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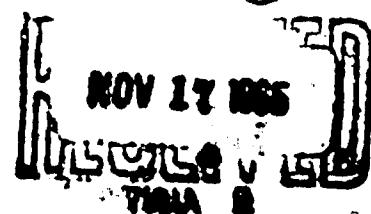
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ABSTRACT

The principal development during the past year has been the production of a variety of "antigenic hybrids" - influenza virus recombinants that can be shown to possess antigens typical of both parents. Such hybrids may be produced by matings among any of the human influenza A subtypes and by matings of swine influenza virus and an A strain. It has also been shown that the proportion of parental antigens of such recombinants may vary and that mating of recombinant virus with the parent that contributed the minor antigenic component may increase the recognizable amount of such component in subsequent progeny. Antigenically hybrid viruses are not heterozygote and they are stable and they can be used to infect mice, in which they produce at least partial immunity to both parental viruses. The implications for immunization of man are clear. It also appears possible that the reshuffling of demonstrable antigens in viruses by recombination may lead to the production of antigenically novel viruses - particularly if it is assumed that all antigens in differing amounts are present in all human influenza A strains. Evidence of this is provided by the induction of heterotypic immunity in mice by a single infection.

In our epidemiologic model of influenza virus infection of the mouse the major developments have been 1) reduction in susceptibility to transmitted infection by the induction of endogenous interferon in mice by intravenous NDV 2) actual demonstration of airborne virus in the vicinity of infector mice and 3) an analysis of the viral factors responsible for transmissibility by the use of recombinant viruses with defined genetic traits.

GENETIC STUDIES OF INFLUENZA VIRUSES

I. FURTHER STUDIES OF ANTIGENIC HYBRIDIZATION

A. The range of antigenic hybridization among human influenza A virus subtypes.

It was reported last year that definite evidence of recombination of NWS (A) and RI/5⁺ (A2) viruses has been found by two separate techniques of antigenic analysis - by viral specific complement fixation and by a new method developed in this laboratory of plaque size reduction or PSR. Such AA2 recombinants had been derived in two separate experiments so that it was suspected that such antigenic hybridization might be a general phenomenon. This impression has since been substantiated and we now have in hand recombinants that are AA1, A1A2, or SwA in antigenic structure as ascertained by the plaque size reduction (PSR) method. Thus, there seems to be no doubt of the potentiality of the human subtype strains to derive parental antigen from both parents. These recombinants may be isolated and purified by plaque cloning and they are stable on serial passage in our clone 1-5C-4 (conjunctival cell) variant and in the chick embryo or in mice. They are obviously not mixtures of viruses as they are doubly reactive with antisera against both parental viruses rather than being incompletely reactive with either parental antiserum. There is no evidence that they are heterozygotes that may tend to segregate genomes on passage with or without selective pressure. If an AA2 recombinant is used to infect 30 separate 60 mm petri dishes in a dilution calculated to produce between 100-200 plaques per dish, and if agar overlays containing antiserum to either parental virus is then employed, no segregants or mutants resistant to one or the other antiserum appear among the 3000-6000 plaques available for study.

B. Independence of the antigenicity of other genetic markers.

It had been thought at one time that antigenicity might be linked to virulence or perhaps to inhibitor susceptibility. However, our extensive analysis of many recombinants produced by recombination of RI/5⁺ and NWS demonstrates that none of the several markers studied is linked to any other. Thus, we have recombinants containing A2 as the major antigen that are inhibitor resistant and others that are inhibitor susceptible.

This fact argues against the theory proposed separately by both Burnet and Hirst of recombination of influenza viruses involving the pasting together of "sub-genomic pieces".

C. The recombination system - methods for obtaining antigenic hybrids.

We are now studying newly created recombinants and recombinants derived in this laboratory six or seven years ago with reference to their antigenic composition. In the older experiments, the system had involved reactivation of heat or ultraviolet irradiated virus with an infective virus. In most instances, selection against the infective parent had been used so that all virus available for study would then be recombinant by definition. This method has now been used in the conjunctival cell culture system as well as in the chick embryo with and without the use of inhibitor or antibody for selection of recombinant progeny. Identification and selection of recombinants may be accomplished on the basis of the characteristic plaque morphology of such recombinants - differing from that of the parents (see below).

We have also obtained an antigenic hybrid of A and A1 strains by infection of conjunctival cells with two infective parent viruses. Therefore, we conclude that this sort of recombination (i.e., antigenic hybridization) is not necessarily the result of repair of an inactivated viral genome by recombination.

D. The varying proportion of antigens in doubly antigenic recombinants.

It has been a striking fact that all of the recombinants thus far obtained that manifestly share antigens from each parent have been "asymmetrical" - at least in terms of antigenic reactivity with specific antiserum. In HI tests or conventional egg neutralization tests most of these recombinants behave like one or the other parent and evidence of the other parental antigen must be obtained either indirectly by immunization of rabbits with the recombinant or by infection of mice with the recombinant and subsequent challenge with parent viruses. Direct evidence is provided by the subtler techniques of plaque size reduction or the viral specific CF test. Recently we have been able to "build up" the minor antigenic component by re-recombination of the recombinant with the parent that has contributed to its minor antigen. This build up in

antigenic component is still difficult to detect by conventional serologic techniques, but is clearly detectable in the conjunctival cell system, in that antibody to the minor parent is now able to completely inhibit plaque formation rather than merely to reduce plaque size (PSR). Such recombinants are now being studied by Dr. Schulman as vaccines for mice with the expectation that even broader double protection against both parental viruses will be demonstrable.

The critical question raised by these experiments is whether all recombinants are in fact recombinant in greater or lesser degree for antigenic character and that perhaps because of steric or other considerations a demonstration of minor antigen may be difficult. It is possible if not probable that this is the case despite the fact that we now have in hand recombinant progeny of the A - A2 recombination system that are indistinguishable from either parent - i.e., one reacts as an A2 virus and the other as an A. However we do not have the results of immunization of mice and rabbits with these recombinants nor have we tried marker rescue experiments to determine whether a latent minor antigenic component may be detectable in these viruses. In addition we must reexamine these recombinants by our plaque size reduction (PSR) technique using periodate treated antisera to be sure that in fact plaque size reduction (previously masked by inhibitor) does not occur.

E. The number of reactive antigenic sites in the "antigenic hybrids".

Cross absorption of specific antiserum against parental and recombinant viruses and subsequent testing of such antisera in our plaque size reduction (PSR) system reveal that:

- 1) Recombinant or X virus appears to have both A and A2 antigenic components.
- 2) Absence of cross reactivity of A and A2 is conclusively demonstrated.
- 3) A has a component not adsorbed with X; and X has a component not adsorbed by A.
- 4) A2 has a component not adsorbed by X; and X has a component not adsorbed by A2. (The latter conclusions must be verified further because of the fact that A2 is a minor

component and adsorption may have to be more extensive with the recombinant virus).

These experiments suggest the possibility of an antigenic mosaic of the recombinant influenza virus capsid with two separate reactive sites involving those antigens that we term "A2" and those we term "A".

II. STUDIES ON THE MECHANISM OF PLAQUE SIZE REDUCTION (PSR).

Our studies indicate that reduction of influenza virus plaque size without reduction in the number of plaques, or complete plaque inhibition, (PI) correlates with the viral specific CF test only and not with HI or neutralization either in the chick embryo or in the conjunctival cell system. On the other hand complete plaque inhibition (PI) does correlate (in one direction) with neutralization or with HI - i.e. antisera that neutralize virus in the chick embryo always completely inhibit plaque formation (plaque inhibition, or PI), whereas some antisera that cause PI do not significantly neutralize virus in the egg. Therefore we conclude that the plaque size reducing effect is not the result of true neutralization or irreversible bonding of the virus particle by an antibody molecule. Rather it seems more probable that aggregation or agglutination of virus particles occurs when recombinant virus is placed in the presence of antiserum directed against its minor component. Such aggregation of virus would inhibit its migration from cell to cell in the monolayer under agar and thus result in no diminution in the number of detectable plaques but rather a diminution in plaque size, as we described. This explanation would explain the great sensitivity of the PSR system as a method for antigenic analysis and one would expect it to be even more sensitive than the virus-specific CF test. Our evidence at the moment indicates that this is the case, although further studies must be done.

III. ANTIGENIC VARIATION IN INFLUENZA B VIRUSES STUDIED BY PI AND PSR.

Prototypes of the major antigenic variants of influenza B viruses (Lee, BGL54, and IBI 1950) all produce clear plaques in the clone 1-5C-4 monolayer culture system. When comparative studies of cross neutralization are carried out in this system some cross reactivity among these three strains can be discerned

on the basis of both PI and PSR. In this respect these "subtypes" of influenza B virus are unlike the more antigenically distinct human influenza A virus subtypes and thus constitute a more homogeneous group antigenically. These observations are confirmatory of similar conclusions already published by Davenport and Hennessy.

IV. GENETIC VARIATION IN PLAQUE TYPE.

Just as recombinants can be shown to differ in the proportion of antigens that they contain, so do they differ in the size and type of plaques that they produce in the conjunctival cell (clone 1-5C-4) system. Studies are being initiated of the relevance of plaque size and type to the virulence of recombinant strains in mice. At the moment it can be stated that a crude direct correlation of plaque size and virulence can be made.

Of special interest are the so-called "red plaque"-forming viruses (those in which neutral red uptake is increased at the plaque borders). Microscopic study of such plaques discloses that islands of surviving cells exist at the borders of the plaques and it can also be shown by hemadsorption that virus production is going on far beyond the borders of the plaques. It is possible that such viruses are better interferon formers than the clear plaque producers so that cells within the plaque center or at the border may be transiently spared from the cytonecrotizing effect of virus. This question is under investigation.

V. RECOMBINATION FOR PLAQUE TYPE.

Recombination of A (NWS) and A1 (CAM) viruses has been studied in some detail by Dr. Akira Sugiura in this laboratory. The A strain produces a large clear plaque while the A1 produces a somewhat smaller red or r^+ plaque. We have shown that these plaque characteristics are stable on serial transfer of virus and that they are exchangeable genetic traits. Thus an NWS r^+ and a CAM r^- may be isolated after recombination, and reciprocal recombinants may be obtained on the back cross that resemble the parents as far as plaque type is concerned.

In the course of these experiments some quantitative information has been obtained about the efficiency of recombination

using two infective parents and also using one inactivated and one infective parent. In these experiments inoculation of conjunctival cell monolayers with multiplicities of 12 PFU of NWS per cell and 8 PFU of CAM virus per cell were carried out under conditions in which a one-step growth curve was obtained (See Table I). When the mixed yield was then plated in the presence of specific antisera it was possible to determine the proportion of recombinants by the use of the antigen and plaque morphology markers. On this basis the proportion of recombinants was shown to be 22.8%.

TABLE 1

PLATING OF THE MIXED YIELD¹ (NWS X CAM)

Dilution of the mixed yield -log ₁₀	Overlay media	Plaque numbers ² of the character of			Total plaque no.
		r ⁻	r ⁺	? ³	
2.3	Regular	106	107	0	213
	anti NWS	17	53	4	74
	anti CAM	28	7	0	35

Proportion of recombinants among progeny virus

$$24/105 = 22.8\%$$

- ① The cells were infected NWS (12 PFU/cell) and CAM (8 PFU/cell)
- ② Plaque numbers listed are the totals from 3 replicate plates for each medium group.
- ③ Plaques which could not be classified into either type

The yield of the two types of plaque recombinants was unequal with r⁻ plaques occurring more frequently than the r⁺ ones. This may be explained by the assumption that the phenotypic expression of the r⁺ character requires more than one

cistron. The recombinational frequency varied from experiment to experiment and appeared to be affected in part by the multiplicity of infection with the parental types (See Table II). Thus in eight separate experiments performed in the same way, the frequency varied from 6-23% with the NWS-CAM cross and from 16-34% with the CAM r^- and NWS r^+ cross.

TABLE 2

SUMMARIZED RESULTS OF MIXED INFECTIONS

Mixed yields from infection of	Input viruses PFU/cell		Ratios of each serotype among progeny		Proportion of recombinants (%)
	CAM	NWS	CAM serotype	NWS serotype	
NWS + CAM	8	12	92%	8%	6.0
	7	12	67.5%	32.5%	22.8
	7	12	67.5%	32.5%	11.1
	CAM r^-	NWS r^+	CAM serotype	NWS serotype	
CAM r^- + NWS r^+	12	12	68%	32%	31.4
	12	12	54%	46%	27.6
	6	12	38%	62%	20.1
	6	12	29%	71%	34.2
	4	12	6%	94%	16.5

The effect of UV inactivation of one of the two parents upon the frequency of recombination was also studied. NWS r^+ was partially inactivated by UV-irradiation with a dose of 3.2 hits/particle. Either active or partially inactivated NWS r^+ was then inoculated into cultures together with active CAM r^- .

The results were somewhat similar to experiments in which unequal growth of two types had been observed - namely there was a preferential growth of the active parental type over the inactive one among the progeny. The overall recombination frequency did not differ whether or not one of the parents was inactive. An essentially similar result was obtained using NWS r^+ subjected to further irradiation (See Table III).

TABLE 3

EFFECT OF UV-IRRADIATION OF ONE OF TWO PARENTS
UPON PROGENY VIRUS AND RECOMBINATIONAL FREQUENCY

Input viruses PFU/cell			Ratios of each serotype among progeny		Proportion of recombinants
CAM r^-	NWS r^+	UV-NWS r^+ ①	CAM serotype	NWS serotype	(%)
6	12		29 %	71 %	34.2
6		0.5 (12) ②	78 %	22 %	30.0
6		1 (24)	38 %	62 %	26.8

① The virus was irradiated for 5 seconds. The average dose of UV was approximately 3.2 hits/particle

② Values in parenthesis are PFU/cell calculated from pre-irradiation titer.

Recombination frequency at different time intervals after infection was also compared. With each of two combinations of input viruses a significant increase in recombination frequency was observed from the early phase of infection (10-14 hours) to the later stage (16-30 hours), at which time one multiplication cycle was completed. This suggests that recombination is neither an exclusively initial event that has to precede nor an exclusively terminal event which has to follow the replication of the viral genomes.

VI. RECOMBINATION FOR INCREASE IN GROWTH RATE IN THE CHICK EMBRYO

A practical problem in the production of vaccine to newly derived strains may be presented by the poor capacity of certain viruses to grow in the chick embryo used for vaccine production. Experiments performed in this laboratory some five years ago indicated that recombination of a poorly growing A2 strain with a rapidly growing high titer A strain resulted in the production of a recombinant A2 virus of enhanced growth potential. At the suggestion of Dr. Davenport we have studied a strain of A2 isolated in Washington in 1964 that grows extremely poorly in the chick embryo. We have recombined this in the chick embryo with a 1953 A1 strain (CAM MT) that grows well in the chick embryo. From this recombination virus with A2 as the major serotype has not been isolated but there was a suggestion that in parallel control serial passage the A2 parental virus had increased in its capacity to grow in the chick embryo. This proved to be due to the influence of cortisone used to prevent autointerference in low dilution passage. Thus, the result of this particular experiment is negative except for the interesting suggestion that cortisone may speed the rapid adaptation of poorly growing strains. However, in other recent experiments with other recent A2 strains it has been possible by plaque selection and use of the PSR test to isolate viruses with A2 as the major antigenic component that grow to 16-64 fold higher titers in eggs.

TRANSMISSION OF INFLUENZA VIRUS INFECTION IN MICE

I. VIRAL FACTORS

Previous studies have shown wide variation in transmissibility among different strains of influenza virus. Furthermore, these studies have shown that transmissibility correlated poorly with other indications of virulence for mice such as peak pulmonary virus titers, lung lesions and mortality.

A. Use of recombinant viruses.

Previous attempts in this laboratory to enhance the transmission of a poorly transmitted virus (NWS) by recombination with a moderately well transmitted one (RI/5⁺) were unsuccessful.

1. Recombinants of CAM and NWS were tested to determine if the transmissibility of the CAM parent could be demonstrated in

recombinant offspring. The results of four experiments are summarized in Table IV.

TABLE 4

<u>aerosol</u> <u>infection</u>	<u>48 hr. pulmonary</u> <u>virus titer*</u>	<u>lung lesion</u> <u>score (day 7)</u>	<u># contacts</u> <u>infected</u>
NWS	7.2		10/100
NWS r ⁺	7.5		25/100
CAM r ⁻	7.6	32%	14/100
CAM	8.5	90%	49/100

* Log₁₀ EID/50. Individual titers 5 animals in each group.

In each of the four experiments the rank order of transmissibility was identical - CAM, NWS r⁺, CAM r⁻, and NWS. These results demonstrate that the NWS r⁺ recombinant virus which is antigenically primarily NWS-like has inherited at least some of the transmissibility-associated properties of its CAM parent without significant change in other mouse virulence characteristics such as pulmonary virus titers or lung lesions.

2. Attempts to obtain a very highly transmissible virus by recombination of the two most transmissible viruses tested so far - mouse adapted Jap. 305 (A2) and mouse adapted CAM (A1) - produced a recombinant which was antigenically CAM-like and was transmitted to the same extent as its CAM parent.

B. Survival of Aerosolized Virus.

Identical quantities of NWS (poorly transmitted) and mouse adapted Jap. 305 virus (well transmitted) were aerosolized in a closed chamber under identical conditions. After discontinuing nebulization, samples of air were removed and passed through glass impingers, immediately, and 5, 15, 30 and 60 minutes later, to titrate the virus concentration remaining in the air of the chamber. Results for the two viruses were identical, suggesting that the greater transmissibility of the A2 virus was not related to greater survival in the airborne state.

II. HOST FACTORS

A. Effect of NDV-Induced Interferon.

Previous studies in this and in other laboratories have shown that mice inoculated intravenously with NDV had high titers of interferon in their sera, lungs, and spleens 6-24 hours later. It was of interest to determine whether NDV-induced interferon would protect mice against influenza virus infection transmitted by other mice.

1. Mice inoculated IV with NDV or saline were placed in contact (one each in a series of cages) three hours later with infector mice infected 24 hours earlier with A2 virus. After the usual 24 hour contact period, the contact mice were removed, separated, and their lungs tested 48 hours later for the presence of virus. Twenty four hours after inoculation with NDV or saline, lungs and sera of five mice in each group were pooled for interferon assay in L cell cultures with VSV. The results of six experiments are summarized in Table V.

TABLE 5

<u>IV inoculation of contact + 21 hrs.</u>	<u>Interferon titer* +45 hrs.</u>		<u>Contacts infected</u>
	<u>Serum</u>	<u>Lung</u>	
NDV	4000	776	42/117 - 34.2%
Saline	< 10	< 10	83/120 - 69.2%

* Reciprocal of dilution of test material producing 50% reduction in number of VSV plaques on L cell cultures.

In each experiment contact mice given NDV intravenously three hours before initiation of contact were less susceptible to transmitted infection.

- a) Lungs of positive contacts in both (NDV and saline) contact groups were titrated for infectious virus to determine whether intravenous NDV inoculation resulted in lower pulmonary virus titers in positive contacts as well as fewer positive contacts. The mean titer was $10^{4.8}$ in control contact mice and $10^{4.6}$ in NDV inoculated contacts.

- b) To calculate quantitatively the reduced susceptibility to infection induced by IV NDV inoculation, NDV and saline inoculated mice were challenged four hours later with serial four-fold dilutions of A2 virus by aerosol. Two times as much A2 virus was required to infect 50% of NDV inoculated mice. Studies are in progress to measure differences in MID/50 at intervals following NDV inoculation.

2. It has been shown in this and other laboratories that NDV induced interferon in mice could be partially blocked by prior inoculation with cortisone.

Attempts were made to determine whether the decreased susceptibility of NDV inoculated contact mice to transmitted infection also could be reversed by cortisone. One day prior to and on the day of exposure to transmitted infection mice were inoculated subcutaneously with 5 mg of hydrocortisone or with saline. Three hours before the initiation of contact, half of each contact group was given NDV intravenously and the other half saline IV. Interferon in the lungs and serum were measured 24 hours later. The results of three such experiments are summarized in Table VI.

TABLE 6

<u>Sub. cut. inoc.</u> <u>0 hrs. & 24 hrs.</u>	<u>I V inoc.</u> <u>21 hrs.</u>	<u>Interferon titer</u> <u>+ 45 hrs.</u>		<u># Contacts</u> <u>infected</u>	
		<u>Lung</u>	<u>Serum</u>		
Saline	Saline	< 10	< 10	46/60	76.7%
Saline	NDV	776	7440	24/57	42.1%
Cortisone	NDV	426	1000	25/57	43.9%
Cortisone	Saline	< 10	< 10	35/54	64.8%

Although subcutaneous inoculation with hydrocortisone lowered interferon titers in the lung and serum of NDV inoculated mice, increased resistance to transmitted infection

in NDV-inoculated mice was not reversed. These data are interpreted as indicating that enough interferon is produced in mice given NDV and cortisone to maintain the increased resistance to transmitted A2 infection.

3. In earlier experiments concerning viral interference in mice it was found that inactivated NDV given intranasally or by aerosol could protect contact mice against transmitted CAM virus infection. Attempts were made to repeat these observations and to measure pulmonary titers of interferon following the local administration of inactivated NDV. Inactivated NDV was given to contact mice intranasally or by aerosol 3 hours or 21 hours before initiation of contact with A2 virus infectors. Although pulmonary interferon titers of 716 and 32 were found 24 and 48 hours after NDV inoculation, reduced susceptibility to transmitted infection was not found. The discrepancy between these results and the earlier findings may be explained by the fact that higher concentrations of inactivated NDV was employed in the earlier experiments. The lack of protection against transmitted infection despite the presence of significant titers of interferon in the lung may be due to the fact that following intranasal or aerosol administration of virus only a minority of cells may be induced to produce interferon and are therefore protected, whereas following IV administration of NDV, interferon is uniformly distributed reducing the susceptibility of all cells.

4. Infector mice given NDV intravenously 24 hrs. after initiation of A2 virus infection (at the beginning of the contact period) did not have lower pulmonary virus titers 24 hrs. later and transmitted infection as readily as control infectors. However, the NDV inoculated infectors had less extensive pulmonary lesions 96 hrs. but not at 7 days after the initiation of infection. The data indicate that the NDV probably resulted in a slight, transitory reduction in virus multiplication that did not affect transmission.

B. Estimation of quantitative differences among infector mice in the ability to transmit infection.

Previous experiments have demonstrated that paired contact mice tend to share similar fates (both mice or neither animal acquiring infection). The interpretation of these data has been that the risk of infection is greater in some cages than in others and therefore that some infectors are better transmitters than others. Attempts to derive quantitative estimates of these differences have been made in two ways.

1) Instead of employing the usual two infectors and two contacts in each cage, in one experiment three contacts and one infector were placed in each cage. The results were as follows:

	number of cages	
	<u>infected</u>	<u>observed</u>
3 contacts infected	3	7
2 contacts "	7.7	4
1 contact "	6.5	2
0 contacts "	1.8	6

The disproportionate number of cages ($P < .001$) where all or none of the contacts were infected confirms the previous hypothesis that the risk of infection is greater in some cages than in others. Furthermore, the data suggest that the risk in some cages where all contacts were infected was more than three times as great as in some cages where none of the contacts was infected.

2) It was found that mice immunized with a single IP infection of inactivated A2 vaccine required 10 times the concentration of aerosolized A2 virus for infection to occur as was required to infect 50% of non-immune animals. In three experiments, mice were immunized IP with inactivated A2 virus four weeks before being exposed to unimmunized infector mice. One immunized and one unimmunized contact was placed in each cage. The 8/62 positive immunized contacts and the 31/62 positive unimmunized contacts were distributed as follows with regard to pairing.

							<u># pairs</u>	
Immune contact +	:	non-immune contact +					7	
"	"	+	:	"	"	"	-	1
"	"	-	:	"	"	"	+	27
"	"	-	:	"	"	"	-	27

If immunized contact mice require exposure to 10 times as much transmitted virus as normal contacts to become infected in the same way that a 10-fold difference has been shown in the MID/50 by aerosol challenge, then certain deductions can

be made from the above data. In those cages where both contacts were infected, the concentration of virus must have been 10 times as high - sufficient to infect the immunized contact - as in those cages where only the non-immune contact was infected. Therefore, some infectors are at least 10 times as infective as others.

III. DEMONSTRATION OF AIRBORNE VIRUS IN AIR SURROUNDING INFECTOR MICE

Thirty mice were infected with A2 virus and 24 hrs. later were placed again inside the closed aerosol chamber. A continuous air flow of 12 liters/min. through the chamber was passed through a glass impinger attached to a port in the chamber. The impinger was changed every 20 min. and fluid samples for the 1st 12 hrs. and for the 2nd 12 hrs. were pooled to form 2 specimens which were concentrated in the ultracentrifuge and inoculated into eggs. Infective virus was demonstrable in both specimens. The total for both specimens was measured as 60 egg infective doses giving an average output of 2.0 infective doses/infectors in 24 hours. These results were of interest not only because infective virus could be demonstrated in the air surrounding infectors, but also because the quantity of such virus was sufficient to explain the frequency of transmitted infection during contact experiments carried out in the closed chamber. (In such contact experiments the numbers of infectors and contacts, and the ventilation rate in the chamber are all known, the respiration rate of contacts can be estimated, and the number of contacts which acquire infection is determined. From these data the mean output of infectious doses for each infector can be calculated.)

BROADENED ANTIGENICITY OF RECOMBINANT VIRUSES AS DEMONSTRATED IN MICE

The broadened immunity observed in mice infected with a recombinant of A (NWS) and A2 (RI/5⁺) viruses has been commented on in a previous report. Since that time, experiments with a 2nd recombinant of the same two subtypes have produced identical results. Mice immunized by infection with the recombinant virus were partially protected against challenge with either parental subtype.

I. IMMUNIZATION OF MICE TO A2 VIRUS BY A COMBINATION OF
OF RECOMBINANT (AA2) VIRUS INFECTION AND INACTIVATED
A2 VACCINE.

In two experiments, mice were infected with B (Lee) virus, A (NWS) virus or with one of two AA2 recombinants. Two weeks later each group was given a single IP injection of inactivated A2 vaccine. All groups were challenged two weeks later with A2 virus infection. The results of the two experiments were identical. Mice originally infected with influenza A virus or with either of the recombinants had a greater response to the inactivated A2 vaccine than mice initially infected with influenza B virus given A2 vaccine before challenge. Titers of HI antibody to A2 virus and pulmonary virus titers and lung lesions following A2 infection were reduced to a greater extent than in animals infected with B virus and later given inactivated A2 vaccine. The increased response to the A2 vaccine was greatest in the animals initially infected with AA2 recombinant virus. These experiments tend to confirm earlier experiments in this laboratory which demonstrated heterotypic immunity in mice infected with A virus and later challenged with A2 virus and also demonstrate a greater protection against A2 challenge when the initial infection was with recombinant (AA2) virus.

II. BROADENED IMMUNITY WITH VIRAL RECOMBINANTS OF OTHER
SUBTYPES

The preparation of recombinants of A (NWS) and A1 (CAM) is described in another portion of this report. Mice were infected with a hybrid virus (Nc) of double antigenicity or with NW3 or were exposed to a saline aerosol. Four weeks later they were challenged with A virus (NWS) or with A1 virus (CAM) by aerosol. Pulmonary virus titers 48 hrs. after challenge and lung lesions 7 days after infection were assessed. The results are shown in Table VII.

TABLE 7

<u>Initial Infection</u>	<u>Challenge</u>	<u>Pulmonary Virus titer (48 hrs.)</u>	<u>Lung Lesions (%) (7 days)</u>
A	A	< 2.0*	0
AA1	A	< 2.0	0
Saline	A	7.2	43
A	A1	5.3	10
AA1	A1	< 2.1	0
Saline	A1	8.6	100

* Log_{10} EID/50 5 animals in each group.

Mice infected with the recombinant virus were completely protected against challenge with either A (NWS) or A1 (CAM) virus, whereas mice initially infected with A (NWS) were only partially protected against A1 (CAM) virus challenge.

RELEVANT PUBLICATIONS SINCE LAST ANNUAL REPORT

- Schulman, J. L. and Kilbourne, E. D., Experimental influenza virus infection of mice in A Symposium on Aerobiology, Proc. First Intern. Symp. Aerobiol., University of California Press, 1963, 141.
- Sugiura, A. and Kilbourne, E. D., Genetic Studies of Influenza Viruses: II. Plaque formation by influenza viruses in a clone of a variant human heteroploid cell line. Virology, 1965, 26, 478.
- Kilbourne, E. D. and Schulman, J. L., The induction of broadened (multitypic) immunity with doubly antigenic influenza virus recombinants. Trans. Assoc. Amer. Phys., 1965, 78.

SUMMARY AND CONCLUSIONS

The evidence presented in earlier reports suggesting the production of antigenic hybrids of influenza virus by recombination has now been abundantly and repeatedly confirmed. Recombinants have been produced that share antigens derived from both parental viruses, whether such viruses are A-A2, A-A1 or A1-A2 pairs and an antigenic hybrid of swine influenza virus and PR8 has also been prepared. Thus far, the hybrids that have been selected from our recombinational systems appear to possess the parental antigens in unbalanced or asymmetrical proportions. This finding may represent merely an artifact of selection and this interpretation is suggested by the fact that with closer scrutiny of recombinant progeny the expression of the minor antigen phenotype appears to vary. Thus, although the usual AA2 recombinant isolated contains A and A2 antigens in a proportion of eg. 9-1, in some it may be 7-3 - particularly after re-recombination. It is our current hypothesis that recombination of influenza viruses without some exchange at loci controlling the production of antigens does not occur. The fact that in some recombinants (recombinant for other markers than antigenicity) the antigen of only one parent is expressed does not invalidate this hypothesis, as it is possible either that antigen is present but is sterically masked or that genetic exchange has been completely symmetrical at the antigenic loci. Such "singly antigenic" recombinants would be expected to be in the minority if multiple antigenic loci are involved.

The new serologic technique of PSR (or plaque size reduction) is proving fruitful in the analysis of recombinants and it may also prove useful in redefinition of strain relatedness with respect to laboratory strains or wild-type viruses. By this method, for example, we have confirmed Davenport and Hennessey's finding of the closer antigenic relatedness of the B strains among themselves than obtains with the A group.

The use of plaque type recombinants has already proved of value in the study of recombination frequency in the conjunctival cell system, and the recognition of many plaque type variants provides not only useful genetic markers but the opportunity to correlate in vitro cytopathic effects with in vivo virulence. There is also a suggestion that plaque size may be directly correlated with the ability of a virus to multiply in other host systems - including the chick embryo. This observation makes it possible to select large plaque A2 strains with enhanced chick embryo growth potential.

Summary & Conclusions (Cont'd.)

Recombinant viruses have continued to be useful in the analysis of factors governing transmissibility of influenza virus in our mouse model. Although we suspect that transmissibility is a complex and polygenic property of the virus, we have evidence that recombination of a poorly-transmitted virus with a well-transmitted virus produces progeny of intermediate transmissibility. We hope to segregate the factors responsible for transmissibility through differential transfer of other markers.

The induction of endogenous interferon in mice by inoculation of NDV intravenously has increased their resistance to contact infection.

Airborne virus from infector mice has now been isolated with impingers and titrated. An estimate of the amount of virus put out may be made and it proves to be quite small, i.e., two infective doses per 24 hrs. This small amount of virus correlates well with our indirect evidence on the frequency of infection.

The use of antigenic hybrids as live virus vaccines in mice has been extended and the broader immunity produced has been confirmed. This effect can be enhanced by the use of monovalent vaccine from the parent making the minor antigenic contribution to the recombinant.

It is concluded that continuation of these studies should aid in delineating the range of antigenic variation of which the human influenza viruses are capable and may even result in the production of antigenically novel viruses. It may be possible thus to anticipate nature in the evolution of the next mutant strain.