Further Studies on Serial Passage of a Mixture of Influenza Viruses in Embryonated Eggs.* (18435)

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It has been reported previously(1) from this laboratory that a mixture of the PR8 strain of influenza A virus and the Lee strain of influenza B virus was carried through 10 serial passages in embryonated eggs. In those experiments, passage was always made by the intra-allantoic inoculation of a 10^{-3} dilution of allantoic fluid, and in the fluids of all harvests both viral agents were present in sufficient concentration to agglutinate chicken erythrocytes. In the present report it will be shown that the PR8 and the Lee viruses can be maintained as a mixture through at least 52 egg to egg transfers. In these experiments passage was made by the inoculation of undiluted instead of diluted fluid and occasionally one or the other of the viruses was not detected by hemagglutination for as many as two consecutive passages, but, on the next passage, reappeared in sufficient concentration to cause hemagglutination in relatively high dilution.

Experimental. Allantoic fluids obtained from eggs inoculated 48 hours previously with either PR8 or Lee virus were combined and used as starting material. Passage was always made by inoculating 0.2 ml of undiluted allantoic fluid, to which penicillin had been added, into the allantoic sac of 2 or 3 eggs containing 10- to 12-day-old embryos. The inoculated eggs were incubated for 48 hours at 35°C, and were then chilled at 4°C for 4 to 24 hours before the allantoic fluids were harvested. The capacity of each fluid to agglutinate chicken erythrocytes in the presence of PR8 antiserum, Lee antiserum, and saline was determined. This procedure was conducted by a pattern test(2) in which equal volumes (0.2 ml) of serial 2-fold dilutions of virus suspension, antiserum or saline, and 0.5% chicken erythrocytes were combined and incubated at room temperature for approximately 1 hour. In addition, the fluids of the first 11 passages were tested in exactly the same manner against a 0.3% suspension of guinea pig erythrocytes. The latter tests were then discontinued since the results were essentially the same as those obtained with chicken cells.

Results. In Table I are shown the data obtained with the fluids used for the first 11 passages. It will be seen that there was considerable variation in the capacities of the different fluids to produce agglutination in the presence of the different antiserums. If that agglutination is an indication of the concentration of the heterologous virus particles, it is obvious that not only the concentration of the different viruses varied from passage to passage, but that the variation of the 2 agents was not always in the same direction so that the PR8/Lee ratio fluctuated over a wide range. This is well illustrated by a comparison of passages No. 2 and 3; in the first instance Lee was present in relatively high concentration whereas PR8 was barely detectable, but in the next passage (No. 3) the relationship was reversed. The data in Table I are representative of the results of the hemagglutination tests with all of the subsequent fluids; the agglutinin titers continued to rise and fall, and on several occasions one or the other of the viruses was not detected by this method during two consecutive passages, but on the next passage reappeared in sufficient concentration to produce hemagglutination in relative high dilution (*e.g.* passages 7-9). Both agents persisted through 52 egg to egg transfers, and the impression was obtained that the presence of one virus had little, if

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1. Sugg, J. Y., and Magill, T. P., *J. Bact.*, 1948, v56, 201.

2. Salk, J., *J. Immunol.*, 1944, v49, 87.

TABLE I. Examples of Agglutination of Chicken* Erythrocytes by Mixtures of Dilutions of Allantoic Fluids and Constant Amounts of Antiserum.

Passage No.	Antiserum	Results 2-fold dil. of allantoic fluid 1	2	3	4	5	6	7	8	9	10
0	PR8	++	++	++	++	++	++	++	+	0	0
	Lee	++	++	++	++	++	++	++	0	0	0
	Saline	++	++	++	++	++	++	++	++	0	0
1	PR8	++	++	++	++	++	++	++	0	0	0
	Lee	+	0	0	0	0	0	0	0	0	0
	Saline	++	++	++	++	++	++	++	++	0	0
2	PR8	++	++	++	++	++	++	++	0	0	0
	Lee	+	0	0	0	0	0	0	0	0	0
	Saline	++	++	++	++	++	++	0	0	0	0
3	PR8	0	0	0	0	0	0	0	0	0	0
	Lee	++	++	++	++	++	0	0	0	0	0
	Saline	++	++	++	++	++	++	0	0	0	0
4	PR8	++	++	+	0	0	0	0	0	0	0
	Lee	++	++	++	++	++	++	++	++	++	0
	Saline	++	++	++	++	++	++	++	++	++	+
5	PR8	++	++	++	++	++	0	0	0	0	0
	Lee	++	++	++	++	+	0	0	0	0	0
	Saline	++	++	++	++	++	++	++	0	0	0
6	PR8	++	++	++	++	++	++	++	++	0	0
	Lee	++	+	0	0	0	0	0	0	0	0
	Saline	++	++	++	++	++	++	++	++	++	0
7	PR8	++	++	++	++	++	++	++	0	0	0
	Lee	0	0	0	0	0	0	0	0	0	0
	Saline	++	++	++	++	++	++	++	0	0	0
8	PR8	+	0	0	0	0	0	0	0	0	0
	Lee	0	0	0	0	0	0	0	0	0	0
	Saline	+	0	0	0	0	0	0	0	0	0
9	PR8	0	0	0	0	0	0	0	0	0	0
	Lee	++	++	++	++	++	++	++	++	0	0
	Saline	++	++	++	++	++	++	++	++	0	0
10	PR8	++	++	+	0	0	0	0	0	0	0
	Lee	++	++	++	++	++	++	++	++	+	0
	Saline	++	++	++	++	++	++	++	++	++	+
11	PR8	++	++	++	++	++	++	+	0	0	0
	Lee	++	++	++	++	++	+	0	0	0	0
	Saline	++	++	++	++	++	++	++	++	0	0

++, +, and 0 complete, partial, and no agglutination, respectively.

* In parallel tests with 0.3% guinea pig RBC all the fluids gave essentially the same results as those presented for the chicken RBC.

any influence upon the multiplication of the other.

However, a rise in hemagglutinins is not always accompanied by a corresponding rise in infectivity when undiluted allantoic fluid is used as inoculum(3,4). Therefore, in order to obtain more information on the relative proportions of the two viruses, the egg-infectious titers were determined for a number of the dually infected fluids when combined with PR8 or with Lee antiserum. For these tests, equal volumes of serial 10-fold dilutions of the test fluids, prepared in infusion broth, and of a 1:15 dilution of antiserum were mixed, and sufficient penicillin was added to provide 100 units per ml. After the mixtures had been incubated 30 minutes at 37°C, 0.2 ml of each mixture was inoculated into the allantoic sac of each of 3 9- to 12-day-old embryonated eggs. The inoculated eggs were incubated for 48 to 72 hours at 35°C and were then tested for hemagglutinins; in the

3. Von Magnus, P., *Arch. f. kemi, mineralogi och geologi*, 1947, v24, 1.

4. Henle, W., and Henle, G., *J. Exp. Med.*, 1949, v90, 23.

TABLE II. Comparison of Egg-infectious Titer and Hemagglutinating Capacity of Different Allantoic Fluids When Mixed with PR8 or Lee Antiserum.

Allantoic fluid from passage No.	Antiserum added to test	Egg-infectious titer	Hemagglutinating capacity (chicken erythrocytes) 2-fold dil. of allantoic fluid									
			1	2	3	4	5	6	7	8	9	10
44	PR8	4.3*	0	0	0	0	0	0	0	0	0	0
	Lee	7.0	++	++	++	++	++	0	0	0	0	0
	Saline	†	++	++	++	++	++	+	0	0	0	0
45	PR8	6.3	++	++	++	++	+	0	0	0	0	0
	Lee	6.7	++	++	++	++	++	++	++	++	0	0
	Saline	†	++	++	++	++	++	++	++	++	++	0
46	PR8	5.1	++	++	++	++	++	++	+	0	0	0
	Lee	6.3	++	++	++	++	++	++	0	0	0	0
	Saline	†	++	++	++	++	++	++	++	++	0	0
47	PR8	2.5	++	++	++	++	++	+	0	0	0	0
	Lee	4.5	0	0	0	0	0	0	0	0	0	0
	Saline	†	++	++	++	++	++	++	0	0	0	0
48	PR8	4.8	0	0	0	0	0	0	0	0	0	0
	Lee	4.5	0	0	0	0	0	0	0	0	0	0
	Saline	†	0	0	0	0	0	0	0	0	0	0
49	PR8	4.5	++	++	++	++	++	++	0	0	0	0
	Lee	8.5	++	++	++	++	++	++	++	+	0	0
	Saline	†	++	++	++	++	++	++	++	++	++	0
50	PR8	3.0	++	++	++	++	++	0	0	0	0	0
	Lee	6.8	++	++	++	++	++	++	++	+	0	0
	Saline	†	++	++	++	++	++	++	++	++	++	0
51	PR8	3.0	0	0	0	0	0	0	0	0	0	0
	Lee	5.3	++	++	++	++	++	++	0	0	0	0
	Saline	†	++	++	++	++	++	++	++	+	0	0
52	PR8	6.8	++	++	++	++	++	++	++	0	0	0
	Lee	5.5	++	++	++	++	++	0	0	0	0	0
	Saline	†	++	++	++	++	++	++	++	++	0	0

* Reciprocal of the log of 50% end point.
† No test.

absence of agglutination, the eggs were considered free from infection. The titers are expressed as the reciprocal of the log of the 50 per cent end point as determined by the method of Reed and Muench(5).

The data in Table II show that the PR8/Lee ratios differed greatly in the various fluids when determined by egg-infectious titer, as well as when determined by hemagglutination. However, the results obtained by the two tests did not always correspond. For example, in passage 47 the infectivity tests indicated a higher concentration of PR8 than of Lee, whereas the hemagglutination tests indicated the opposite relationship. But the ratio of the 2 viruses in the inoculum, as determined by either method, was not an indication of the relative concentrations of the agents that would be present in the harvested allantoic fluids. This is particularly well illustrated by a comparison of passages 50 and 52. In the former instance, both tests indicated a higher concentration of PR8 than of Lee in the inoculum (passage 49), and the same relationship was found in the harvest (passage 50); whereas in the latter instance, both tests again showed a higher PR8/Lee ratio in the inoculum (passage 51), but there was a definitely lower concentration of PR8 than of Lee in the harvested fluid (passage 52). It also will be seen that in some passages the drop in virus activity was greater than could be accounted for on the basis of dilution alone, but whether or not either agent failed to multiply during any one passage cannot be determined with certainty from the present data. Nevertheless, sufficient multiplication did occur to maintain the mixture through 52 consecutive egg passages, and at the end of

5. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

the experimental period each agent was recovered in an apparently pure culture from eggs that had been inoculated with the dually infected allantoic fluid plus either PR8 or Lee antiserum.

Discussion. The results of previous experiments(1) had indicated that a mixture of the PR8 strain of influenza A virus and the Lee strain of influenza B virus could be maintained indefinitely by serial passage in embryonated eggs, and the present report has provided additional evidence in support of that conclusion. This finding is in contrast to what might be expected. In the chick embryo PR8 has a faster growth rate than Lee(6) and under proper conditions the two viruses reciprocally interfere with the multiplication of each other(7), and it might be expected that Lee would have been eliminated on serial passage. Hence, the continued persistence of the two viruses as a mixture raises the question as to whether or not the same host metabolic systems that are required for the multiplication of PR8 are likewise required for the multiplication of Lee. In that connection it is significant that the propagation of the mixture has been accomplished by the use of diluted(1) as well as undiluted allantoic fluids. It is well known that the latter method does not produce maximum virus multiplication, and, although this did not alter the final results (*i.e.*, the successful passage of the mixture), it may have been responsible in large part for the pronounced differences observed among the PR8/Lee ratios of the various fluids.

Recent observations(3,4,8) indicate that if high concentrations of influenza virus are used as inocula, one can obtain what appear to be "immature" virus particles that are noninfectious, but that are capable of agglutinating erythrocytes and of interfering with multiplication of active virus. In all probability the concentrations of both PR8 and Lee particles of that sort varied greatly from passage to passage: when the concentration was high there was little if any multiplication of the homologous virus, but when the concentration was low (due to dilution on additional passage) the virus again multiplied to high titer, and a new cycle was started. But, in that event, the "immature" PR8 and Lee particles must have been more effective in interfering with the multiplication of one (presumably homologous) virus than the other, since it is obvious that the rise and fall in the concentrations of the two viruses did not always parallel, whether based on hemagglutination or on egg-infectious titer.

Summary. Undiluted allantoic fluids were used as inocula for serial passage of a mixture of the PR8 and Lee strains of influenza virus in embryonated eggs. Although wide differences were found in the relative concentrations of the two viruses in the various fluids, the mixture was successfully carried through 52 consecutive passages, and each agent was recovered in an apparently pure culture at the end of the experimental period. Since the two viruses have different growth rates and are capable of interfering with the multiplication of each other, the persistence of both agents during serial passage of the mixture suggests that multiplication of the PR8 and of the Lee viruses may not require the same host factors.

6. Henle, W., Henle, G., and Rosenberg, E. B., *J. Exp. Med.*, 1947, v86, 423.

7. Ziegler, J. E., Jr., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1944, v79, 361.

8. Bernkopf, H., *J. Immunol.*, 1950, v65, 571.

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