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Complement Fixation Antigens of Influenza Viruses Type A and B.*

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High speed centrifugation of allantoic fluid infected with the virus of influenza A and B at speeds sufficient to sediment almost completely the agent as contained in infected mouse lungs frequently leaves a more or less substantial portion of the virus in the supernate.[1,2] This has suggested that the elementary infectious unit of the virus of influenza was distinctly smaller (about 10 mμ in diameter) than was thought formerly (80-100 mμ[3,4]). This concept seemed to receive support when analytical centrifugation revealed the presence in infected allantoic fluid of a small component with a sedimentation constant of approximately 30 S in addition to material sedimenting at a faster rate (sedimentation constant 800 ± 100 S). Electronmicroscopy confirmed this finding.[5]

Recent studies of the influence of convection on sedimentation in the angle centrifuge showed that complete settling of suspended particles could not be atttained unless convection was counteracted.[6] The addition of a synthetic density gradient to influenza virus preparations was found to prevent resuspension of the agent by convection and it was shown that practically all of the infectivity and hemagglutinating property was linked to the larger particles.[7,8] This fraction on more extended studies by various workers was found to possess a sedimentation constant of between 600 and 800 S[2,7,8,9] and will be referred to hereafter as 600 S component. It was prepared from infected allantoic fluid by 2 cycles of alternate centrifugation at 20,000 and 3,000 r.p.m. for 20 minutes each.

The smaller material (30 S component†) obtained from allantoic fluid after removal of the 600 S component by alternate centrifugation at 30,000 and 20,000 r.p.m. for 60 and 20 minutes, respectively, failed to produce measurable hemagglutination, and possessed only a fraction of the infectivity of the 600 S component as shown by Stanley.[8] Additional purification by centrifugation decreased the infectivity further and it appeared likely that this property in preparations of the 30 S material was due to contamination by small amounts of the larger particles.[8] However, it was obvious that most of the 30 S material in the allantoic fluid resulted from infection with the influenza virus since it was found in high concentrations in cultures of recently isolated strains (F-12 and F-99 of influenza A), in smaller quantity in those of the Lee strain of influenza B or the PR-8 strain of influenza A virus, while very little

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1 Chambers, L. A., and Henle, W., *J. Exp. Med.*, 1943, **77**, 251.

2 Taylor, A. R., Sharp, D. G., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1943, **47**, 261.

3 Elford, W. J., Andrewes, C. H., and Tang, F. F., *Brit. J. Exp. Path.*, 1936, **17**, 51.

4 Elford, W. J., and Andrewes, C. H., *Brit. J. Exp. Path.*, 1936, **17**, 422.

5 Chambers, L. A., Henle, W., Lauffer, M. A., and Anderson, T. F., *J. Exp. Med.*, 1943, **77**, 265.

6 Pickels, E. G., *J. Gen. Physiol.*, 1943, **26**, 341.

7 Friedewald, W. F., and Pickels, E. G., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 261; *J. Exp. Med.*, 1944, **79**, 301.

8 Stanley, W. M., *J. Exp. Med.*, 1944, **79**, 267.

9 Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., Beard, J. W., Feller, A. E., and Dingle, J. H., *J. Immunol.*, 1944, **48**, 129.

† The material sedimentable from allantoic fluid at 90,000 g for 60 minutes after previous removal of the 600 S component has been called 30 S component for the sake of brevity although we are aware that it may be a mixture of a number of different materials (*cf.* Knight[10]).

10 Knight, C. A., *J. Exp. Med.*, 1944, **80**, 83.

material was sedimented from normal allantoic fluid of 10- to 13-day embryos under similar conditions.

Further information was gained as to the nature of these components by using the complement fixation test. The technic used has been described previously.[11] Both the 600 and 30 S components were serologically active *in vitro* and in addition the supernate of infected allantoic fluid after removal of these 2 components still contained some antigen which could not be sedimented by repeated centrifugation at 30,000 r.p.m.,[11] or by centrifugation in the presence of synthetic density gradients. This antigen will be called <30 S component. All human sera used in these tests were free of antibodies against Forssman antigen and chick proteins and they were Wassermann-negative.

The complement fixation tests showed that the reactions with the 600 and 30 S components from infectious allantoic fluids were specific. The tests were positive in the presence of human influenza convalescent serum but not with serum taken before or during the acute stage of the disease. Furthermore, the reactions were type-specific in that the 600 and 30 S components derived from influenza A virus preparations reacted only with influenza A and not with influenza B convalescent serum and conversely, the corresponding component prepared from Lee cultures gave positive tests only with influenza B and not influenza A convalescent serum. Evidence to support this statement is included in Table II. Some preparations of the 600 and 30 S components were kindly supplied by Dr. W. M. Stanley. These had been studied in the analytical centrifuge and had shown single boundaries.

The serological activity of the 30 S material could not be due to contamination of these preparations by small amounts of the 600 S component since comparison of the reactivities of the 600 and 30 S fractions on a N-basis revealed similarly strong serological potencies per mg of protein for both preparations. Further purification of the 30 S material by repeated centrifugation at 20,000 r.p.m. for 20-30 minutes, which removed small additional amounts of the 600 S component from suspension did not change the results of the complement fixation test. Furthermore, attempts to adsorb the antigen from 30 S preparations onto chick red cells failed while the 600 S component could be removed from suspension by this technic as shown by complement fixation as well as hemagglutination tests.

These experiments clearly demonstrated physical differences between the 2 antigen fractions. In addition, striking serological differences were noted between the 600 and 30 S components when their optimal antigen-antibody relationships were compared. An experiment with these preparations derived from the Lee strain of influenza B, summarized in Table I, serves to demonstrate this point. Furthermore, absorption of human convalescent sera with the 600 S component on the one hand, and the 30 or <30 S material on the other, clearly showed the occurrence of two distinct antigens in influenza virus preparations. When the sera were absorbed with small quantities of the 600 S component they no longer reacted with up to 32 units of this antigen while the reaction with an aliquot of the original allantoic fluid from which the absorbing material had been prepared, as well as with the homologous 30 S component, was only slightly changed. However, when high concentrations of the 600 S antigen (more than 32 units) were added to the absorbed serum positive results were obtained and the optimal antigen-antibody relationship was similar to that found with the 30 S fraction (*cf.* Table I). In agreement with this observation absorption of the convalescent sera with large amounts of the 600 S component removed all antibodies to the large particles as well as to the 30 S material. On the other hand, absorption of the convalescent sera with the $(NH_4)_2SO_4$ precipitate (<30 S) of the supernate of allantoic fluid after sedimentation of the 600 S and 30 S components removed all antibodies to the <30 S material and to the 30 S component but left a strong reaction with the 600 S material and the original allantoic fluid. These relations are demonstrated in Table II. Control tests

[11] Henle, W., Henle, G., Groupé, V., and Chambers, L. A., *J. Immunol.*, 1944, **48**, 163.

TABLE I.
Optimal Antigen-Antibody Relationships Encountered with 600 and 30 S Components.

Antigen	Serum dilution	Antigen dilution									
		und.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
600 S	1: 8	0	0	0	0	0	0	0	0	wk	c
LEE	1: 16	0	0	0	0	0	0	0	tr	ac	c
	1: 32	0	0	0	0	0	0	tr	st	c	c
	1: 64	0	0	0	0	0	tr	ac	c	c	c
	1:128	st	tr	0	tr	wk	c	c	c	c	c
	1:256	c	ac	ac	c	c	c	c	c	c	c
	1:512	c	c	c	c	c	c	c	c	c	c
30 S	1: 16	0	0	0	0	st	st	c			
LEE	1: 32	0	0	0	0	st	ac	c			
	1: 64	0	0	0	0	ac	c	c			
	1:128	wk	tr	0	0	ac	c	c			
	1:256	c	ac	st	st	ac	c	c			
	1:512	c	c	c	c	c	c	c			

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

showed that absorption of an influenza A convalescent serum with the influenza B fractions did not affect the homologous reactions and vice versa. The inhibition of red cell agglutination and neutralization of mouse infectivity by the sera after absorption with the 600 S component was negative, while absorption with the <30 S material altered only slightly the results as compared with the control tests using the unabsorbed sera.

The finding of 2 serologically distinct antigens in allantoic fluids infected with influenza A or B is in agreement with former observations.[11] It had been noted before that material sedimentable at 90,000 g in 1 hour (containing both the 600 S and 30 S components) possessed 2 distinct antigens while the supernate contained only one which was similar to one of the sedimentable antigens. Friedewald[12] noted recently that the "soluble antigen" of influenza showed different properties in many respects from the antigen associated with the virus. The relation between the 30 S component and the 600 S material requires further investigation. In view of the above absorption experiments the 30 S material probably constitutes only a small fraction of the 600 S particle but may be continuously released from it into the surrounding medium; or the 30 S material may not be exposed at the surface of the large component to any great extent but is freed only upon its disintegration. In this regard it is of interest to note that vibration of the 600 S component in the treatment vessel of a magnetostriction oscillator[13] at 90,000 cycles per second released some antigen from the large component which reacted serologically like the 30 S antigen.

Since sera of human convalescents from experimental infection with 2 distinct strains of influenza A virus (PR-8 and F-99) were available the strain specificity of the various components was studied by serum absorption tests. No difference was noted between the 30 S components of the PR-8 and F-99 strains, but the 600 S materials yielded strain-specific complement fixation tests in addition to a common antigenic activity. As shown in Table III, absorption of convalescent sera with the 600 S component of the homologous virus removed the complement-fixing antibodies to both the homologous and heterologous antigens, whereas the heterologous 600 S material absorbed only the heterologous antibodies but left some activity with the homologous 600 S antigen. Strain-specific dominance of certain immune or convalescent sera in the complement fixation test had been noted previously.[12,14,15] Similar results of

[12] Friedewald, W. F., *J. Exp. Med.*, 1943, **78**, 347.

[13] Chambers, L. A., and Gaines, N., *J. Cell. and Comp. Physiol.*, 1932, **1**, 451.

[14] Lush, D., and Burnet, F. M., *Austral. J. Exp. Biol. and Med. Sc.*, 1937, **15**, 375.

[15] Eaton, M. D., *J. Immunol.*, 1941, **41**, 383.

TABLE II.
Demonstration of 2 Antigenic Components in Allantoic Fluid Infected with Influenza A or B Virus.

Antigen Strain fraction	Optimal titer of serum									
	Convalescent influenza A absorbed with					Convalescent influenza B absorbed with				
	—	PR-8 600S	PR-8 <30S	Lee 600S	Lee <30S	—	PR-8 600S	PR-8 <30S	Lee 600S	Lee <30S
PR-8 Orig. all. fl.	1:32	1:16	1:16	1:32	1:32	1:4	<1:4	<1:4	1:4	1:4
600S	1:32	<1:4	1:16	1:32	1:32	1:4	<1:4	<1:4	1:4	1:4
30S	1:16	1:16	<1:4	1:16	1:16	<1:4				
<30S	1:8	1:8	<1:4	1:8	1:8	<1:4				
Lee Orig. all. fl.	<1:4					1:64	1:64	1:64	1:32	1:32
600S	<1:4					1:32	1:32	1:32	1:4	1:32
30S	<1:4					1:64	1:64	1:64	1:32	1:8

1:32 = highest initial serum dilution giving complete fixation of complement in the presence of from .5-16 units of antigen. All serum and antigen controls were completely hemolyzed.

TABLE III.
Strain-Specificity of Fractions Derived from 2 Strains of Influenza A Virus.

Antigen Strain fraction	Optimal Titer of serum									
	Convalescent PR-8 absorbed with					Convalescent F-99 absorbed with				
		600S		<30S			600S		<30S	
	—	PR-8	F-99	PR-8	F-99	—	PR-8	F-99	PR-8	F-99
PR-8 Orig. all. fl.	1:32	1:16	1:16	1:16	1:16	1:64	1:32	1:32	1:16	1:32
600S	1:32	<1:4	*1:16*	1:16	1:32	1:32	<1:4	<1:4	1:8	1:32
30S	1:16	1:16	1:16	<1:4	<1:4	1:64	1:64	1:64	<1:4	<1:4
F-99 Orig. all. fl.	1:32	1:16	1:16	1:8	1:16	1:128	1:64	1:64	1:32	1:32
600S	1:16	<1:4	<1:4	1:8	1:8	1:64	*1:8*	1:4	1:16	1:32
30S	1:32	1:32	1:16	<1:4	<1:4	1:128	1:128	1:64	<1:4	<1:4

1:32 = highest initial serum dilution giving complete fixation in the presence of from 0.5-16 units of antigen. All serum and antigen controls were completely hemolyzed.

absorption tests were reported recently by Friedewald.[16]

Summary. Allantoic fluids infected with influenza A or B virus contain 3 antigenic fractions as measured by complement fixation technic: 1. sedimentable at relatively low speeds and directly linked with virus activity (600 S); 2. sedimentable at 90,000 g (30 S) possibly identical with the antigen observed in mouse lungs by Lennette and Horsfall;[17] 3. non-sedimentable at 90,000 g upon repeated centrifugation or in the presence of a sucrose density gradient (<30 S). The serological activity of fraction 2 and 3 appears to be based on one antigen, while the activity of fraction 1 is mainly due to another antigen distinct from fractions 2 and 3, as shown by serum absorption technic. The 30 S components of 2 strains of influenza A virus were found indistinguishable by serum absorption technic while the 600 S components revealed strain-specificity in addition to antigenic activity common to both strains.

[16] Friedewald, W. F., *J. Exp. Med.*, 1944, **79**, 633.

[17] Lennette, E. H., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1940, **72**, 233.