STUDIES ON THE COMPLEMENT FIXATION ANTIGENS OF INFLUENZA VIRUSES TYPES A AND B*

BY MARIA WIENER, PH.D., WERNER HENLE, M.D., AND GERTRUDE HENLE, M.D.

(From the Department of Pediatrics, School of Medicine, University of Pennsylvania, the Children's Hospital of Philadelphia, and the Department of Bacteriology, Woman's Medical College of Pennsylvania, Philadelphia)

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Allantoic fluids collected from chick embryos infected with the viruses of influenza have been shown to contain at least two types of specific particles which can be separated by differential centrifugation. The larger of these particles possesses a sedimentation constant of between 600 and 800 S (1–4) and is referred to as "600 S component" in the present report. From the sedimentation constants and filtration data (3, 5) the diameter of the particle has been calculated to measure between 80 and 120 mμ, a value which has been confirmed by electron microscopy. The second component is smaller and has been found to possess a sedimentation constant of approximately 30 S (2, 6) indicating a diameter of about 10 mμ in agreement with electron microscopical measurements (6, 7). Difficulties in the sedimentation by centrifugation of the smaller component have been reported (8) and it is possible that the constant of 30 S may change with increased understanding of the experimental conditions. However, the term "30 S component" has been adopted for the present report as a convenient designation for the small particle. In addition, some antigen was found to remain in the allantoic fluids after removal of the 600 S and 30 S components (9, 10) which, for the time being, has been called "<30 S fraction." This material remained in the supernatant fluid upon repeated centrifugation or after centrifugation against sucrose density gradients. This fraction, which behaves serologically like the 30 S component, has not been studied to any extent.

Analysis of the properties of the 600 S and 30 S components has revealed marked differences. The 600 S fraction carries the infectivity (1–4), it agglutinates chicken red cells (1–4), it produces toxic lesions in mice (11, 12), it is responsible for the interference phenomenon (13), possesses immunizing capacity (14), and acts as antigen in the complement fixation test (10, 15). In short, the 600 S material has all the attributes of the virus. The 30 S component, on the other hand, shows little infectivity, and this little presumably caused by contamination with 600 S particles (2), it does not agglutinate red cells, it is not toxic, does not cause interference, and is active only in the complement fixation test (10). The immunizing capacity of the 30 S material has not been studied as yet. This summary reveals only one common property

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between the two components, their ability to react as antigens in the complement fixation test with specific immune sera against influenza. However, even this property is based predominantly on different antigenic constituents, as has been briefly reported in a previous communication (10). It is the aim of this report to extend this observation and to explore the rôle played by the two components in the serological response of human beings to infection with or vaccination against influenza viruses.

Materials and Methods

Antigens.—(*a*) Allantoic fluid. The PR8, F-12, and F-99 strains of influenza A and the Lee strain of influenza B virus were used in these experiments. The virus was propagated in the allantoic cavity of 10-day-old chick embryos during an incubation period of 48 to 72 hours at 36-37°C. Thereafter the eggs were chilled, and the blood-free allantoic fluids were collected. After centrifugation at 2000 R.P.M. for 10 minutes, to remove larger aggregates of debris, the supernatant fluids were saved and stored in suitable volumes at −10°C. until needed for the tests. In later tests the fluids were dialyzed aganst 0.01 M phosphate-buffered saline solution of pH 7.0 before storage, which reduced the amount of precipitate formed on thawing of the frozen preparations. Normal allantoic fluid was collected and prepared in a similar manner.

(*b*) 600 S component. Part of the allantoic fluids, collected as described, were subjected to high speed centrifugation at 20,000 R.P.M. for 20 minutes, a speed sufficient for sedimentation of most of the virus. The supernatant fluids were saved for preparation of the 30 S components. The sediments were resuspended in buffered saline solution in a fraction of the volume of the original allantoic fluid. The particles were sedimented again by centrifugation at 20,000 R.P.M. for 20 minutes, resuspended, and stored in suitable volumes at −10°C. until used. The final preparation contained per milliliter the virus particles obtained from 32 to 64 ml. of allantoic fluid.

(*c*) 30 S component. The supernatant fluids, after removal of the bulk of the 600 S particles, were centrifuged at 30,000 R.P.M. for 60 minutes. The sediment was suspended in 1/32nd to 1/64th of the original volume using buffered saline solution and centrifuged at 20,000 R.P.M. for 20 minutes to reduce contamination with 600 S material. This procedure was repeated once or twice until the hemagglutination test became negative. The antigen was stored at −10°C.

(*d*) Particulate components of normal chorio-allantoic membranes. Membranes from normal 12-day-old chick embryos were emulsified in buffered saline solution by means of a Waring blendor. After preliminary centrifugation at 5000 R.P.M. for 30 to 60 minutes the supernatant fluid was again centrifuged at 20,000 R.P.M. for 20 minutes. The pellets were suspended in buffered saline solution so that 1 ml. contained the particles obtained from 100 mg. of wet tissue. The material was stored at −10°C. until needed. The antigen was titrated against a rabbit serum prepared by injection of these particles. The optimal dilution of antigen giving the highest serum titer was used in the tests.

(*e*) Alcoholic guinea pig kidney extract (Forssman antigen). The kidneys of several guinea pigs were finely minced with scissors and for every gram of tissue 10 ml. of 95 per cent ethyl alcohol were added. The mixture was left at room temperature with frequent shaking for 6 days. The extracted tissue was then filtered off and the alcoholic extract stored. For the complement fixation tests it was diluted six-fold by adding saline solution to the extract drop by drop under constant agitation. Further dilutions, if required, were made in the usual manner.

Sera.—Sera were obtained from subjects exposed experimentally to active influenza viruses (PR8, F-12, F-99, and Lee) (16) as well as from a few patients convalescent from the epidemic disease. Specimens taken from the various individuals before exposure or during the acute stage of the disease were likewise tested. Sera were also obtained from subjects before and after vaccination with various influenza vaccines. Among the vaccines used were preparations of allantoic fluid, concentrates prepared by the precipitation with protamine or by sedimentation in the Sharples centrifuge. In some cases the effect of adjuvants on vaccination had been studied (17, 18). All sera were inactivated at 56°C. for 30 minutes and stored at −10°C. until needed.

Absorption of the Sera.—For the absorption of sera with the 600 S component an adequate amount of this material was sedimented by centrifugation at 20,000 R.P.M. for 20 minutes. The sediment was resuspended in fourfold diluted serum and left at 4°C. for 48 hours. After centrifugation of the mixture at 32,000 R.P.M. for 90 minutes the supernatant fluid was saved and tested for the absence of hemagglutinins and of anticomplementary activity.

Absorption with the <30 S fraction was carried out according to the technic described elsewhere (9). In a few instances absorption with the 30 S fraction was employed using a technic similar to that used for absorption with the 600 S material except for sedimentation of the 30 S fraction at a higher centrifugal speed (30,000 R.P.M.).

Unabsorbed serum was carried along in all these procedures as a control for the possible loss of antibodies during some of the steps.

Complement Fixation Test.—Dilutions of antigen, serum, and complement were mixed in equal amounts. On account of the difficulties in obtaining some of the antigens in sufficient quantity, and the number of tests planned for individual sera the volume for the various reagents had to be decreased to 0.1 ml. each instead of the 0.2 ml. usually used. After preliminary incubation of the antigen-serum-complement mixtures for 60 minutes at 37°C. 0.2 ml. of sensitized sheep cells (2.5 per cent suspension) was added by automatic pipette. The test was read after further incubation for 1 hour and the results recorded as 0 = no hemolysis. tr = trace; wk = weak; st = strong; ac = almost complete; and c = complete hemolysis; Two units of amboceptor and 1.3 units of Sharp and Dohme lyovac complement as determined by optimal titration technic were employed. All the usual controls were included in each test.

Most of the tests concerning the reactivities of the 600 S and 30 S antigens were carried out according to optimal titration technic; *i.e.*, varying dilutions of antigen were tested with varying dilutions of serum on account of the zone phenomena observed with influenza antigens (19–21). In the study of large numbers of sera for the presence of antibodies to the various components only optimal dilutions of the antigens were employed (usually 4 to 8 units of the 600 S and 2 units of the 30 S components) since it had been found in preliminary tests that the optima were identical for a number of different sera.

All other methods have been described elsewhere. These included agglutination of chicken red cells as an indication of viral concentration (Hirst and Pickels (22)), the inhibition of agglutination of chicken red cells by immune serum (16), and the neutralization test in mice (23).

EXPERIMENTAL

Serological Properties of 600 S and 30 S Components

In a previous publication differences have been demonstrated between the 600 S and 30 S components of influenza virus preparations by serological means (10). These became particularly apparent upon careful absorption of sera of patients convalescent from epidemic or experimental influenza with one or

the other of the components. Table I summarizes an experiment of this kind with two specimens of serum taken from a patient before and 2 weeks after experimental infection with the Lee strain of influenza B virus. The various preparations, *i.e.* the preinhalation serum, the non-absorbed convalescent serum, and the samples absorbed with either a small amount of the homologous 600

TABLE I

Effect of Absorption of Human Convalescent Serum from a Case of Experimental Influenza B with Various Fractions Derived from Influenza A and B Preparations

Serum	Serum absorbed with	Titer of serum as measured by							
		Inhibition of agglutination of chicken erythrocytes	Neutralization test in mice (1,000 LD_{50}) Lee virus	Complement fixation test with					
				Influenza B (Lee)			Normal membrane particles	Alcoholic guinea pig kidney extract	Saline solution
				Allantoic fluid	600 S component	30 S component			
85 A (Before inhalation)	—	<1:16	<1:4	<1:4	<1:4	<1:4	<1:8	<1:8	<1:4
85 B (2 wks. after inhalation)	—	1:256	1:128	1:64	1:32+	1:64	<1:8	<1:8	<1:4
	600 S Lee	1:8	<1:4	1:32	<1:4	1:32			<1:4
	<30 S Lee	1:96	1:96	1:32	1:32	1:8			<1:4
	600 S PR8	1:256	1:64	1:64	1:32	1:64			<1:4
	<30 S PR8	1:128	1:64	1:64	1:32	1:64			<1:4
	Normal membrane particles	1:256	1:128	1:64	1:32	1:64	<1:8	<1:8	<1:4
Rabbit serum against normal membrane particles	—			<1:20	<1:20	<1:20	1:320	1:80	<1:20
	Sheep erythrocytes						1:80	<1:20	<1:20
Saline solution				c*	c	c	c	c	c

* c = complete hemolysis.

S or the 30 S fraction, were tested for their capacity to inhibit the agglutination of chicken red cells by the homologous virus, their ability to neutralize the infectivity for mice, and to fix complement in the presence of infected allantoic fluid, the 600 S and 30 S components. A comparison of the data listed in Table I shows that exposure to active Lee virus resulted in rises of the antibody titer as measured by all tests. Whereas the various reactions proved negative with the preinhalation serum, high antibody titers were recorded by the various tests

using the convalescent serum. Absorption of the convalescent serum with a small amount of the homologous 600 S component removed not only the ability of the serum to react with the usually employed concentration of the 600 S particles (4 to 8 units) in the complement fixation test but also its capacity to inhibit the agglutination of chicken erythrocytes and to neutralize the infectivity of the Lee virus for mice. On the other hand, the titer of complement-fixing antibodies against the original allantoic fluid and the 30 S component was only slightly reduced by absorption of the serum with the 600 S component. After absorption of the convalescent serum with the <30 S material of the homologous virus the complement fixation reaction with the 30 S component showed a marked decrease in serum titer, but not when the original allantoic fluid or the 600 S fraction were used as antigens. The capacity to inhibit the agglutination of red cells and to neutralize the infectivity for mice was only slightly impaired in the serum absorbed with the <30 S fraction. It should be noted that in other, less extensive experiments antibodies against the 30 S component have been removed completely by absorption of the sera with the <30 S fraction without appreciably altering the reaction of the serum with the 600 S component. Similar results were obtained, using the various fractions of the PR8, F-12, and F-99 strains of influenza A.

In control tests the convalescent serum was absorbed with the two corresponding components of the heterologous type of virus; *i.e.*, in the cited experiment with the fractions derived from the PR8 strain of influenza A. No significant changes occurred in the various tests following absorption with these materials. Such slight decreases in titer as were noted in these control tests as well as in some tests with the components of the homologous virus may well be explained by the limits of the experimental technics. The small volumes of the reagents necessarily employed in the various tests did not favor their accuracy. Furthermore, preparations of one of the particles were never entirely free of those of the other size.

In other sets of controls the absence from the sera of antibodies to normal chick and Forssman antigens was ascertained. In the experiment recorded in Table I the patient's sera did not react with particulate components of normal chorio-allantoic membranes nor with alcoholic extract of guinea pig kidney (Forssman antigen). On the other hand, serum of a rabbit immunized with normal membrane particles reacted in high dilution with the homologous antigen or with the kidney extract but not to any appreciable extent with the dilutions of the influenza preparations used in the recorded test. Absorption of the rabbit serum with sheep erythrocytes removed all antibodies against the guinea pig kidney extract but only part of the antibodies to the normal membrane particles, indicating that both Forssman and particle-specific antibodies were involved in the reaction. It should be stated here that other preparations of the influenza fractions have given reactions with the rabbit anti-normal

particles serum in agreement with the observations of others (8, 24) and that a few human sera were encountered in this study which contained complement-fixing antibodies against particles derived from normal membranes but rarely against Forssman antigen. Some of these were preinhalation or prevaccination sera. In other instances these antibodies developed as a result of vaccination. These occasional findings demonstrate the possible interaction of such non-specific reactions in complement fixation tests for influenza and necessitate adequate controls to guard against them. All sera, therefore, were tested against the normal membrane particles as well as with Forssman antigens. A sample of protein prepared by high speed centrifugation from normal allanotic fluid (8) was kindly supplied by Dr. Knight. It did not react in the complement fixation test using human influenza convalescent sera.

The above experiment demonstrated that definite serological differences existed between the two components. However, in further tests, employing various technics, it could be shown that the 600 S component contains some of the 30 S-type antigen. Fresh samples of the convalescent serum used in the preceding experiment were absorbed either with a small amount of the 600 S component or with the 30 S fraction. The absorbed sera were then tested in varying dilutions with increasing concentrations of the two fractions. The results of this experiment are recorded in Table II. The unabsorbed serum showed the different relationships frequently encountered in the optimal titrations with the two fractions. With increasing concentration of the 600 S component the antibody titer rose until the maximum was reached with the use of 8 to 16 units of antigen. The opposite held true for the 30 S component since the minimal reacting dose of antigen gave the highest serum titer. A line drawn through the last dilutions of serum giving complete fixation of complement ("O" in the table) in the presence of various concentrations of the 600 S particles described a wide curve, whereas a similar line drawn through the end points in the 30 S reaction revealed a sharply angular shape.

Absorption of the serum with the 600 S fraction produced a decrease in antigen titer (shift of the minimal dose from 1:64 to 1:4), whereas the optimal serum titer showed practically no change. This finding could be explained by the presence of at least two distinct antigens in the 600 S particle. One of these exceeded the other markedly in quantity or sensitivity. Therefore, removal of antibodies against the dominant antigen could be achieved without markedly altering the reaction with the lesser antigen. The pattern of this remaining activity did not show the "falling off" curve considered characteristic of the 600 S-specific antigen but revealed rather the same optimal relationships observed with the 30 S component. Absorption of the serum with the 30 S component again, removed the antibodies against these particles but left the reaction with the 600 S-specific antigen unaltered.

The reaction pattern of the 600 S component was not always as different from

that of the 30 S fraction as the one recorded in Table II. Different preparations were found to vary somewhat in this respect (see Table V). This variation may depend in part on the amount of the 30 S-type antigen linked to the 600 S particle. The quantity of the 30 S antigen in the 600 S component may depend, in turn, on the conditions under which the virus was cultivated; *i.e.*,

TABLE II

Demonstration of 30 S Antigen in Higher Concentrations of 600 S Particles

Convalescent serum 85 B (experimental influenza B)		Antigens													
		600 S component (Lee) in dilutions								30 S component (Lee) in dilutions					Saline solution
Absorbed with	Initial dilution of serum	Undiluted	1:2	1:4	1:8	1:16	1:32	1:64	1:128	Undiluted	1:2	1:4	1:8	1:16	
—	1:4	0	0	0	0	0	0	0	tr	0	0	0	tr	ac	c
	1:8	0	0	0	0	0	0	0	tr	0	0	0	tr	ac	c
	1:16	0	0	0	0	0	0	0	tr	0	0	0	tr	ac	c
	1:32	0	0	0	0	0	0	tr	st	0	0	0	tr	ac	c
	1:64	0	0	0	0	0	tr	ac	c	tr	0	0	wk	ac	c
	1:128	st	tr	0	tr	wk	c	c	c	c	st	0	ac	c	c
600 S component (Lee)	1:4	0	0	0	tr	st	st	c		0	0	0	wk	ac	c
	1:8	0	0	0	tr	st	c	c		0	0	0	wk	ac	c
	1:16	0	0	0	tr	st	c	c		0	0	0	wk	c	c
	1:32	0	0	0	wk	ac	c	c		tr	0	0	wk	c	c
	1:64	tr	0	0	ac	c	c	c		c	tr	tr	st	c	c
30 S component (Lee)	1:4			0	0	0	0	0			tr	st	ac	c	c
	1:8			0	0	0	0	0			wk	ac	c	c	c
	1:16			0	0	0	0	0			st	c	c	c	c
	1:32			0	0	0	0	wk			c	c	c	c	c
	1:64			tr	0	0	tr	ac			c	c	c	c	c
Saline solution		c	c	c	c	c	c	c		c	c	c	c	c	c

0 = no hemolysis; tr = trace; wk = weak; st = strong; ac = almost complete; c = complete hemolysis.

on the concentration of the inoculum used for the chick embryos, the duration of the incubation period, and possibly other factors.

The experiment recorded in Table II indicated, then, that 30 S material formed part of the 600 S component. Absorption of convelescent sera with an excess of the 600 S fraction should, therefore, remove the antibodies against both components. This was actually the case as shown in Table III. On the other hand, absorption of the convalescent serum with an excess of 30 S ma-

terial removed the reaction with the 30 S component only and not that with the 600 S-specific antigen.

Further proof of the presence of 30 S antigen in the 600 S particles was obtained by liberating 30 S material from the 600 S component by mechanical means. A large batch of the 600 S component was prepared in the usual man-

TABLE III

Effect of Massive Absorption with the 600 S Fraction on 30 S Antibody

Convalescent serum 97 B (experimental influenza A)		Antigens								
		600 S component (F-99) in dilutions				30 S component (F-99) in dilutions				Saline solution
Absorbed with	Initial dilution of serum	1:8	1:16	1:32	1:64	1:8	1:16	1:32	1:64	
—	1:8	0	0	0	0	0	0	0	0	c
	1:16	0	0	0	0	0	0	0	0	c
	1:32	0	0	0	0	0	0	0	0	c
	1:64	0	0	0	tr	tr	tr	0	0	c
	1:128	tr	tr	wk	wk	ac	st	tr	0	c
	1:256	st	st	ac	ac	c	c	ac	wk	c
600 S component (F-99) in excess	1:4	st	ac	ac	ac	st	st	wk	wk	c
	1:8	ac	ac	c	c	c	c	c	c	c
	1:16	c	c	c	c	c	c	c	c	c
	1:32	c	c	c	c	c	c	c	c	c
	1:64	c	c	c	c	c	c	c	c	c
	1:128	c	c	c	c	c	c	c	c	c
	1:256	c	c	c	c	c	c	c	c	c
30 S component (F-99)	1:8	0	0	0	0	st	c	c	c	c
	1:16	0	0	0	0	ac	c	c	c	c
	1:32	0	0	0	0	c	c	c	c	c
	1:64	tr	0	0	0	c	c	c	c	c
	1:128	st	st	wk	wk	c	c	c	c	c
Saline solution		c	c	c	c	c	c	c	c	c

ner and divided into two lots. One part was subjected to vibration for 1 hour in the treatment vessel of a magnetostriction oscillator at 90,000 cycles per second (25), the other part was used as control. Both the untreated and the vibrated suspensions were centrifuged at 20,000 R.P.M. for 20 minutes, a procedure insufficient for the sedimentation of the 30 S material but adequate for the almost complete removal from suspension of the 600 S component. The supernatant fluids of the native and sonically treated material, as well as the untreated and vibrated 600 S components were used as antigens in the comple-

ment fixation test. The results are recorded in Table IV. It is evident from the table that sonic vibration released more serologically active material from the 600 S particle than was obtained in the untreated specimens. This released material behaved like the 30 S component since it was not sedimented by centrifugation at 20,000 R.P.M. and reacted with antibodies to 30 S material; *i.e.*, with a serum from which antibodies to the 600 S-specific antigen had been removed by absorption. This result furnishes evidence that the 30 S component can be released from the 600 S particle.

TABLE IV

Liberation of the 30 S Antigen from the 600 S Fraction by Supersonic Oscillation

Convalescent serum 85 B (experimental influenza B)		Antigen in optimal dilution					
		600 S component (Lee)		Supernatant fluid		30 S component (Lee)	Saline solution
Absorbed with	Initial dilution of serum	Native 1:8	Sonic 1:4	Native Undiluted	Sonic 1:2	Native 1:4	
—	1:4	0	0	0	0	0	c
	1:8	0	0	tr	0	0	c
	1:16	0	0	tr	0	0	c
	1:32	0	0	tr	0	0	c
	1:64	0	wk	tr	0	0	c
	1:128	st	c	st	st	0	c
	1:256	c	c	c	c	c	c
600 S component (Lee)	1:4	st	wk	0	0	0	c
	1:8	ac	ac	tr	0	0	c
	1:16	c	c	tr	0	0	c
	1:32	c	c	wk	0	0	c
	1:64	c	c	c	tr	tr	c
	1:128	c	c	c	c	c	c
Saline solution		c	c	c	c	c	c

Differences in the Antibody Response of Human Beings to the 600 S and 30 S Components

The data presented thus far demonstrate the existence of serological differences between the two components, the 600 S and 30 S fractions. In further studies it became apparent that the antibody response of human beings varied in regard to these two components. Table V shows, as an example, optimal antigen-antibody titrations with sera of two human subjects taken before and 2 weeks after experimental exposure to active influenza A virus. Patient 110 responded to the exposure with high fever (104°F.) and developed antibodies against both the 600 S and 30 S components. On the other hand, the convales-

cent serum of patient 108, who showed no febrile reaction following the exposure, contained antibodies against the 600 S component only and not against the 30 S fraction.

Further tests furnished ample evidence that the formation of antibodies to the 30 S component was a fairly regular result of the natural or experimental

TABLE V

Difference in Antibody Response to the 30 S Component after Experimental Exposure to Active Virus of Influenza A

Serum	Inhibition of hemagglutination titer	Initial dilution of serum	Antigens														
			600 S component (F-99) in dilutions							30 S component (F-99) in dilutions							Saline solution
			1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:8	1:16	1:32	1:64	1:128	1:256	1:512	
108 A (before inhalation)	1:32	1:4	c	c	c					c	c	c					
108 B (2 wks. after inhalation)	1:256	1:8	0	0	0	tr	wk	ac	c	st	st	st	st	st	c		c
		1:16	0	0	0	tr	wk	ac	c	st	st	st	st	st	c		c
		1:32	0	0	0	0	tr	ac	c	st	st	c	c	c	c		c
		1:64	st	st	st	st	st	ac	c	st	c	c	c	c	c		c
		1:128	c	c	c	c	c	c	c	ac	c	c	c	c	c		c
		1:256								c	c	c	c	c	c		c
110 A (before inhalation)	1:16	1:4	c	c	c					c	c	c					c
110 B (2 wks. after inhalation)	1:64	1:8	0	0	0	0	tr	ac	c	0	0	0	0	tr	ac	c	c
		1:16	0	0	0	0	tr	ac	c	0	0	0	0	tr	c	c	c
		1:32	0	0	0	0	tr	c	c	0	0	0	0	wk	c	c	c
		1:64	0	0	0	0	st	c	c	0	0	0	0	tr	ac	c	c
		1:128	0	0	0	0	st	c	c	wk	0	0	0	tr	ac	c	c
		1:256	tr	tr	tr	wk	c	c	c	c	c	ac	st	st	(c)	c	c
		1:512	st	st	st	c	c	c	c	c	c	c	c	c	c	c	c
Saline solution			c	c	c	c	c	c	c	c	c	c	c	c	c	c	c

disease, whereas failure of antibody production against the 30 S component was rather the rule following vaccination and only infrequently noted after exposure to active virus. Support for this statement is given in Table VI. This table shows the incidence of reactions with the 600 S and 30 S components of the PR8 and Lee strains in human sera, taken either before and after epidemic infection or inhalation of active virus of the homologous type (90 pairs of sera) or before and after vaccination with inactivated viruses of influenza A and B (69 pairs). The individual sera were tested simultaneously with the PR8 and

Lee components in optimal dilution as well as with normal membrane particles and Forssman antigen. As can be seen in the table slightly more than 50 per cent of the preinhalation and prevaccination sera reacted with the 600 S components of influenza A and B. After the experimental or epidemic disease, or after vaccination between 93 and 100 per cent of the sera gave positive tests with this fraction. On the other hand, the incidence of reactions with the 30 S components of influenza A virus rose from 24 to 76 per cent following inhalation of active virus of the homologous type or epidemic disease, but only from 15 to 23 per cent following vaccination. In the case of exposure to the Lee

TABLE VI

Incidence of Reactions with 600 S and 30 S Components in Human Beings Following Epidemic and Experimental Exposure to Active Virus or Vaccination with Inactive Virus

Sera	Virus used for antigens	Antigens							
		600 S component				30 S component			
		Positive		Negative		Positive		Negative	
		No. of subjects	Per cent	No. of subjects	Per cent	No. of subjects	Per cent	No. of subjects	Per cent
Preinhalation (or pre-epidemic)	PR8	27	46.5	31	53.5	14	24	44	76
Postinhalation or post-epidemic)		54	93	4	7	44	76	14	24
Prevaccination	PR8	37	54.5	31	45.5	10	15	58	85
Postvaccination		65	96	3	4	16	23	52	77
Preinhalation	Lee	16	50	16	50	3	9	29	91
Postinhalation		32	100	0	0	31	97	1	3
Prevaccination	Lee	41	59.5	28	40.5	15	22	54	78
Postvaccination		69	100	0	0	28	40.5	41	59.5

strain of influenza B the incidence of positive tests with the homologous 30 S component rose from 9 to 97 per cent following inhalation of active virus, whereas the corresponding figures for vaccination showed only an increase from 22 to 40 per cent. In examining the clinical records of the fourteen subjects, whose sera did not react with the 30 S component after inhalation of influenza A virus it was found that eight had no signs or symptoms, the other six reacted with an elevation of temperature but showed only insignificant or no rises in neutralizing antibodies as measured by the inhibition of hemagglutination. The one individual in the Lee inhalation series showed a febrile response and a slight rise in neutralizing antibodies. On the other hand, not all subjects responding with 30 S antibodies had febrile reactions following the inhalation of virus.

These figures suggest that antibodies to the 30 S component are formed in a

high percentage of subjects following exposure to active virus under either experimental or epidemic conditions, but only rarely following vaccination with inactivated virus. These differences in the two groups of sera were actually more striking. Table VI gives merely the incidence of sera reacting with the two components regardless of the concentration of the antibodies. However, considering the titers against the various antigens further differences became

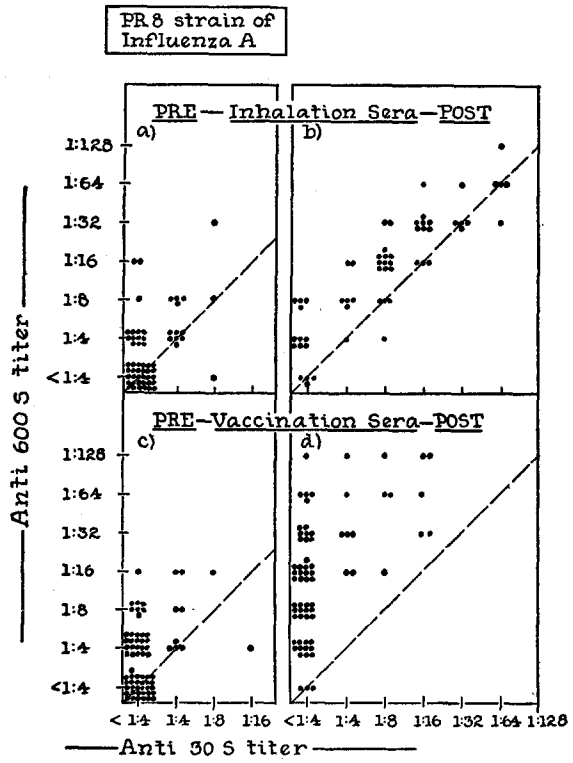

FIG. 1. Correlation between 600 S and 30 S antibodies in sera taken from subjects before and after exposure to or vaccination with influenza A virus.

apparent among the two groups. In Figs. 1 and 2 the 600 S antibody titers were plotted against the 30 S titers of individual sera. Each dot represents, therefore, the results obtained with one serum, giving the anti-600 S titer on the ordinate and the titer of 30 S antibodies on the abscissae. Fig. 1 records the results of the influenza A reactions separately for the different types of sera; *i.e.*, specimens taken before and after experimental exposure and epidemic disease and sera taken before and after vaccination. The corresponding values for the influenza B series are shown in Fig. 2.

The conclusions drawn from these two figures may be summarized as follows:

The preinhalation and prevaccination sera showed a similar incidence of sera with 600 S and 30 S antibodies, or with 600 S antibodies only. Postinhalation sera possessed anti-30 S titers equal to or not less than ¼ that of the 600 S antibody levels. This is demonstrated by the fairly narrow diagonal band of dots in Figs. 1*b* and 2*b*. Among the postvaccination sera only a few showed anti-30 titers of similar height as the

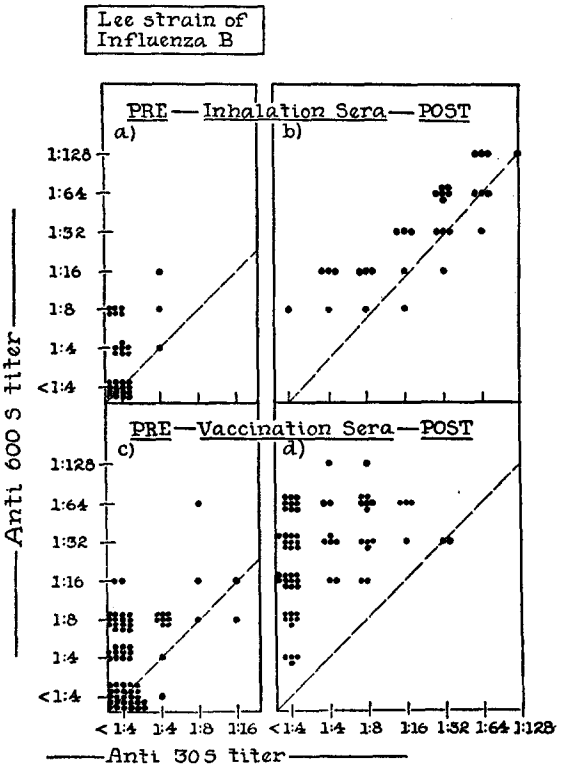

FIG. 2. Correlation between 600 S and 30 S antibodies in sera taken from subjects before and after exposure to or vaccination with influenza B virus.

anti-600 S levels and the dots were spread, therefore, over the whole upper triangular field of the chart (Figs, 1*d* and 2*d*). If there were 30 S antibodies they were present usually only in low titer as compared to the levels observed in the postinhalation sera. Some of the cases in the vaccination group who possessed antibodies against the 30 S component before vaccination maintained the same antibody level to the 30 S fraction after vaccination but showed an increase in the anti-600 S titer. Only a relatively small number of individuals, as shown in Table VI, acquired anti-30 S by vaccination. It should be pointed out that, thus far, only sera from individuals injected with one of three

different vaccines have been studied. It is possible that other vaccines prepared by the same or different methods may induce formation of 30 S antibodies more readily.

The Significance of 600 S and 30 S Antibodies

Discrepancies between the results of neutralization tests in mice and the complement fixation reaction have been reported repeatedly (26–31). No such

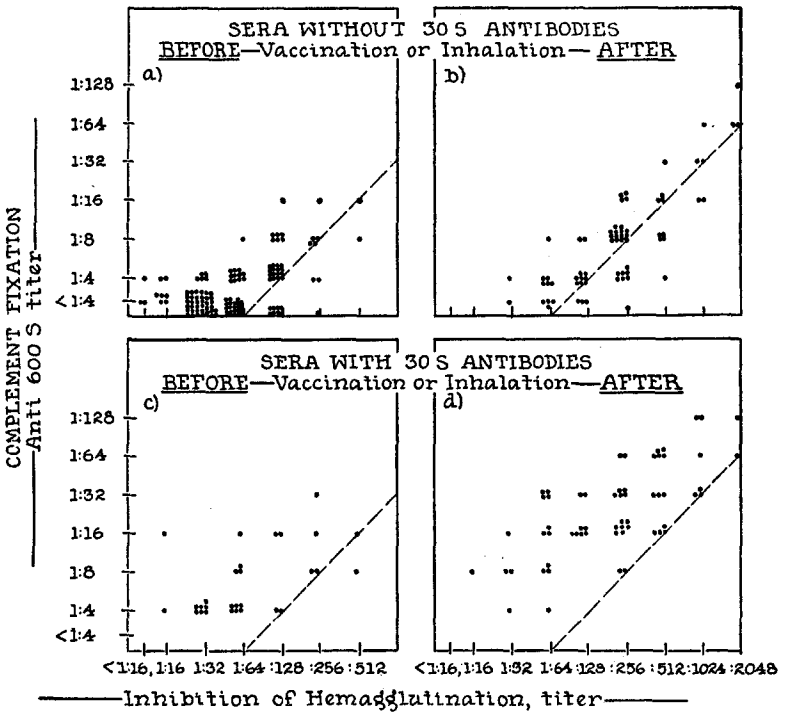

FIG. 3. Correlation between antibody titers obtained in the inhibition of hemagglutination and complement fixation with the 600 S component in the case of influenza A.

studies are available as yet as to comparisons between the inhibition of the hemagglutination and the complement fixation reactions. However, since the neutralization and inhibition tests were found to be correlated (32) it may be expected that similar discrepancies, as noted between neutralization and complement fixation tests, will be observed also between the inhibition and complement fixation reactions.

The data obtained with the two separate components of influenza virus preparations as antigens were analyzed in regard to these relationships. In Figs. 3 and 4 the anti-600 S titers as determined by complement fixation tests were plotted against the antibody levels measured in the inhibition of hemagglu-

tination. Fig. 3 contains the data obtained in the influenza A reactions, Fig. 4 those for influenza B. Differentiation was made in these figures between sera containing 30 S antibody and those lacking measurable amounts of it, as well as between sera taken before and after experimental exposure and vaccination. In contrast to the postinhalation and postvaccination sera, specimens taken before the exposure to or immunization with the influenza viruses represent samples from populations with unknown influenza histories, involving possibly a variety of different strains of the agents. It is possible that for this reason sera

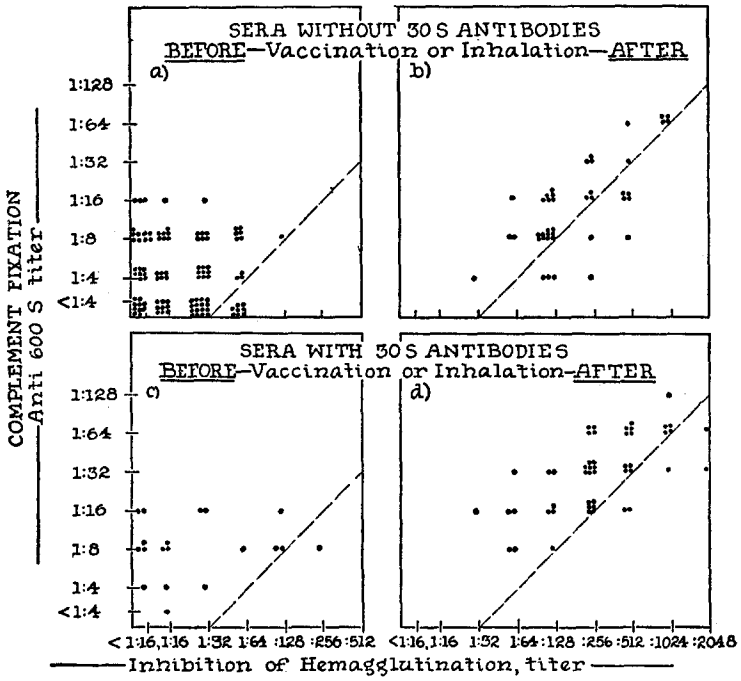

FIG. 4. Correlation between antibody titers obtained in the inhibition of hemagglutination and complement fixation with the 600 S component in the case of influenza B.

taken at a given time interval following contact with a known strain of virus yielded clearer results. In the absence of 30 S antibodies these sera gave a fairly close correlation between the inhibition and the 600 S antibody titers which is expressed in the rather narrow diagonal band of dots in Figs. 3*b* and 4*b*. The number of sera studied, thus far, is limited and does not justify calculation of the geometric mean anti-600 S levels for a given inhibition titer. In spite of this limitation it seems clear that a linear relationship exists between the two reactions approximately expressed in the diagonal lines. Most dots fall either on or one step above or below these lines. Only three dots, both in the PR8 and Lee series, were outside this range. It is felt, that these irregu-

larities may be the result of variations in the individual tests and of the technical difficulties discussed above. Under the experimental conditions employed, the titer obtained by the inhibition of hemagglutination was about 32 times that of the anti-600 S titer in the case of the tests with PR8 virus and about 16 times greater in the case of the Lee strain.

In contrast to the postvaccination or postinhalation sera which were free of measurable quantities of anti-30 S, those containing this antibody did not show such a close relationship between the two tests. In comparing these charts (Figs. 3*d* and 4*d*) with the diagonal band of dots discussed above, it can be seen that a much wider spread of dots was obtained particularly in the case of

TABLE VII

Incidence of Significant Rises in Antibody Titers as Measured by Various Tests in Febrile Cases Following Experimental Exposure or Epidemic Disease*

Inhibition of hemagglutination	Complement fixation with		PR8 antigens		Lee antigens		Total	
	600 S component	30 S component	No. of cases	Per cent	No. of cases	Per cent	No. of cases	Per cent
+	+	+	17	48.5	18	75	35	60
+	+	−	3	8.5	0		3	5
+	−	+	0		0		0	
+	−	−	0		0		0	
−	+	+	4	11	1	4	5	8
−	+	−	2	6	1	4	3	5
−	−	+	2‡	6	2‡	8.5	4	7
−	−	−	7	20	2	8.5	9	15
Total No. of cases.....			35	100	24	100	59	100

* Fourfold rise or better.

‡ One of these sera showed a high titer in the inhibition of hemagglutination before exposure.

the reactions with the PR8 preparations. No attempt was made to differentiate between different heights of the anti-30 S titers in these charts.

In studying the preinhalation and prevaccination sera, which did not contain anti-30 S (Figs. 3*a* and 4*a*) it may be seen that many do not seem to follow the same pattern as observed with the postinhalation and postvaccination sera. Some of the sera contained appreciable concentrations of antibodies to the 600 S component but very low inhibition titers, particularly in the case of the influenza B tests. Non-specific reactions with chick proteins and Forssman and Wassermann antigens have been excluded since only very few of the sera reacted with these antigens. The possibility of other specific antigens remains to be explored.

The above data indicate that the inhibition of hemagglutination and the complement fixation reactions with 600 S-specific antigens measure probably

the same antibodies. Discrepancies between the two tests may be observed if the sera contain anti-30 S. The rôle played by the two components was further studied in comparing the various reactions in their diagnostic value. Since only ten pairs of sera from epidemic cases were available and most tests were performed with sera taken from subjects with febrile reactions after experimental exposure to active influenza virus (54 cases) the results recorded in Table VII must be considered as preliminary until further studies in large series with sera from epidemic cases have been completed. As can be seen in the table, 65 per cent of all epidemic and experimental cases showed significant rises in antibodies of four fold or greater in the inhibition of hemagglutination as well as in the complement fixation tests with the 600 S and 30 S preparations as antigen. Fifteen per cent of the cases failed to show a response in any of the tests or gave only suggestive results. Twenty per cent of the cases gave significant increases in the complement fixation reaction but only doubtful or negative responses in the inhibition test. In four of these subjects, who possessed partly high initial titers in the inhibition test, the increase in the complement fixation reaction was based solely on the 30 S component. All individuals who failed to show a significant response by complement fixation technic likewise revealed no significant increase in titer in the inhibition test. The complement fixation test, therefore, may be of additional value in the diagnosis of influenza.

DISCUSSION

By using centrifugation technic Hoyle and Fairbrother (33) showed that the supernatant fluid of suspensions of mouse lungs infected with influenza A virus contained a "soluble antigen" whereas the separated infectious "elementary bodies" failed to react in the complement fixation test. Lennette and Horsfall (34) confirmed the presence of a soluble antigen in mouse lungs but noted in addition that the virus particles always contained some complement fixation antigen. No serological differences between the two fractions were noted. The reported sedimentation data strongly suggest that this "soluble antigen" is identical with the 30 S component of infected allantoic fluids. Observations made simultaneously by Friedewald (15) and in this laboratory (9) showed definite physical and biological differences between two antigenic fractions derived from allantoic fluids by differential centrifugation. The data summarized in the present paper demonstrate distinct serological differences between the two particulate components isolated from infected allantoic fluids. The larger of the particles, the 600 S component, which possesses all the attributes of the virus, as stated in the introduction to this paper, can be differentiated from the 30 S component by the pattern of the optimal antigen-antibody reactions, by cross-absorption of sera with the two particles, and by the use of human sera reacting either with the 600 S component only (mostly post-vaccination sera), or with both particles (mostly convalescent sera).

These findings imply that the major part of the antigens present in the 600

S component is different from that of the 30 S fraction. It was possible, however, to demonstrate in the larger particle the presence of some antigen identical with that of the 30 S component. This was accomplished by the use of high concentrations of the 600 S component in the complement fixation tests with sera absorbed by small amounts of 600 S particles, which retained antibodies to the 30 S component; or by the release of 30 S antigen from preparations of the 600 S particle by intense sonic vibration. Furthermore, in earlier experiments strain-specific as well as type-specific reactions have been shown in tests with the 600 S components of influenza virus by the use of animal or human sera (10, 35). Thus the 600 S particles contain a number of antigenic constituents, which are in part specific for the 600 S component (type-specific as well as strain-specific), in part identical with the antigen of the 30 S fraction.

The 30 S antigen appears to be type-specific only and not strain-specific (10). However, 30 S preparations of a greater number of strains have to be studied before this point can be accepted. Since it seems very likely that the "soluble antigen" in suspensions of infected mouse lungs is identical with the 30 S preparation of allantoic fluid the data reported by Lennette and Horsfall (36) can be taken as additional evidence for the absence of strain-specificity of this fraction.

The relation of the complement fixation tests with the various components to other serological reactions has not been studied to any extent. However, absorption of sera with the 600 S particles rendered them ineffective in the neutralization test and in the inhibition of hemagglutination. Removal from the sera of the 30 S antibodies causes only insignificant changes in these reactions. It is obvious, then, that the complement fixation reaction with the 30 S component does not assay antibodies involved in the tests of neutralization or inhibition of hemagglutination. On the other hand, complement fixation tests with the 600 S component with sera free of 30 S antibodies gave a fairly close correlation to the inhibition test indicating that identical antibodies were determined under these conditions. This correlation seems to be practically independent of the strain of virus used since human sera show only slight strain-specific differentiation which can be demonstrated in some instances only after absorption of the sera with heterologous strains (10, 35).

It is likely that the differences in the relative concentration of the two components in infected allantoic fluids or other virus preparations, used as antigens in the complement fixation tests, as well as the variation in the response of human beings to these antigens can account for some of the discrepant findings reported in the literature. Thus, Smith (26) and Fairbrother and Hoyle (27) in presenting the first two reports on complement fixation reactions in influenza, showed differences between the neutralization and complement fixation tests. They suggested that neutralization of the virus was caused by a specific antibody which did not cause complement fixation, while a "group antigen" was

active mainly in the complement fixation and to a slight extent only in cross-neutralization. Similar discrepancies were subsequently reported by others (28–31). Differences in the zone phenomena encountered in sera of different species by Nigg *et al.* (21) may also depend on the predominance of one or the other of the antigenic components or on the presence or absence of the 30 S antibody.

Differences in the response of human beings to either infection or vaccination have been noted in a few cases by Smith (26), and by Fairbrother and Hoyle (27) in comparing human convalescent with horse hyperimmune sera. In the light of the above experiments it appears likely, that the antigens used by these authors were predominantly composed of 30 S and insufficient in 600 S particles. The same may account for the failure to demonstrate rises in complement-fixing antibodies in man following vaccination as reported by Morrison, Shaw, Kenney, and Stokes (37).

The fact that the 30 S antibodies appear most frequently upon exposure to active virus and only occasionally following vaccination may be of assistance in epidemiological surveys, when it may be necessary to differentiate between recent infections and residual antibodies from previous vaccinations. The 30 S reaction may be of additional value too, in the diagnosis of influenza since it may give a greater incidence of positive results (significant rises in antibodies) than the test for inhibition of hemagglutination. It may show an increase in titer, on occasion, in subjects with high titers in the inhibition test which did not increase after infection. These suggestive observations made mainly in cases of experimental influenza require confirmation on larger scale with sera from cases of the epidemic disease.

SUMMARY

Two types of specific particles can be obtained from allantoic fluid preparations of influenza A and B virus. The larger particle which possesses all the attributes of the virus and which shows a sedimentation constant of about 600 S was compared with the smaller component (30 S) by complement fixation technic.

The 30 S component can be differentiated from the 600 S particle by the patterns of the optimal antigen-antibody relationships and by cross-absorption of the sera with the two particles.

Some of the 30 S-type antigen can be demonstrated in higher concentrations of the 600 S particles by the use of sera containing only anti-30 S; *i.e.*, sera carefully absorbed with 600 S component. Also, upon sonic vibration of the 600 S particle, serologically active material was released which behaved in every respect like the 30 S antigen.

The response of human beings to these antigens was found to vary. Antibodies to the 30 S component developed in the majority of subjects exposed to

active virus either under epidemic or experimental conditions, but only rarely following vaccination with three different vaccines.

In selecting sera without 30 S antibodies a fairly close correlation between the antibody titers obtained by inhibition of hemagglutination and complement fixation with the 600 S component was obtained. The presence of 30 S antibodies prevented such a correlation.

The reaction with the 30 S antigen may be of value in the diagnosis and study of the epidemiology of influenza.

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