Biological and Antigenic Characteristics of an A2 Influenza Virus Isolated in 1965

RAUL WALDER, M.P.H., and G. D. HSIUNG, Ph.D.

IN THE WINTER of 1964 to 1965, localized outbreaks of upper respiratory illness in the Eastern United States were reported, and type A influenza infections were diagnosed by serologic means (*1*). An epidemic of influenza-like illness was observed among the staff of the Yale Medical School in January and February 1965. The presence of the A2 strain of influenza virus was confirmed by virus isolation and antibody studies. Our report is concerned with some of the biological properties of the 1965 influenza A2 virus and an analysis of the antigenic properties of the newly isolated A2 virus strain.

For more than a decade, various investigators have noted differences in antigenic properties among strains of the influenza virus group. Recent reports indicate that antigenic changes have been observed within the A2 virus type, especially among the 1963 isolates (*2–4*). Since different animal species have shown variations in the specificity of antibody response to infection by members of the parainfluenza virus group (*5*), we decided to characterize the antigenic properties of the new A2 virus strain, using antiserums produced in different animal species, and to compare these data with those of two older A2 strains isolated in 1957 and 1961.

At the time of the study, the authors were with the department of epidemiology and public health, Yale University School of Medicine, New Haven, Conn. Mr. Walder is now with the respiratory unit of the Instituto Venezolano de Investigaciones Cientificas, Ministerio de Sanidad y Asistencia Social, Caracas, Venzuela. Dr. Hsiung is now an associate professor of medicine (microbiology) at the New York University School of Medicine, New York City.

Materials and Methods

Virus isolation and virus strains. During the epidemic in New Haven in January and February 1965, we attempted to isolate influenza virus from throat swabs of selected patients. All isolations were made in primary cultures of rhesus monkey kidney cells by the hemadsorption technique (*6*). Two A2 virus strains, prototype Jap/305/57 and Conn/1/61 virus, were used for comparison. The Conn/1/61 virus was also isolated in cultures of rhesus monkey kidney cells, but it was subsequently adapted to embryonated eggs (*7*).

Tissue culture. Rhesus monkey kidney cells were grown in roller tubes or in 3-ounce prescription bottles. All cultures were maintained in Earle's balanced salt solution containing 0.5 percent lactalbumin hydrolysate, 2 percent calf serum, and 0.2 percent hyperimmune SV5 antiserum. The methods for kidney tissue trypsinization and for preparation of cultures have been described previously (*8*).

Virus assay. Virus infectivity was determined by the amount of hemadsorption of guinea pig erythrocytes in tube cultures and by the plaque formation in bottle cultures. The use of 0.5 percent freshly obtained guinea pig erythrocytes helped to avoid nonspecific hemadsorption (*9, 10*). The constituents of the agar

overlay medium and the technique for plaque assay were the same as previously described (*8*). Viral agglutinins were assayed with 0.5 percent human type O erythrocytes in plastic panels.

Preparation of antiserums. Roosters, ferrets, Syrian hamsters, and rhesus monkeys were used in preparing the antiserums. Both infectious tissue culture fluids and egg-adapted virus stocks were employed as immunizing antigens. Each animal or bird was given two doses of infectious virus 9 days apart in two sites. Blood samples were collected before the inoculation and 21 days after the first dose. Ferrets and hamsters were inoculated intramuscularly and intraperitoneally; monkeys, intranasally and intramuscularly; and roosters, intramuscularly and intravenously. All serums were kept frozen until tested.

Collection of human serum. On February 8, 1965, at the time of the suspected outbreak, 93 single blood samples were collected from staff members of the department of epidemiology and public health, Yale Medical School. An additional 75 blood specimens were obtained in March 1965 from the second-year Yale Medical School students for whom pre-epidemic serum specimens collected in October 1964 were available. All serums were stored at −20° C. until tested. All persons from whom blood specimens were obtained were asked whether they had been ill or evidenced symptoms suggestive of influenza during the study period.

Antibody determination. Three serologic methods were used for the determination of antibody titers. The group-specific antibody titers were assayed by the complement-fixation (CF) test, and the type-specific antibody titers were determined by the hemagglutination-inhibition (HI) test and the neutralization test, or by both.

The CF test was conducted in disposable plastic panels (*8*), basically following the technique introduced by Fulton and Dumbell (*11*). Influenza virus type A soluble antigen was purchased commercially. All serums were inactivated at 56° C. for 30 minutes before being tested.

In the HI test, titrations were performed according to standard methods (*12*). A 0.2 ml. portion of serum in serial twofold dilutions (initial dilution 1 to 8) was placed into each well of plastic panels. Then 0.2 ml. of virus-infected pooled egg fluid was added to each serum dilution, and mixtures were shaken in the panels. To remove nonspecific serum inhibitors, the serums were kept at a temperature of 56° C. for 30 minutes, then treated with potassium periodate, and subsequently inactivated by the addition of 1 percent glycerine-saline (*13*). Naturally occurring agglutinins for human O red blood cells in animal serums were absorbed by mixing diluted test serum with 0.1 ml. of washed and packed human O erythrocytes.

Neutralization tests were performed on rhesus monkey kidney tissue cultures, either in tube cultures by the hemadsorption-inhibition method or in bottle cultures by the plaque-reduction method. Serums were inactivated at 56° C. for 30 minutes for both tests. To serial twofold dilutions of serums, an equal volume of approximately 150 TCD_{50} per 0.1 ml. of the virus suspension was added. This mixture was left at room temperature for 60 minutes. Then, 0.2 ml. of each virus-serum mixture was inoculated into each of three tubes of monkey kidney cell cultures and incubated at 37° C. for 5 days before being examined for hemadsorption. The neutralizing antibodies were expressed as the reciprocal of the highest dilution of serum which completely inhibited the growth of influenza virus.

For determining the rate of neutralization of the virus by the specific antiserum, an initial master dilution was made by adding 2.0 ml. of antiserum (titer 1 to 1,024), diluted to 1 to 1,000, to 2.0 ml. of virus suspension containing either 100 plaque-forming units or 100 TCD_{50} per 0.1 ml. From this master dilution, further tenfold dilutions of the virus-serum mixture were made at 0, 15, 30, and 90 minutes after the virus and serum were mixed. Each mixture was inoculated into bottle or tube cultures and adsorbed for 1 hour; the cultures were then overlaid with nutrient agar medium or fluid medium. The end point was considered the highest serum dilution resulting in an 80 percent or greater reduction of plaques or in inhibition of hemadsorption.

Results

Virus isolation and characterization. During the epidemic, three virus isolations were made from throat swabs of 10 selected persons who were in the acute stage of illness. Final identification of these isolates was confirmed by the

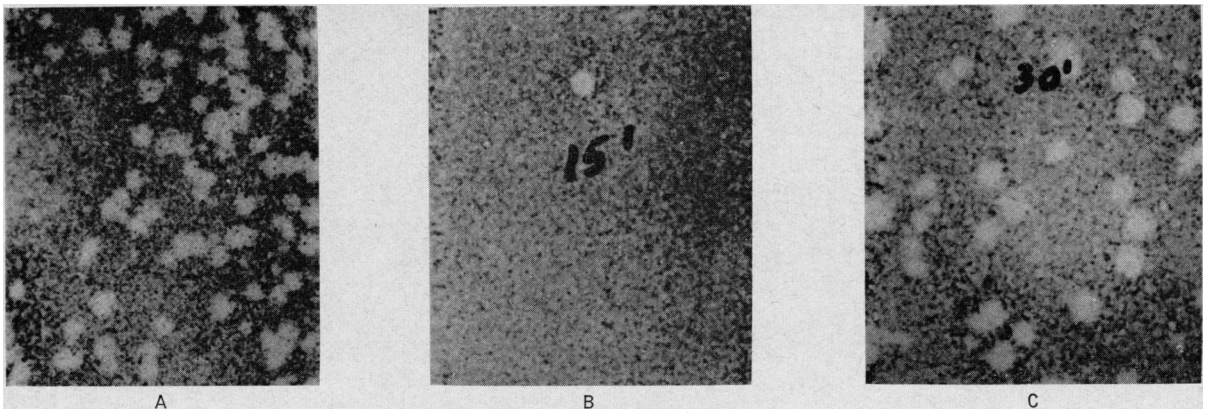


Figure 1. Plaque-formation and plaque-reduction neutralization of A2/Conn/1/65 virus in rhesus monkey kidney cell monolayer 10 days after innoculation: *A.* A2/65 virus control without antiserum; *B.* A2/65 virus antiserum (serum-virus mixtures incubated 15 minutes at room temperature before inoculation); *C.* A2/61 virus antiserum (serum-virus mixtures incubated 30 minutes at room temperature before inoculation).

International Influenza Center for the Americas at the Communicable Disease Center, Atlanta, Ga., which is a World Health Organization Virus Reference Center. One of the new isolates, the JRH strain, was designated as A2/Conn/1/65 influenza virus. This strain, isolated in a primary culture of monkey tissue, was subsequently adapted to the allantoic sac after one passage in the amniotic cavity of 10-day-old embryonated eggs. Infected egg fluid harvests titered $10^{6.4}$ embryo infectious dose $_{50}$ per milliliter with a hemagglutinin titer of 1 to 512. No hemagglutinin titers were measurable from the infected tissue culture fluid although the infected cultures showed hemadsorption. The JRH virus strain produced plaques in cultures of monkey kidney cells under agar overlay medium. Distinct plaques could be counted from the 6th through the 8th day after inoculation with the virus strain (fig. 1A). The ease with which the A2/65 virus produced plaques in cell cultures facilitated the characterization of the A2 virus strains in the neutralization tests.

Antibody response in humans. Serums collected from 168 persons immediately after the epidemic were assayed for antibody titers group-specific for influenza A; 63, or 58 percent, showed positive titers ranging from 1 to 16 to 1 to 256 as determined by the CF test. Only 34, or 20 percent, of the 168 persons were reported as having clinical influenza-like syndromes, and paired serums were available from 14 of them (table 1). The first serums were taken during the pre-epidemic period, and the second serums were obtained after the epidemic. As shown in table 1, all but two persons showed high CF titers in their second serums, and none showed significant CF titers in their first serums. All 14 persons with confirmed cases showed fourfold or greater increases in HI antibody titers to the new A2/Conn/1/65 JRH virus strain; 11 showed fourfold or greater antibody titer rises to prototype Jap/305/57; only 6 showed rises to A2/Conn/1/61 strain. Patients I.L. and M.L. had each received a dose of multivalent influenza vaccine before the epidemic and showed greater than fourfold antibody rise only to the A2/Conn/1/65 virus strain.

Antibody response in experimental animals. Five hamsters, four ferrets, four roosters, and four monkeys were immunized with the A2/Conn/1/65 virus strain. Two of each kind of animal or bird received infected tissue culture fluid; the remaining nine each received infected egg fluid. Representative antibody responses of the individual animals are shown in table 2. Neither HI nor neutralizing antibody could be demonstrated in any of the preserums. Regardless of the source of the immunizing virus A2/65—whether obtained from infected egg fluid or from infected tissue culture fluid—all subjects showed substantial antibody titer rises to the immunizing viruses (table 2). It was noted that serums of monkeys immunized with A2/65

showed no HI antibody titer when tested against A2/57 and A2/61 strains. In general, hamsters and ferrets showed lower levels of homologous antibody than roosters and monkeys. The nonspecific inhibitors occurring in ferret serums could not be removed by treatment with heat, receptor-destroying enzyme, or potassium periodate, and consequently hemagglutination-inhibition test data were not obtainable.

Antibody responses in roosters and monkeys following inoculation of A2/61 or A2/65 virus were compared (table 3). Serums of roosters immunized with A2/61 virus showed little difference in HI titers or neutralizing antibody titers to the three antigens used. On the other hand, serums of roosters immunized with A2/65 virus showed high-level homotypic antibody titers, but the same serums showed low levels of inhibition or neutralization against the A2/61 strain. When serums of monkeys inoculated with the A2/61 virus were tested by the HI or neutralization test against the A2/57, A2/61, and A2/65 antigens, slight differences in antibody titers to these viruses were noted. Serums of monkeys immunized with A2/65 virus, however, showed no detectable HI titers against A2/57 or A2/61 antigens, although significant HI antibody titers were obtained in the homologous system. The same serum sample showed a lesser degree of antigenic specificity when the neutralization test was used.

Differences in A2/61 and A2/65 strains. Antigenic differences between the A2/61 and A2/65 influenza virus strains were determined quantitatively by the rate of neutralization. The distinct plaques produced by A2/65 virus (fig. 1A) were reduced completely after 15 minutes of reaction at room temperature with monkey

Table 1. Antibody responses of 14 persons reported ill with influenza

Date patients' serums were taken [1]	Antibody titers to antigens			
	Complement fixation test	Hemagglutination-inhibition test		
		Jap/305/57	Conn/61	Conn/65
A. L.				
1964	4	128	128	64
1965	16	1, 024	1, 024	1, 024
B. W.				
1964	8	8	16	8
1965	32	8	16	64
B. W. E.				
1964	4	64	64	16
1965	16	512	256	128
D. M.				
1964	4	32	16	16
1965	64	128	64	256
I. L. [2]				
1964	16	256	128	128
1965	32	512	128	1, 024
L. S.				
1964	8	128	64	64
1965	16	256	64	256
L. P.				
1964	4	64	32	32
1965	64	256	64	256
M. K.				
1964	4	128	64	32
1965	64	512	256	1, 024
M. L. [2]				
1964	4	64	16	32
1965	32	256	64	256
R. J.				
1964	4	64	32	16
1965	8	256	64	256
S. R.				
1964	8	32	32	8
1965	8	256	128	512
P. J.				
1964	4	32	([3])	16
1965	64	512	([3])	1, 024
N. J.				
1964	4	128	([3])	16
1965	64	512	([3])	1, 024
F. R.				
1964	4	8	([3])	8
1965	256	512	([3])	1, 024

[1] The 1964 serums were taken in October, during the pre-epidemic period; the 1965 serums were taken in March, immediately after the epidemic.

[2] Patient had received 1 dose of multivalent influenza vaccine in October 1964.

[3] Not done.

homologous immune serum (fig. 1B). On the other hand, only a 50 percent reduction in plaques occurred after 30 minutes of reaction when the same A2/65 virus suspension was Conn/1/65 virus strain. Two of each kind mixed with A2/61 monkey antiserum (fig. 1C); even after 90 minutes of reaction, only 80 percent of the plaques were reduced.

The results of one representative experiment on the rate of neutralization of the A2/61 and A2/65 virus strains and their type-specific antiserums are shown in figure 2. The A2/61 virus did not produce plaques; therefore, the hemadsorption-inhibition method was used for neutralization of this virus strain. With the homologous system, complete neutralization oc-

Table 2. Representative antibody responses in animals immunized with A2/65 virus

Animal and assigned number	Virus suspension inoculation [1]	Antibody titers to antigens after inoculation [2]				
		Hemagglutination-inhibition test			Neutralization test	
		A2/57	A2/61	A2/65	A2/61	A2/65
Hamster:						
3	MKTC	64	32	128	[3]	[3]
10	AA	32	16	128	16	256
Ferret:						
1	MKTC	[4]	[4]	[4]	0	16
4	AA	[4]	[4]	[4]	16	256
Rooster:						
1	MKTC	32	0	128	16	1,024
4	AA	512	256	1,024	64	4,096
Monkey:						
600	MKTC	0	0	256	64	1,024
611	AA	0	0	512	64	1,024

[1] Monkey kidney tissue culture containing infectious virus, $10^{5.5}$ plaque-forming units per milliliter, or amniotic-allantoic fluid from infected embryonated eggs containing $10^{6.4}$ embryo infectious doses per milliliter.
[2] All preinoculation serum samples showed hemagglutination-inhibition titers of $\leqq 8$ and neutralization titers of $\leqq 4$; these titers were the lowest serum dilutions tested.
[3] Not done.
[4] Nonspecific inhibitor, which could not be removed by heat, potassium periodate, or treatment with receptor-destroying enzyme.

Table 3. Antibody responses in roosters and monkeys after immunization with 1961 and 1965 A2 influenza virus strains

Assigned number of rooster or monkey	Virus suspension inoculation (amniotic-allantoic fluid) [1]	Antibody titers to antigens after inoculation [2]				
		Hemagglutination-inhibition test			Neutralization test	
		A2/57	A2/61	A2/65	A2/61	A2/65
Rooster:						
7	A2/61	256	512	256	64	64
9	A2/61	128	256	128	64	64
4	A2/65	512	256	1,024	64	4,096
6	A2/65	256	64	1,024	16	1,024
Monkey:						
659	A2/61	1,024	1,024	256	1,024	64
660	A2/61	512	512	256	256	256
609	A2/65	0	0	256	64	1,024
611	A2/65	0	0	512	64	1,024

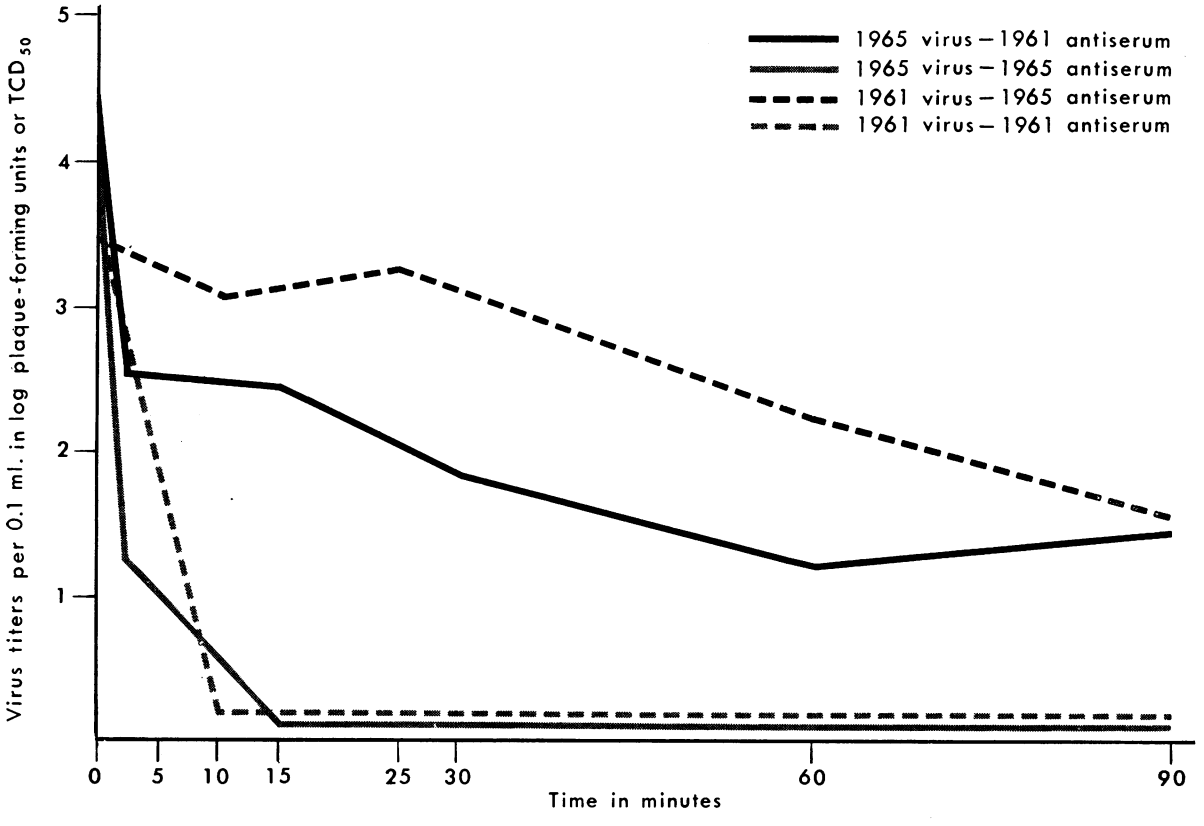
[1] Amniotic-allantoic fluid from infected embryonated eggs containing $10^{6.4}$ infectious dose per milliliter.
[2] All preinoculation serum samples showed hemagglutination-inhibition titers of $\leqq 8$ and neutralization titers of $\leqq 4$; these titers were the lowest serum dilutions tested.

curred 10 to 15 minutes after the 100 plaque-forming units of A2/65 or 100 TCD_{50} of A2/61 virus suspension were mixed with antiserum. Nevertheless, as was apparent from the heterologous system, the A2/65 virus was neutralized by the A2/61 antiserum somewhat faster than was the heterologous A2/61 virus by the A2/65 antiserum. The latter observation may be due, at least partially, to the sensitivity of the plaque method. Furthermore, significant numbers of infectious virus remained detectable in the heterologous systems when the mixtures were kept at room temperature for as long as 90 minutes (fig. 2).

Discussion

The data in our study indicate that the influenza epidemic that occurred in a particular segment of the population of New Haven, Conn., from January to March 1965 was caused by the A2/65 influenza virus. The biological properties of the newly isolated A2/65 virus are similar to those which have been reported earlier for A2 strains. The newly isolated A2/65 strain appears to be relatively resistant to any nonspecific inhibitors in the agar medium; distinct plaques were easily obtained with this virus in monkey kidney cultures under agar overlay medium. Although plaque-reduction neutralization tests have been used extensively by many investigators for various viruses, such techniques have not been generally applied to the study of the influenza virus group. Most of the influenza virus strains were not plaque-producers, especially those isolates obtained from embryonated eggs. Previous investigators (*2*, *3*) have stressed the advantage of tissue-culture neutralization tests conducted by the hemadsorption-inhibition method. Results obtained in our study by the plaque-reduction test show

Figure 2. Antigenic differences between A2/61 and A2/65 influenza viruses as revealed by rate of neutralization



NOTE: Time in minutes indicates incubation period of mixtures before inoculation into test cultures. Plaque-reduction neutralization method was used for 1965 virus; hemadsorption-inhibition test used for 1961 virus.

that a more quantitative method is available for the A2/65 virus. As the kinetics of the viral neutralization test revealed, significant antigenic differences between the influenza strains may be detectable if the plaque technique can be applied more generally.

Antigenic drift among the influenza viruses has been noted ever since the early work of Magill and Francis (*14*), but the consequence of minor antigenic variation within a subtype may be significant in a rapidly spreading influenza epidemic (*15*). As shown in our study, an attack rate of symptomatic influenza infection of 20 percent (34 of the 168 persons studied) was produced by the A2/65 virus. In past studies, antigenic variations of influenza virus were recognized primarily because the new isolates were not inhibited, or were poorly inhibited, by antiserums to older strains. In our study, a different situation was noted. The newly isolated A2/65, JRH strain, was inhibited by antiserum to older A2 strains, including those of 1957 and 1961, at a titer comparable to the homologous system. Antiserums prepared against the A2/65 virus strain, however, were poor inhibitors of older A2 viruses. Weinberger and co-workers have reported similar results with their 1963 isolates (*3*, *4*). The significance of minor antigenic differences between the A2 strains is uncertain, but it is worthwhile to note the distinctiveness of the A2/65 virus.

In previous studies, antiserums to influenza viruses were usually prepared in roosters. Ferrets and mice were also frequently used, but monkeys were not commonly used. In our study, the antigenic distinctiveness of the 1965 virus was clearly demonstrated in monkey serum. Apparently, the monkeys were highly selective to influenza virus infection and were satisfactory for the production of antibodies to the influenza virus strains used.

Summary

During the winter months of 1964 to 1965, an epidemic of influenza occurred in the area of New Haven, Conn. A total of 93 single and 75 paired blood specimens were collected from 168 members of the Yale Medical School staff and students. The virus responsible for the outbreak belonged to the A2 type of influenza virus but was found to differ antigenically from the A2 strains isolated in earlier years.

Roosters, ferrets, hamsters, and monkeys were used for immunization. Antiserums produced by older A2 strains inhibited the new isolate in both hemagglutination-inhibition and neutralization tests, but serums produced in animals with the A2/65 virus proved to be poor inhibitors of the older virus strains. This observation was demonstrated most clearly in serums obtained from monkeys immunized with the A2/65 virus. The A2/65 virus produced distinct plaques under agar medium, and the plaque-reduction neutralization method was found to be useful for the study of the antigenic properties of A2 influenza viruses.

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