in whole chick embryo cultures, was inoculated intracerebrally into mice and serial passages from brain to brain were carried out (Experiment 2, Table II). Mice of the 9th passage exhibited distinct neurological signs and deaths occurred in the 10th passage. Intranasal inoculations and neutralization tests made during this period demonstrated that the pneumotropic influenza virus was constantly present in the brain. This strain (WS-TC) has now been through 70 consecutive passages in the brains of mice. A similar procedure was also carried out with the W.S. strain of virus from the 230th passage in the regular culture medium. Here again the virus multiplied, produced neurological signs in mice of the 4th passage and death in mice of the 10th passage (Experiment 3, Table II). This series was discontinued.

In contrast to the results obtained with the W.S. strain, following the introduction of the PR8 strain to the brains of mice directly from the regular culture medium, the virus failed to survive through four serial passages (Experiments 16 and 17, Table II). Negative results were obtained with the Talmey strain as well (Experiment 18, Table II).

The Melbourne strain was the only one besides W.S. to survive in brain tissue culture for 3 or more transfers, suggesting that the neurotropic

tendency was present. In seven separate attempts to adapt this strain to the brains of mice, however, the virus failed to survive through 4 pas-

TABLE II

Fate of Strains of Influenza Virus after Intracerebral Passage in Mice

Strain	Experiment No.	Source of virus	Results			Comments
			Passage symptoms began	Passage death occurred	Number of passages virus survived	
W.S.	1	7th passage, brain TC medium	11	11	67	Continuing
	2	194th passage, regular TC	9	10	70	"
	3	230th " " "	4	10	12	Discontinued
	4	9th i.n. passage from TC	—	—	<5	Passed at 7 day intervals
	5	13th " " " "	—	—	<4	
	6	14th " " " "	4	6	12	Continuing
	7	15th " " " "	3	3	11	"
Melbourne	8	110th passage, regular TC	—	—	<4	Carried 22 passages, no symptoms
	9	190th " " "	—	—	<4	
	10	5th passage, mixed TC	—	—	<4	
	11	8th passage, mixed TC, 3rd brain culture	—	—	<4	
	12	6th passage, mixed TC, 6th brain culture	—	—	<4	
	13	6th passage, brain culture	6	8	14	Continuing
	14	6th " " "	—	—	<4	
	15	3rd " " "	—	—	<4	
PR8	16	489th passage, regular TC	—	—	<4	Carried 22 passages, no symptoms
	17	506th " " "	—	—	<4	
	18	7th passage, brain culture	—	—	<4	
Talmey	19	3rd passage mixed TC	—	—	<4	
Control	20	Uninoculated TC	—	—	—	Carried 41 passages

i.n. = intranasal. TC = tissue culture.

sages. In one experiment (Experiment 13, Table II) in which virus was present in the 6th transfer in chick embryo brain cultures, subsequent serial intracerebral passages resulted in the beginning of irritative signs in mice of the 6th passage and deaths in those of the 8th passage. The modified

Melbourne strain is now in its 14th intracerebral passage and is regularly lethal. This demonstration would appear to validate the suggestions of neurotropism offered by the limited ability of the Melbourne strain to propagate in brain culture medium, occupying in this respect an intermediate position between the W.S. strain and the other strains studied.

Three series, comprising 22, 22, and 41 (Experiments 8, 16, 20) serial brain to brain passages in normal mice, served as controls and have revealed no evidence of an extraneous neurotropic agent occurring spontaneously in the course of these experiments.

Since the present experiments have dealt solely with strains of virus grown in tissue culture, it was of interest to know whether the W.S. strain of virus maintained by intranasal infection of mice also possessed neurotropic attributes. Accordingly, virus of the W.S. strain, which had been carried for 9 to 15 intranasal passages since removal from regular tissue culture medium, was tested. Suspensions were made of the infected lungs and with them mice were inoculated intracerebrally. Virus prepared from lungs of the 9th and 13th passages failed to survive serial intracerebral passage—in the first instance probably because passages were made at 7 day intervals. With virus from the 14th and 15th intranasal passages, however, fatal nervous infections were obtained after 6 and 3 intracerebral passages, respectively (Experiments 4, 5, 6, 7, Table II).

On the basis of the foregoing investigations, it seems clear that a period of prior adaptation in embryonic chick brain is not required in order to bring out the neurotropic qualities of the W.S. strain of influenza virus. On the contrary, serial passages in the brains of mice with virus previously maintained in whole embryonic culture or in the lungs of mice, indicate that the neurotropic capacity is an inherent property of that strain. The Melbourne strain appears also to possess a neurotropic tendency but to a much lesser degree than the W.S. That this property has not been acquired during cultivation in the regular tissue culture medium is indicated by its absence in other strains which have been carried much longer under the same conditions.

The evidence available, therefore, indicates that the capacity of the W.S. and Melbourne strains of epidemic influenza virus to become pathogenic for mice by the intracerebral route represents merely an enhancement of a basic characteristic of these strains which also manifests itself in an original capacity to multiply in culture medium containing only embryonic brain tissue. When once developed the neurotropic activity in mice appears to persist indefinitely. It has been maintained through 70 passages in the brains of mice. Furthermore, no significant change in

intracerebral virulence was observed when the adapted virus was subsequently passaged by the intranasal route for as long as 25 successive transfers.

Evidence That the Neurotropic and the Pneumotropic W.S. Strains Are Identical

The possibility was immediately apparent that the neurotropic virus recognized in the course of the experiments might represent an extraneous contamination with a virus differing from that of epidemic influenza. The evidence does not, however, appear to support such a conclusion.

1. The neurotropic activity through 53 and 57 successive intracerebral passages of two series has been uniformly associated with the presence of pneumotropic influenza virus (Table III).

The comparative virulence of the virus by the two routes has remained essentially parallel. For example, a titration with strain WS-TC in its 67th brain passage was made by both the intranasal and intracerebral routes. The results are as follows:

Dilution of brain	Route of inoculation	
	Intracerebral	Intranasal
10^{-1}	3, 4, 4	5, 5, 0
10^{-2}	3, 5, 6	6, 7, ±
10^{-3}	6, 6, 6	7, 8, ++++
10^{-4}	4, 5, 8	6, 9, 11
10^{-5}	6, 8, 11	6, 7, 8
10^{-6}	S, S, S	−, −, −
10^{-7}	S, S, S	−, −, −

The mice receiving the virus intranasally died with the pulmonary lesions typical of infection with influenza virus; those inoculated intracerebrally died with typical neurological signs. There have been at times, however, suggestions that in the course of intracerebral passage some decrease in intranasal virulence occurs. This decrease consists for the most part in a prolongation of the pulmonary disease.

2. The neurotropic effect of the W.S. strain is neutralized by serum developed against PR8, a strain which has no demonstrable neurotropic tendency. Since the PR8 and W.S. strains are serologically different, it is necessary to employ a hyperimmune PR8 serum to obtain complete neutralization of the W.S. strain by the intranasal route and a serum of this caliber has, therefore, been used.

When the tests are done by the intracerebral route, uniform protection is not always obtained even with hyperimmune PR8 serum. While the mice usually survive, virus may be demonstrated by passage from their

TABLE III

Comparison of Neurotropic and Pneumotropic Activity of Virus in the Course of Intracerebral Passage

Passage series	Passage No.	Results of inoculation	
		Intranasal	Intracerebral
WS-7	1	4, 6, 6	k2, k2, k2
	4	5, k5++	k3, k3
	12	6, 7, ++	4, 5, 6
	14	4, 4, 7	3, 3, k4
	17	7, 7, 7	5, 5, 6
	24	7, 9, 9	5, 5, 6
	35	6, 7, 7	5, 6, 7
	41	k9+, +, 0	6, 6, 7
	43	k6++++, ++++	3, 4, 14
	48	4, 5, ++++	4, 5, 5
	51	5, 7, 7	5, k5, s16
	57	5, 5, 5	3, 4, 5
WS-TC	1	3, 3, 4	k2, k2, k2
	4	4, 5	k3, k3, k3
	10	3, 4, 5	3, 5, 10
	12	7, ++++, ++	3, 6, 6
	17	6, 6, ++++	3, 4, s10
	24	6, 9, 9	3, 3, 5
	31	6, 8, 8	3, 5, 5
	33	3, 5, 6	3, 3, 5
	35	4, 5, 6	5, 6, s14
	43	++++, +++	6, 6, 7
	47	2, 6, 7, 7, 7, 7	6, 6, 6, 6, 6, 6
	53	7, 7	5, 5, 6

Numeral indicates day of death of individual mouse. Mice were killed on the 10th day unless indicated otherwise.

k = sacrificed. s = survived.

0 = no pulmonary lesions at autopsy.

+ to ++++ = increasing degrees of pulmonary involvement.

brains and under these conditions the pneumotropic and neurotropic activities are found to have survived to a parallel degree.

When the same serum-virus mixture is given by the intranasal route complete protection without pulmonary lesions results. Even with repeated serial passages from the lungs of these mice to normal mice by

intracerebral or intranasal route no virus is detected, thus showing that both the pneumotropic and neurotropic activities are eliminated simultaneously (Table IV).

Specific, low titer, rabbit serum against the regular W.S. strain has also been employed. In intranasal neutralization tests the neurotropic virus is completely neutralized but by the intracerebral route rabbit serum protects in an irregular fashion. Rabbit serum similarly produced against the PR8 strain affords no protection against the W.S. strain by either route.

3. Mice immunized with neurotropic W.S. strain by intracerebral or intraperitoneal routes are then resistant to intranasal infection with either the regular W.S. or the neurotropic W.S. strain. Mice vaccinated

TABLE IV

The Effect of Hyperimmune PR8 Serum upon Neurotropic and Pneumotropic Capacities of Virus

Strain	Route of neutralization test	Day sacrificed	Gross evidence of infection	Virus in brain		Virus in lung	
				Neurotropic	Pneumotropic	Neurotropic	Pneumotropic
WS-TC-33	i.c.	3	0	+	+		
	i.n.	3	0			0	0
WS-7-35	i.c.	5	Slight symptoms	+	+		
	i.n.	5	0			0	0

i.n. = intranasal. i.c. = intracerebral.

intraperitoneally with the non-neurotropic PR8 strain are protected against intranasal or intracerebral infection with the neurotropic W.S. strain, though less completely in the latter instance in that all mice may not survive.

4. The only neurotropic viruses maintained in this laboratory are those of meningo-pneumonitis and choriomeningitis. Mice vaccinated and immune to the neurotropic W.S. strain died uniformly when tested intracerebrally with either of these two viruses. Mice vaccinated and immune to meningo-pneumonitis also failed to survive intranasal or intracerebral infection with the neurotropic W.S. strain. Not only the immunological evidence but the clinical picture produced by neurotropic influenza virus in mice differs sharply from that exhibited in infection with either of the other agents. Furthermore, the neurotropic virus passes through Berkefeld W filters which eliminates the much larger virus of meningo-pneumonitis.

5. The continued passage from brain to brain in three extended series

of normal mice has yielded no evidence of a neurotropic passenger virus present in the normal stock of mice used in these experiments.

These observations point sharply to the conclusion that the neurotropic and pneumotropic W.S. strains of epidemic influenza virus are identical and that they merely represent different qualities of the same agent made apparent by different routes of inoculation. Such an interpretation is further supported by the experiments recorded in Table V. In this instance attempts were made to recover virus by serial passages from the lungs and brains of mice at different intervals after intranasal and intracerebral inoculation of 10 per cent suspensions of neurotropic virus. In contradistinction to the reports of other investigators, virus was not found in the brains after intranasal injection. Nor was virus recovered from the

TABLE V

Presence of Virus in Brain and Lung of Mice after Intracerebral and Intranasal Inoculation of the Neurotropic Strain

Experiment No.	Route of original inoculation of virus	Virus in brain			Virus in lung		
		Hours after inoculation			Hours after inoculation		
		8	24	72	8	24	72
1	Intracerebral	+	+	+	0	0	0
	Intranasal	0	0	0	+	+	+
2	Intracerebral	+	+	+	0	0	0
	Intranasal	0	0	0	+	+	+

lungs after intracerebral injection. The results offer an explanation for the fact that neurological signs have not been observed in mice receiving neurotropic virus intranasally, nor pulmonary lesions in mice injected intracerebrally. In each case the pathological picture is determined by the route of administration of the virus.

DISCUSSION

The results of the present investigations have confirmed the observations of Stuart-Harris: (*a*) that the W.S strain of the virus of epidemic influenza can be induced to produce infection in the brains of mice inoculated by the intracerebral route; (*b*) when once developed the neurotropic quality persists indefinitely; (*c*) the neurotropic activity is not due to contamination with a passenger virus of laboratory origin. This latter statement is based on the facts that the pneumotropic and neurotropic capacities parallel one another, that they survive to a similar extent in the case of incomplete neutralization, that both factors may be eliminated in neu-

tralization tests with hyperimmune serum derived against the PR8 strain which has exhibited no neurotropic tendencies, and that proper vaccination of mice with the non-neurotropic PR8 strain protects them against intranasal or intracerebral infection with the neurotropic W.S. strain.

The evidence reported here, however, does not support the suggestion of Stuart-Harris' experiments that the development of neurotropism for mice resulted from the adaptation of virus to the central nervous system of the chick embryo following serial cultivation on the chorioallantoic membrane of the egg. On the contrary, it was found that the W.S. strain which had been maintained in this laboratory in ordinary tissue culture medium for several years became directly pathogenic for the central nervous system of mice following serial intracerebral transfers in that species of animals. It was shown, in addition, that without any previous treatment the stock W.S. strain multiplied freely and could be maintained indefinitely by transfer in cultures which contained no tissue other than embryonic chick brain. These observations point strongly to the conclusion that the neurotropic capacity is an inherent property of the W.S. strain of virus, which is enhanced quantitatively by passage through the brains of mice, and does not represent the development of a variant strain possessing properties of which the original was entirely devoid. The Melbourne strain of virus has the same neurotropic tendency as shown by the ease with which it could be cultivated in brain culture medium. In this instance, however, the characteristic was somewhat less well developed in that the strain failed in all but one experiment to survive for a significant period through transfers in the brains of mice. It is interesting that four other strains of human influenza virus and two of swine influenza virus failed completely to survive in brain culture medium or in passage through the brains of mice, although these strains had been maintained in the regular culture medium as long or longer than the two strains found to be potentially neurotropic. The differences appear, therefore, to be related to fundamental differences in the strains studied.

The neurotropic activity does not develop at the expense of the pneumotropic. Where one exists, there the other is found in equal concentration. The effect produced is, however, strictly governed by the route of inoculation. While both pneumotropic and neurotropic virus can be recovered from the brain after intracerebral inoculation or from the lung after intranasal inoculation, the neurotropic effect is not produced by intranasal injection nor is the pneumotropic effect observed after intracerebral injection of the virus. It seems only reasonable to conclude then that the neurological disease produced in mice by the W.S. and Melbourne strains

of influenza virus represents merely the different picture produced by the usually pneumotropic virus acting in a different medium.

SUMMARY

The demonstration by Stuart-Harris that the W.S. strain of epidemic influenza virus can induce a fatal nervous disease in mice has been confirmed.

In contrast, however, no previous period of adaptation to chick embryonic brain was required. By serial brain to brain passages in mice originally inoculated with the virus cultivated in the usual chick embryo culture medium a fatal disease, essentially meningeal in character, is produced.

The Melbourne strain has been similarly enhanced while other strains have failed to reveal any neurotropic tendencies.

The evidence indicates that the neurotropic characteristic is present in the two strains as an inherent quality which is quantitatively heightened and does not represent the acquisition of a property not previously present.

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