Experimental Production of Combination Forms of Virus.* IV. Mixed Influenza A-Newcastle Disease Virus Infections. (21016)

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Previous papers from this laboratory(1,2) have established the fact that when two strains of influenza virus (A-A or A-B) are inoculated together into the allantoic sac, some of the progeny therefrom may have antigenic characteristics of both parent types. This doubly antigenic virus was called X virus and was recognizable by the fact that it reacted with specific antisera of both parent types in the hemagglutination inhibition (HAI) and neutralization tests. The fact that two antigenically unrelated viruses could participate in forming the coat of single particles led us to study combined Influenza A-Newcastle Disease Virus infections. The following account shows that these two strains can contribute to the antigenic make-up of single particles.

Materials and methods. The Melbourne strain of influenza A and the Beaudette strain of Newcastle Disease Virus were used throughout this study and are referred to as M and N. All antisera were prepared in rabbits and before use in HAI tests were treated with RDE and absorbed with Lee virus(3) until non-specific inhibitors were completely removed. Untreated sera were used for *in ovo* neutralization tests. In all HA (hemagglutination) and HAI tests, the virus used was first treated with sodium periodate which had a stabilizing effect on the NDV hemagglutinin and this was essential in order to get valid and reproducible results with this virus. To 0.5 ml of infected allantoic fluid was added 0.1 ml of M/10 $NaIO_4$. After 30 minutes at room temperature, 0.1 ml of 40% glucose was added. This treatment was applied routinely to all fluids, many of which contained mixtures of M and N. It did not lower the HA titer of either agent. HA and HAI tests were carried out by the pattern method(1). Virus titrations *in ovo* were usually done with 3-fold, sometimes 10-fold, dilution steps, and 10 eggs per dilution. Incubation for 40 hours was followed by testing the allantoic fluids for HA after treating them with periodate.

Search for mixed antigenic particles with the HAI test. The M and N strains were found to have very similar growth rates in the allantoic cavity (roughly 30 hours for a maximum HA yield from 1 ID_{50}) and, hence, there was no consistent tendency for one particle to outgrow the other in mixed infections. Two sets of experiments were carried out initially, both with mixed inocula. In the first, the number of particles injected gave a particle/host cell ratio of more than one, while in the second experiment the input was small, about one ID_{50} per egg. 1) For the large inoculum, both viruses were concentrated 100 times from allantoic fluid by centrifugation. Each concentrate was diluted in 10-fold steps and various M-N pairs of these dilutions were made by combining equal volumes and these mixtures were inoculated into 5 chick embryos each. After 24 hours' incubation at 37°C, the fluids were harvested and tested for the type of the predominating virus by means of the HAI test. 2) Mixed infections were also carried out with dilute inocula. Each virus was diluted by twofold steps to the point where each 0.1 ml contained about one ID_{50} and, again, various combinations of dilutions were made and inoculated into eggs. After 40 hours' incubation, the fluids were harvested and tested for the predominant serotype by the HAI test. The results are shown in Table I.

In general, the virus type which predominated in the inoculum also predominated in the final yield, whether the total amount injected was large or small. However, among the eggs that received nearly equal amounts of the two infecting strains, were a number that yielded fluids containing HA that was not inhibited by either parent antiserum. This was very different from what has been obtained with

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TABLE I. Simultaneous Inoculation of Allantoic Sac with an Influenza A and a Newcastle Disease Virus.

Exp. No.	Inoculum total ID_{50}, log		Ratio ID_{50} inoculated, Mel/NDV	Predominant virus type in fluid after incubation				
	Mel	NDV						
1	5.95	8.53	1/400	N	N	N	N	
	6.95	8.53	1/40	N	N	N	++	++
	7.95	8.53	1/4	N	++	++	++	++
	8.95	8.53	3/1	M	M	M	M	M
	8.95	7.53	30/1	M	M	M	M	
2	.3	1.3	1/10	M	N	N	N	N
	.5	1.3	1/4	M	N	N	++	
	1.0	1.3	1/2	M	++	++		
	1.3	1.3	1/1	M	M	M	M	++
	1.3	1.0	2/1	M	M	M	M	++
	1.3	.5	4/1	M	M	M	N	
	1.3	.3	10/1	M	M	M	M	

Both viruses were inoculated together in 0.1 ml. After 24 (Exp. 1) and 40 (Exp. 2) hours of incubation, allantoic fluids were removed and tested for the predominating type of virus by means of the HAI test, in which 4 HA units were tested against a 1/64 dilution of NDV and Mel antiserum. M indicates predominance of the Melbourne strain, N of Newcastle Disease Virus, and ++ indicates that the two agents were present in equal (HA) or nearly equal titer.

A-A and A-B influenza virus infections, which yielded fluids containing an HA that was inhibited by both parent type antisera. This behavior (no inhibition) is what one could expect from a simple mixture of equal amounts of the 2 parent viruses and this similarity was confirmed by further tests. HA titrations on these fluids done in anti-M and anti-N sera showed that the M and N HA titers were equal and that added together they equalled the total HA titer. There was no titer discrepancy in these fluids and, hence, no evidence of the presence of doubly antigenic or X virus.

Some of these fluids in which the HA titers were equal for M and N (++ fluids) were passed to other eggs in an attempt to pick out an X form. When fluids from individual eggs in titrations were tested for the predominant serotype, ++ fluids were found in eggs that received low as well as high dilutions. It was not necessary to use a large inoculum to perpetuate mixed infections and ++ fluids were found in a number of passages, even when the inoculum contained just a few ID_{50} of virus. A few characteristic passages are shown in Table II. It appeared that M and N could be readily propagated together indefinitely.

The results up to this point failed to give any indication of the occurrence of virus particles having mixed NDV-influenza A antigens, but the evidence showed that in carrying the 2 together equivalent yields of the 2 viruses from single eggs were not uncommon. Previously discovered X forms showed their mixed character by *in ovo* as well as by *in vitro* tests and for this reason a number of ++ fluids were examined for the ability of the virus to be neutralized by both parent antisera.

Demonstration of X virus with in ovo neutralization. A number of ++ fluids from various passages of the M-N combination were tested for neutralization by M and N sera. Each fluid was diluted in threefold steps, and normal and M and N immune sera (1/32) were each added to one set of dilutions. The results are shown in Table III. Fluids 7 and 8 were controls which consisted of artificial mixtures of the M and N strains. The number of ID_{50} of M and of N virus failed to add up exactly to the total number of ID_{50} in each fluid, but the differences were small and could be attributed to the inherent errors of *in ovo* titration.

Some of the ++ fluids showed no significant titer discrepancy(1,2) by these methods but at least half of those tested showed double neutralization of such a degree that there was no doubt that quite a high proportion of particles with a mixed antigenic character were present. Fluid No. 6, for example, showed a

TABLE II. Serial Passage of Influenza A and NDV Together in the Allantoic Sac.

Dilution of AF inoculated, log	Passage No. 1			4				7			11		
0	++	++	++	M	+++	+++	+++	++	++	++	++		
-1				N	+++	+++	+++	++	++	++	++		
-2				++	++	+++	+++	++					
-6	M	++			+++	+++		M	M	++	N	++	++
-7	M	N	++	M	++	++	++	M	++	++	N	N	N
-8	M	N	N	M	M	N	N	M	N	N	N	N	
-9				M	N			M	N		M		

Fluid from individual eggs was tested for predominant virus type as in Table I. M indicates predominance of Melbourne strain, N of Newcastle Disease Virus, and ++ indicates that the two agents were present in equal or nearly equal titer. Transfer from one passage to another was done with ++ material from a 10^{-6} dilution or higher.

reduction in titer of log 1.2 with both antisera. This could hardly have been due to chance since a difference of log 0.6 is ordinarily considered significant, even when the dilutions are made in 10-fold steps. Doubly neutralizable fluids were obtained from a number of different passages. Since these sera were quite specific, it is felt that these results provide excellent grounds for the assumption that doubly antigenic influenza-NDV particles were present.

Inability of M-N Type X particles to initiate mixed infections. The occurrence of virus particles with surface antigens from 2 parents raised the question of whether or not such particles might not individually give rise to both parent serotypes. This question had already been investigated with A-B X forms by a technic which indicated that those particles were of a single parental genotype(2). The test was applied to M-N type X particles with the same result. The primary step, as with other virus combinations, was to determine the frequency of mixed infections in individual eggs after the inoculation of limiting infectious dilutions of *in vitro* mixtures of M and N viruses. These 2 strains were mixed in equal amounts (in terms of ID_{50}) and 2-fold dilutions of the mixture were made between 10^{-8} and 10^{-10}. Forty eggs were inoculated with each dilution. After incubation, all of the fluids which contained detectable amounts of HA were tested for the predominant serotype by the HAI test. An attempt to isolate a second type was made by inoculating the fluids into one or 2 eggs in the presence of antiserum against the predominant strain. The number of mixed infections found is shown in Table IV and in Fig. 1.

In Fig. 1, the dotted line shows the average frequency of mixed infection from 4 previous similar tests with A-A and A-B combinations (2). It will be seen that the frequency of mixed infection with artificial mixtures of M and N was a little less but of the same order of magnitude. It is fairly certain, therefore, that mixed infection was a chance event, dependent on the accidental inclusion of 2 particles of opposite serotype in one inoculum.

A single X fluid of the M-N type was selected for further study. This fluid contained

TABLE III. *In Ovo* Infectivity Titrations on ++ Fluids Done in Presence of Normal and Immune Antisera.

Fluid No.	Passage No.	Dilution of fluid used for infecting egg	ID_{50} of virus tested in presence of serum: Normal	Anti-M	Anti-N	% of virus neutralized by both sera (X virus)
1	1	−8	9.35	9.18	8.63	13
2	6	−6	8.38	8.20	7.66	10
3	4	0	9.07	8.64	7.55	60
4	1	−7	9.75	9.06	8.79	69
5	10	−5	9.44	8.75	8.65	74
6	8	−8	9.64	8.42	8.38	89
7	Control mixture		9.85	9.50	9.11	37
8	" "		9.91	9.54	9.50	19

Fluids were all taken from experiments like those shown in Table II. All were ++ in type. Infectivity titrations were done *in ovo* using 3-fold dilutions and 10 eggs per dilution. Second and third columns of titrations above are the N and M titers respectively. These were added together and subtracted from the first column, the remainder being titer deficiency indicated the amount of virus neutralized by both sera (X virus).

74% X virus as determined by *in ovo* titration and it was inoculated into eggs at high dilution. The number of mixed infections which were found in individual eggs is shown in Table IV and Fig. 1. The ability of these X particles to give rise to mixed infection was as low as the control mixtures and, hence, there is no reason to assume that they possessed intrinsic capability of inducing mixed infection.

Discussion. The foregoing results may be summarized by saying that they are essentially the same as those found with mixed A-B infections, the only exception being that the mixed antigenic character of the M-N X form could be demonstrated by neutralization only and not by hemagglutination inhibition. This is clearly a minor difference. X virus has now been demonstrated with numerous A-A, several A-B, and a single A-NDV combination. The difficulties of demonstrating X virus by the *in vitro* test, increased in that order, and the HAI test failed to reveal these forms with A-NDV combinations. Yet X virus was

TABLE IV. Yield of Single and Mixed Infections in Individual Eggs Inoculated with Small Amounts of Virus.

Exp. No.	Inoculum	Dilution of inoculum	No. of eggs inoculated	No. of eggs infected	No. eggs infected with M and N	% of eggs infected	% infected eggs mixedly infected
1	M and N viruses, artificially mixed in ratio 1/3	8.0	39	39	28	100	72
		8.3	38	31	6	82	19
		8.6	40	18	3	45	17
		8.9	39	17	0	44	0
		9.2	40	7	0	17	0
		9.5	40	2	0	5	0
		9.8	39	0	0	0	0
2	M and N viruses, artificially mixed in ratio 1/2	8.6	40	34	15	85	44
		8.9	40	16	5	40	31
		9.2	40	12	0	30	0
		9.5	40	10	0	25	0
3	X fluid M-N type from passage 10. 74% X virus by neutralization	8.0	39	26	2	67	8
		8.3	40	17	2	42	12
		8.6	40	6	0	15	0
		8.9	40	3	0	7	0
		9.2	40	0	0	0	0

Each infected egg was tested for the predominant serotype by the HAI test and the fluid was then inoculated into 2 eggs in the presence of a serum to suppress the dominant organism, in order to find a second type if present. Infectivity titers for each virus were calculated separately in Exp. 1 and 2 and these were used to determine the ratios M/N.

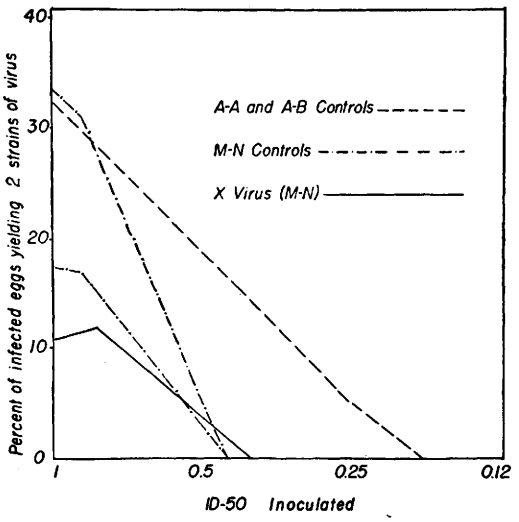

FIG. 1. Effect of number of ID_{50} inoculated on % of infected eggs that yielded 2 strains.

readily demonstrated in all 3 types of mixtures by the *in ovo* neutralization test.

In several previous communications from this laboratory, it was maintained that the occurrence of X_1 virus could best be explained by the phenomenon of phenotypic mixing(4, 5,2). Burnet, *et al.* alluded to X virus (A-A type) as a kind of recombinant(6) but later on discarded this idea in favor of heterozygosis, diploidy, or some type of doublet formation as alternate explanations(7). It is a confusing fact that with A-A infections, X particles and particles heterozygous for antigenic type are found in the same eggs(2). This would lead one to suspect that the two phenomena were connected were it not for the absence of heterozygosis from A-B and A-NDV infections.

The belief that a mixed antigenic condition of the virus surface is due to phenotypic mixing was suggested and supported by phage experimentation(8,9), in which it was shown that the combination of 2 kinds of genotypes with several tail antigens was an almost random process. Luria, in summarizing this work from his laboratory, suggested that one of the implications of phenotypic mixing was a 2-stage process of phage maturation, the first a synthesis of specific materials followed by a less specific assembly of these substances into finished particles(10). He suggested that the phage DNA in the infected cell may induce the formation of specific antigens while in an extended state. The assembly of phage antigens around the condensed phage DNA cores would then follow as a separate step.

Summary. Mixed infection with the viruses of influenza A and Newcastle disease virus was found to result in the formation of some particles which had antigenically mixed surfaces, *i.e.,* which contained components from both parent types. The phenotypic change was transitory and did not appear in the progeny of such a particle. It is suggested that this phenomenon is due to phenotypic mixing.

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