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Antigenic Similarity Between the First and Later Egg Passage Strains of Freshly Recovered Influenza A Viruses. (19788)

IGOR TAMM, HAROLD S. GINSBERG, AND FRANK L. HORSFALL, JR.
From the Hospital of The Rockefeller Institute for Medical Research, New York City.

Analysis of the significance of antigenic variation of influenza viruses in man depends in part on the validity of the assumption that the antigenic properties of strains recovered in the laboratory in unnatural hosts correspond to those of the agents infecting human beings. From this point of view, the use of mice for the recovery or serial passage of influenza viruses appears to be highly undesirable for it is known(1,2) that these agents may show unpredictable changes in antigenic composition when maintained in this host. On the other hand, there is evidence(3) that recently recovered influenza virus strains may be maintained for 6-12 serial passages in the chick embryo without undergoing antigenic variation. The possibility that a change in antigenic composition may occur immediately after recovery during the first few passages in the chick embryo has not been investigated previously.

The present study was undertaken in order to determine whether, after serial embryo passage, influenza virus strains are antigenically similar to or different from the same strains in the 1st egg passage on recovery from throat washings.

Materials and methods. Recovery and serial passage of viruses. During the 1951 epidemic in New York, a number of influenza A virus strains were recovered by inoculation of chick embryos in this laboratory. On the basis of the extent of multiplication obtained during the 1st embryo passage, throat washings from 2 patients (NA and RA) with influenza A were chosen for further study. On 4 different attempts with each throat washing, infection was induced in a considerable proportion of embryos inoculated. The strains of each virus used in the experiments described below were obtained as follows:

NA. 10-day-old chick embryos were inoculated amniotically with 0.2 ml of throat washings from patient NA which contained penicillin, 250 μ, and streptomycin, 2500 μg/ml. After incubation at 35°C for 70 hours, followed by chilling at 4°C overnight, allantoic and amniotic fluids were harvested separately. Agglutination tests with 1% guinea pig RBC (0.5% final) were carried out with fluids from individual eggs employing a final dilution of 1:4. Of 57 amniotic fluids, 12 were positive. All allantoic fluids from these eggs were negative. The hemagglutination titer of the pool of positive amniotic fluids at 24.5°C was 1:64 with guinea pig RBC and 1:8 with chicken RBC. For the preparation of antisera, the pool of positive amniotic fluids was diluted with 5 volumes of amniotic fluids from the same 1st passage eggs which had given negative hemagglutination reactions. 7 serial allantoic passages were carried out in 10- to 11-day chick embryos, starting with a 10^{-1} dilution of the 1st passage amniotic fluid pool and continuing with a 10^{-3} dilution of allantoic fluid at each subsequent passage. Eggs were incubated at 35°C for 48 hours, chilled at 4°C overnight and the allantoic fluids were harvested, pooled, and stored at –65°C.

RA. Primary recovery of this strain from

the throat washing of patient RA was carried out in the allantoic sac. The 1st passage strain was prepared in 2 batches which then were pooled. The 1st batch was obtained by inoculating 28 chick embryos intra-allantoically with 0.2 ml of throat washing containing penicillin and streptomycin as above. After incubation at 35°C for 96 hours, allantoic fluids from 8 embryos showed a positive hemagglutination reaction at a final dilution of 1:4 with guinea pig RBC. With the 2nd batch, allantoic fluids from 12 of 72 embryos handled in an identical manner were similarly positive. Only the positive allantoic fluids from the 2 batches were pooled. Hemagglutination titrations carried out at 24.5°C with the positive fluids from each batch showed titers of 1:64 with guinea pig RBC and 1:16 with chicken RBC. 4 serial allantoic passages then were made in the manner described above.

Virus titrations. Hemagglutination and infectivity titrations *in ovo* were carried out by technics identical with those described previously(4,5).

Immune sera. Roosters and rabbits were injected intravenously with 10 ml of infected amniotic or allantoic fluid pools. Two animals were used for each virus preparation. Roosters were bled 26 days later, and a 2nd intravenous injection of virus was then given. The final bleeding was obtained 46 days after the 1st injection. Rabbits were bled 21 days after the 1st injection.

Antibody titrations. Hemagglutination-inhibition and *in ovo* neutralization titrations were carried out with 2-fold dilutions of inactivated serum by the technics described previously(5). Approximately 1,000 EI_{50} of virus was employed in each of the neutralization tests. Serum antibody titer ratios for heterologous sera were computed as described in a prior paper(3). In computing such ratios, the reciprocals of the titers were used.

Experimental. Identification of NA and RA strains. Hemagglutination-inhibition titrations with the 2 strains were performed with rabbit antisera against various influenza viruses. Guinea pig RBC were employed. The sera were heated at 56°C for 30 minutes, absorbed with 10% guinea pig RBC and treated with PR8 or Lee virus at 42°C for 3 hours as described previously(6) to remove nonspecific inhibitor. The treated sera were then heated at 65°C for 30 minutes to inactivate the added virus. This procedure yielded sera which caused neither agglutination of guinea pig RBC nor nonspecific inhibition of influenza virus hemagglutination.

TABLE I. Identification of NA and RA Strains by the Hemagglutination-Inhibition Procedure.

Rabbit serum vs.	Hemagglutination-inhibition titer* Virus strain NA†	RA‡
Normal	<16	<16
PR8	<16	<16
FM1	512	512
MA§	256	256
Lee, MB‖, 1233	<16	<16

* Titer expressed as reciprocal of dilution at end point.
† Infected allantoic fluid, 2nd passage from throat washing.
‡ Infected allantoic fluid, 1st passage from throat washing.
§ 1950 influenza A virus strain.
‖ " " B " " (6).

The results of hemagglutination-inhibition titrations with the 2 strains at the 1st or 2nd embryo passage are given in Table I. As can be seen, both the NA and RA strains were inhibited by rabbit antisera against FM1 (1947) and MA (1950) influenza A virus strains, but not by antiserum against the PR8 strain. Antisera against influenza B and C (strain 1233) viruses showed no inhibitory effect.

Hemagglutination-inhibiting antibodies against the homologous and a number of heterologous influenza virus strains were measured in the acute and convalescent sera of the patients from whom the NA and RA strains were recovered. Guinea pig RBC were used. The sera were diluted with equal volumes of buffered saline and heated at 65°C for 30 minutes. Table II summarizes the results of these antibody titrations. Both patients showed a rise in antibodies against their own strain as well as against FM1 and PR8, but not against Lee or 1233.

Changes in titer of NA and RA strains during early passages. The titers of the NA and RA strains during early passages in the chick embryo were followed in terms of infectivity *in ovo* and hemagglutinating capacity with

TABLE II. Hemagglutination-Inhibiting Antibody Rise in Patients from Whom NA and RA Strains Were Recovered.

Patient's serum		Hemagglutination-inhibition titer (Virus strain) NA	RA	MA	FM1	PR8	Lee	1233
NA	Acute	<16	<16	<16	32	64	32	256
	Convalescent	32	<16	<16	256	128	32	256
RA	Acute	<16	<16	<16	16	32	32	256
	Convalescent	64	64	64	256	128	32	256

Guinea pig RBC were used except with 1233 in which case chicken RBC were used. 8 units final of each virus was employed.

TABLE III. Increase in Infectivity Titers *In Ovo* of NA and RA Strains During Early Passage.

Virus strain	Embryo passage No. Amniotic sac	Allantoic sac	Infectivity titer* EI_{50}, log
NA	1		−5.3
	1	1	−8
	1	7	−7.7
RA		1	−5.5
		2	−6.3
		5	−7.7

* Geometric mean of 2 titrations.

both guinea pig and chicken RBC. Table III summarizes the results of infectivity titrations carried out in the allantoic sac of embryonated eggs. The NA strain reached maximal infectivity for the chicken embryo after 1 amniotic and 1 allantoic passage whereas the RA strain, which was recovered directly in the allantoic sac, did not show a similar high titer at the 2nd allantoic passage but did so at the 5th passage. Table IV gives the results of hemagglutination titrations. The effect of temperature on the chicken (F)-guinea pig (G) RBC agglutination ratio(7,8), determined with 1st passage fluids, was different for the 2 strains: the 1st amniotic passage NA strain showed a low F/G ratio at both 24.5 and 4.5°C, whereas the 1st allantoic passage RA strain showed a low ratio at 24.5 but not at 4.5°C. It is apparent that as early as the 2nd passage both the NA and RA strains showed high F/G ratios at both 24.5 and 4.5°C.

Antigenic similarity during early passages. Table V summarizes the results of complete cross-neutralization tests *in ovo* with 1st and later passage materials containing the NA or RA strain and corresponding immune sera. Several features require comment. In cross-neutralization tests with the NA strain, sera were used from roosters which had received 2 intravenous injections of virus. After only 1 injection of virus, the antiserum against the 1st passage amniotic strain had a neutralizing titer of but 1:2. This probably was due to the low concentration of virus in the 1st passage amniotic fluid. Cross-neutralization tests with the RA strain and either rooster or rabbit antisera were carried out with serum obtained after but 1 intravenous injection of virus. It is noteworthy that in all cross-neutralization tests 1st passage NA or RA strain appeared to be neutralized by a smaller quantity of antibody than was required with the same strain at a later passage.

As has been shown previously(3,9), the antigenic relation between 2 strains can be expressed in a single figure. To obtain this value, it is necessary to determine the 2 heterologous serum titer ratios from the results of a complete cross-serological experiment. The geometric mean (R) of these 2 ratios may be taken as the best available indication of the extent of the antigenic relation between the strains. R values, computed in this manner, are shown for the data presented in Table V. The fact that each of the R values for both the NA and RA strains closely approximates 1.0 is strong evidence that no definite difference in antigenic composition occurred between the 1st and later embryo passages.

Discussion. The fact that no significant antigenic variation was demonstrable between influenza A virus strains at the 1st and in later passages in the chick embryo is of considerable interest because it provides support for the assumption that strains recovered and passed in the chick embryo are comparable in antigenic composition to those causing dis-

TABLE IV. Changes in Hemagglutinating Capacity of NA and RA Strains During Early Passages.

Virus strain	Embryo passage No.		Hemagglutination titers			
			Temperature			
			24.5°C		4.5°C	
	Amniotic sac	Allantoic sac	Erythrocytes			
			Guinea pig	Chicken	Guinea pig	Chicken
NA	1		64	8	64	16
	1	1	512	256	512	1024
	1	2	512	256	512	512
	1	4	512	256	512	512
	1	7	1024	512	512	1024
RA		1	128	32	128	256
		2	512	512	512	1024
		3	2048	1024	1024	4096
		5	2048	1024	1024	4096

TABLE V. Cross-Neutralization Tests with the NA and RA Strains and Corresponding Immune Sera after 1st and Later Embryo Passages.

Immune serum vs.	Virus strain				R value†
	NA, 1	NA, 8	NA, 1	NA, 8	
	Serum neutralizing titer*		Serum titer ratio		
NA, 1‡	*76*	16	*1*	1/4.7	1/1.58
NA, 8	1000	*523*	1.9	*1*	
	RA, 1	RA, 5	RA, 1	RA, 5	
RA, 1§	*28*	19	*1*	1/1.4	1.07
RA, 5	27	*16*	1.7	*1*	
RA, 1‖	*860*	364	*1*	1/2.3	1/1.08
RA, 5	1450	*726*	2	*1*	

* Each titer represents the geometric mean of 2 titration end points.

† Computed from the 2 heterologous serum titer ratios as follows: $R = \sqrt{r_1 \times r_2}$ (3).

‡ NA, 1 = Rooster serum, 46-day, vs. infected amniotic fl, 1st passage from throat washing.
NA, 8 = " " " " " allantoic 8th " in chick embryo.

§ RA, 1 = " " 26-day, " " " 1st " from throat washing.
RA, 5 = " " " " " " 5th " in chick embryo.

‖ RA, 1 = Rabbit " 21-day, " " " 1st " from throat washing.
RA, 5 = " " " " " " 5th " in chick embryo.

ease in man. It has been demonstrated previously(3,6,9-15) that strains of influenza viruses recovered from patients in different epidemics show evidence of antigenic variation of considerable degree even though maintained exclusively in the chick embryo. It appears now that such variation is not to be attributed to unpredictable factors leading to the selection of variant strains in the chick embryo but instead is due to factors operative during the transmission of influenza viruses in man.

In contrast to the lack of variation in respect to antigenic properties, the reactivity of influenza A virus relative to guinea pig and chicken erythrocytes undergoes a profound and rapid change on passage in the chick embryo as has been emphasized previously (7,8). This alteration may occur as early as the 2nd embryo passage and persists thereafter. In addition, the results of the neutralization tests raise the possibility that the ratio between antibody and virus at the end point may undergo a change as a result of serial passage in the chick embryo. On the average, after 5-8 passages, it was necessary to use approximately twice as much antibody to cause virus neutralization as was required with the same strains after but 1 passage in the chick embryo. Because of the extraordinarily steep slope of the neutralization line *in ovo*(5), it would have been necessary to use consistently about 50 times more later passage than 1st passage virus in order to obtain the ratio differences noted. It should be emphasized that the quantities of 1st and

later passage virus used in each of the neutralization tests were closely similar.

Summary. Cross-neutralization experiments *in ovo* with 1st and later chick embryo passage strains of freshly recovered influenza A viruses showed no definite difference in their antigenic composition. However, the reactivity relative to guinea pig and chicken erythrocytes underwent a rapid and profound change between the 1st and 2nd embryo passages.

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Effect of Relaxin Upon Decidual Reactions in the Rat.* (19789)

EDWARD H. FRIEDEN† AND JOSEPH T. VELARDO. (Introduced by F. L. Hisaw.)
From the Biological Laboratories, Harvard University, Cambridge, Mass.

The most important physiological actions known for relaxin are displayed in effects upon connective tissue(1-5). The range of such effects has been broadened by recent observations(6) that relaxin preparations possess the property of inhibiting the extensive modification of stromal connective tissue which accompanies the development of a deciduoma in the rat uterus. Quantitative comparison of responses to a series of relaxin preparations of widely different specific activities, experiments with relaxin from the blood of pregnant rabbits, and studies of the effects of chemical modification of relaxin all indicate that inhibition of decidual growth in rats is a characteristic property of this hormone. The data also show that some relaxin preparations contain a substance, clearly distinguishable from relaxin (and from the decidual inhibitor), which enhances the action of progesterone in the development of deciduomata.

Experimental. Quantitative study of factors affecting decidual growth requires a rigidly standardized means of producing deciduomata of uniform size. We have routinely employed 100-day-old estrous rats, averaging 200 g in weight, which are made pseudopregnant by electrical stimulation of the uterine cervix. On the fifth day of pseudopregnancy, they are ovariectomized, and the right horn of the uterus traumatized by a needle scratch along its entire length. Daily injections of progesterone (1.5 mg in 0.1 ml sesame oil) are given for 3 days, and the animals autopsied on the fourth.

In some of the earlier experiments, gross comparison of the 2 horns served as a qualitative indication of the response. Subsequently, in order to obtain data which would be susceptible of quantitative comparison, the 2

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† Present address: Department of Biochemistry and Nutrition, Tufts College Medical School, Boston, Mass.