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Apparent Serological Variation Within a Strain of Influenza Virus.*

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During the period of testing preparations of influenza virus for comparative immunizing effect in mice, certain results were encountered suggesting that the PR8 strain of Type A influenza virus maintained in eggs had lost antigenic potency when measured by its capacity to protect mice against the mouse passage line of the same strain. The egg line had been transferred to tissue culture in 1935 from the 70th mouse passage and had been continued in culture for 717 transfers until January, 1942, when it was introduced into fertile hens' eggs. From that time until March, 1944, when the present observations were begun, it had received 53 transfers in eggs. Because of this effect a new line of the PR8 strain was instituted in eggs from the 593rd unbroken mouse passage line. A series of studies was then undertaken to compare the immunological behavior of the 2 lines.

Materials and Methods. Virus. The virus is the PR8 strain of influenza virus, Type A, isolated in 1934 by the inoculation of ferrets. After 7 ferret passages it was transferred to mice. The mouse line was thereafter constantly maintained in mouse passage only. The tissue culture line was established from the 70th mouse passage in 1935. It was then maintained by transfers done twice weekly on the average for 717 generations in culture, until it was transferred to eggs in 1941.

Serological Tests. (a) Hemagglutination and inhibition: This procedure was carried out with a pattern test[1] in which one unit of agglutinin was employed for the titration of serum. Results are given as final dilutions of serum.

* This investigation was conducted under the auspices of the Commission on Influenza, Army Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D.C.

[1] Salk, J. E., *J. Immunol.*, 1944, 49, 87.

TABLE I.
Cross-Immunity Tests in Mice.

Immunized with	3/28/44 Infection with mouse passage 10-1	10-2	10-3	10-4	Protection LD_{50}	Hemagglutinating titer	Mouse infection titer
TC-Egg							
10-0	5/7	6/6	6/6	6/6	>100,000	2560	10-5.15
10-1	2/7	5/6	6/6	6/6	56,230		
10-2	1/6	3/7	5/6	6/6	10,890		
10-3	1/6	0/6	3/7	3/6	560		
10-4	0/6	0/6	0/7	4/6	250		
Mouse-Egg							
10-0	3/3	—	—	—	>100,000	1280	10-6
10-1	7/7	6/6	6/6	6/6	>100,000		
10-2	5/6	6/6	6/6	6/6	>100,000		
10-3	1/6	7/7	6/6	6/6	56,230		
10-4	0/6	6/6	6/6	6/6	45,390		
Control Titration	10-5 0/5	10-6 3/5	10-7 1/5		LD_{50} 1,197,000		

Numerator = Survivors. Denominator = Number inoculated.

(b) Neutralization in mice: For this test the virus suspension and serum dilution were mixed, allowed to stand at room temperature for 30 minutes and each of a group of 3 to 5 mice was given 0.03 cc of a mixture intranasally. The mice were observed for 10 days and the endpoint was recorded as the estimated point of 50% lethality.[2] The dilutions of serum employed varied according to the range of neutralizing titer anticipated in the system. Titers are recorded as final dilutions of serum.

(c) Titrations of virus potency in mice were ordinarily done with serial 10-fold dilutions.

(d) Rabbit sera were generally obtained 10 days after one intraperitoneal inoculation of active virus preparation. In some instances the use of hyperimmune rabbit serum is indicated; animals received multiple inoculations of virus.

Egg Passage. For passage of virus in eggs, inoculations of 0.1 cc volume were made directly into the allantoic sac of 10-day embryos. After 48 hours' incubation at 37°C, the fluid was harvested with needle and syringe in the absence of hemorrhage. Material was titrated for hemagglutinins and, in stoppered containers, was stored in the refrigerator at 4°C until used.

Cross Immunity in Mice. The 2 lines, PR8, 198-M70, TC717-E55, and PR8, 198-M593-E4, were used. Allantoic fluid was harvested from chick embryos inoculated 48 hours earlier. Titers of hemagglutinin were determined with each preparation at the time of harvesting. The fluid was kept at 4°C in the intervals between use. Each of a group of 30 to 35 mice was given 2 intraperitoneal injections of 0.5 cc amounts of undiluted or 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} dilutions of one of the 2 preparations at weekly intervals. Titrations of intranasal infectivity for mice were made at the time of each immunizing inoculation.

One week after the second inoculation each group of mice was subdivided into 5 subgroups and these were inoculated intranasally with 10^{-1}, 10^{-2}, 10^{-3} or 10^{-4} dilutions, respectively, of suspension of lungs of mice of the 597th regular passage of PR8 strain. Inoculated control mice of the same lot were infected at the same time.

The mouse-egg line was approximately 10 times more effective in its lethal titer for mice, but the tissue culture-egg line had a somewhat higher titer of hemagglutinins. As measured by immunizing effect against regular mouse passage virus, the mouse-egg line was at least 100 times as effective as the tis-

[2] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

sue culture-egg line. The effect was emphasized by the fact that in the same test 2 lines of the Weiss strain were also studied, one transferred to eggs directly from ferret passage, the other after 32 mouse passages. The former ordinarily produced fatal infection in mice only with undiluted fluid while the latter had a lethal titer of 10^{-4} approximately. Both lines induced essentially the same degree of active immunity against the PR8 mouse passage virus, somewhat more effectively than the PR8 tissue culture-egg line.

The results were confirmed by a later limited experiment with the 77th egg passage of the tissue culture-egg line and the 11th egg passage of the mouse-egg line. The titers of the 2 preparations were, respectively, by hemagglutination 2560 and 1280; by intranasal infection of mice, $10^{-5.3}$ and $10^{-5.5}$; by infection of fertile eggs allantoically, $10^{-8.48}$ and $10^{-8.41}$. The undiluted tissue culture-egg material protected against 100,000 LD_{50}, the 10^{-1} dilution against 21,000 and the 10^{-3} dilution against less than 1400. The 10^{-1} dilution of the mouse-egg material induced immunity against more than 150,000 and the 10^{-3} dilution against 100,000 LD_{50} of the regular mouse passage virus. Hence, while the virus content of the 2 preparations was closely similar, the immunizing disparity persisted.

The data, in review, suggested that antigenic differences existed between the 2 lines of the PR8 strain maintained under different conditions for several years or that cultivation in eggs had an effect upon virus behavior.

Serological Studies. Experiment I. Rabbit RM 3 received 5.0 cc intraperitoneally of the allantoic fluid containing the tissue culture-egg line in its 54th passage. Rabbit RM 4 received 5.0 cc intraperitoneally of the mouse-egg line in its second egg passage. Serum was obtained from both animals before inoculation and 10 days afterwards. The sera were then used in agglutination-inhibition tests against eluates of the 2 lines and in neutralization tests in mice with approximately 100 LD_{50} of the tissue culture-egg

TABLE II. Serological Tests with Different Lines of Virus.

	Rabbit No.	Date of serum	Stage	Immunized with strain	Passage	Agglutination-inhibition test		Neutralization in mice	
						TC-Egg 43 eluate	Mouse-Egg 3 eluate	TC-Egg 57 10^{-2}	Mouse 597 10^{-3}
Exp. I	RM 3	3/22/44	Norm.			128	64	0	0
		4/ 1	Imm.	TC-Egg	54	512	512	100	8
	,, 4	3/22	Norm.			128	32	0	0
		4/ 1	Imm.	Mouse-Egg	2	2048	4800	280	480
		8/17	Imm.			1024	2048		
						TC-Egg 77 eluate	Mouse-Egg 11 eluate	TC-Egg 77 10^{-1}	Mouse-Egg 11 10^{-2}
Exp. II	RM 12	10/ 9	Norm.			64	32	10	10
		10/27	Imm.	Mouse-Egg	20	1024	2048	140	960
								640	1600
	,, 16	11/22	Norm.			32	32	0	0
		12/ 2	Imm.	Mouse	634	1024	1024	40	480
	,, 21	11/22	Norm.			32	32	10	10
		10/27	Imm.	TC-Egg	75	4096	512	70	35
								280	30

Exp. I: Mouse titer of TC-Egg strain $10^{-4.25}$, 10^{-2} used. Mouse titer of Mouse strain $10^{-6.25}$, 10^{-3} used. Exp. II: Mouse titer of TC-Egg strain $10^{-5.33}$, 10^{-1} used. Mouse titer of Mouse-Egg strain $10^{-5.56}$, 10^{-2} used. Egg titer of TC-Egg strain $10^{-8.5}$; of Mouse-Egg strain $10^{-8.4}$.

and approximately 1000 LD_{50} of the regular mouse passage lines. Equal volumes of 2-fold dilutions of serum and the virus preparation were mixed, incubated for 30 minutes and each mixture was then given intranasally in 0.05 cc amounts to 3 mice.

The results of the agglutination-inhibition test, presented in Table II, gave only an indication that the serum of the rabbit injected with mouse-egg line had a higher titer against the homologous than against the heterologous line. On the other hand, in the neutralization test in mice, each serum gave higher protection against its homologous line although the lethal dosage of mouse-lung virus employed was greater than that of the tissue culture-egg line.

Experiment II. Rabbit RM 12 received intraperitoneally 5.0 cc of allantoic fluid from the 20th egg passage of mouse-egg line. Rabbit RM 16 received intraperitoneally 5.0 cc of suspension of lungs of mice in the 634th passage. Rabbit RM 21 received intraperitoneally 5.0 cc of allantoic fluid from the 75th egg passage of the tissue culture-egg line. They were tested by agglutination-inhibition and neutralization in mice against a 10^{-1} dilution of virus in allantoic fluid from the 77th egg passage of the tissue culture-egg line and against a 10^{-2} dilution of fluid from the 11th egg passage of the mouse-egg line. These materials described in the second immunization test in mice had closely similar infectious titers in eggs and mice.

In this instance the agglutination-inhibiting test was again inconstant in pointing out differences between the lines. The sera of rabbits injected with the mouse-egg and the mouse materials gave much higher neutralization of the homologous mouse-egg line than of the tissue culture-egg line. The latter was neutralized to a higher titer by the homologous serum.

Time of Occurrence of Deviation. Since an obvious explanation of the observed differences is that a contamination of the PR8 strain or a substitution had occurred, tests were made with sera against strains which had been employed in this laboratory in recent years. No sharp differences in neu-

TABLE III.

Serological Tests with Sera Against Different Strains of Virus.

Date of Test 1/9/45.

Rabbit No.	Date of serum	Stage	Immunized with strain	Passage	Agglutination-inhibition test		Neutralization-in-mice	
					TC-Egg	Mouse-Egg	TC-Egg-Mouse	Mouse or Mouse-Egg
R 204	5/21/37	Imm.	Melbourne	217-7			<10	<10
RN 47	10/ 2/39	,,	W.S.				<20	<20
,, 64	4/ 5/41	,,	Baum	M-19			<20	<20
RM 5	3/22/44	Norm.			128	64	0	0
	4/ 1	Imm.	Weiss	M-32, E-11	128	512	15	5
,, 6	3/22	Norm.			128	128	0	0
	4/ 1	Imm.	Weiss	E-17	512	1024	12	5
R 215	5/ 3/37	Imm.	PR8	M-193			18	50
RN 5	1/18/39	Hyper-Imm.	PR8	M-356-360			240	>320
,, 56	10/14/40	Imm.	PR8	M-432			<20	60
RM 16	12/ 4/44	,,	PR8	M-634			35	280
12	10/27	,,	PR8	M-E-20			640	1600
21	12/ 4	,,	PR8	TC-E-75			280	30
RN 3	2/ 6/39	Hyper-Imm.	PR8	TC			80	30
,, 60	2/ 1/41	Hyper-Imm.	PR8	TC			120	50

Mouse titer of Mouse passage virus $10^{-6.5}$, 10^{-3} used. Mouse titer of Tissue Culture-Mouse virus $10^{-5.5}$, 10^{-3} used.

tralization tests with the 2 lines were observed with sera against Melbourne, W. S., Baum or Weiss strains. But when tests were made with sera prepared against PR8 mouse lungs as far back as 1937 or sera available against the tissue culture strain since 1939, it was observed that sera against the mouse line gave better neutralization against the homologous line even though comparatively more mouse virus than tissue culture-egg virus was used in the tests. Sera against the tissue culture line also showed higher titers with the homologous line. It was evident that the difference between the lines had been present for some time.

Tests with Human Sera. Both hemagglutination-inhibiting and mouse neutralization tests were done with sera of human individuals who underwent infection with influenza A in 1943 and of others who received influenza virus vaccine, which probably contained the tissue culture-egg line. The results demonstrated sharply that much higher titers were obtained against mouse line than against tissue culture-egg line with postvaccinal or convalescent sera in both tests while differences in the low titers of acute phase or prevaccinal sera were insignificant. The data clearly indicate that the mouse line was much more readily neutralized by immune sera of human individuals than was the tissue culture-egg line. Differences of 4- to 8-fold were seen in the inhibition tests and from 4- to 10-fold in the neutralization test. It is obvious that differences in serological behavior of this extent could seriously influence impressions gained as to the nature of an epidemic strain.

Discussion. The reported observations clearly indicate that variation in antigenic behavior took place in the line of the PR8 strain of influenza virus, Type A, maintained continuously for a period of years in tissue culture and eggs. It appears that the change from the mouse passage line occurred early in its history outside the mouse. It is interesting in this respect that from the earliest studies with the virus in tissue culture, indications were presented[3,4] that some difference between the virus so maintained and the parent strain existed; and it is likely that lack of refinement in procedures may have prevented an earlier clear-cut demonstration. The evidence supports the conclusion that the difference is not due to a replacement of one strain by another.

Burnet and Bull[5] have reported observations suggesting antigenic variation in the egg passage line of the Melbourne strain, and Stuart-Harris[6] has noted changes in titer obtained with sera tested against the PR8 strain after its adaptation to eggs.

The differences described in the present paper are sufficient to constitute an obstacle to proper evaluation of immunity induced by one line and tested with the other.

Summary. Evidence is presented that antigenic variation took place in a line of the PR8 strain of influenza virus propagated through continuous passage in tissue culture and eggs as compared with a line of the same strain maintained in mice.

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[3] Francis, T., Jr., and Magill, T. P., *Science*, 1935, **82**, 353.

[4] Magill, T. P., and Francis, T., Jr., *J. Exp. Med.*, 1936, **63**, 803.

[5] Burnet, F. M., and Bull, D. R., *Australian J. Exp. Biol. and Med. Sci.*, 1944, **22**, 173.

[6] Stuart-Harris, C. H., *Brit. J. Exp. Path.*, 1943, **24**, 33.