ANTIGENIC STRUCTURE OF INFLUENZA VIRUSES; THE PREPARATION OF ELEMENTARY BODY SUSPENSIONS AND THE NATURE OF THE COMPLEMENT-FIXING ANTIGEN

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There is much evidence to indicate that viruses, in certain cases at least, have an antigenic structure of comparable complexity to that of the bacteria. Hughes (1933) found that the serum of animals immunized with the yellow-fever virus contained two independent antibodies—precipitins and protective antibodies; the precipitinogen was distinct from the virus. Craigie & Wishart (1936) in investigations of the vaccinia virus have shown that, in addition to the elementary bodies, virus suspensions contain two soluble precipitable substances, the "L" antigen which is labile at 56° C. and the "S" antigen which is stable at 95° C. These antigens were readily demonstrated by precipitation, agglutination and complement fixation. Their nature and origin have not, however, been precisely determined. Bedson (1936), working with the psittacosis virus, prepared a soluble antigen, which was independent of the elementary bodies. It was most satisfactorily demonstrated by complement fixation.

We (Fairbrother & Hoyle, 1937) observed a similar diversity of antigenic structure in the human and swine influenza viruses, each of which has a specific antigen involved in the neutralization reaction and a non-specific factor reacting in the complement-fixation test. This paper describes further studies on the structure of the influenza viruses, in particular the preparation of elementary body suspensions and the nature of the complement-fixing antigen.

Preparation and properties of elementary body suspensions

Elford *et al.* (1936) estimated by filtration methods the size of the human and swine influenza viruses to be 80–120$\mu\mu$. The viruses are therefore of a relatively large size and can be readily deposited in the ultracentrifuge. This sedimentation is shown in the following experiment.

A 5 per cent suspension of infected mouse lung was prepared by grinding the lungs with sand and saline and centrifuging at 4000 r.p.m. for 30 min. to deposit gross particles; the

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suspension was then centrifuged at 13,000 r.p.m. for 1 hour in the ultracentrifuge, the supernatant fluid taken off, and the deposit resuspended to the original bulk in saline. The infectivity of the original suspension, supernatant fluid, and resuspended deposit was then tested by the intranasal inoculation of mice with decimal dilutions of the respective fluids, with the following result:

Original suspension ...	Infective in dilution	1 : 1000
Supernatant fluid ...	,,	1 : 100
Resuspended deposit ...	,,	1 : 1000

This experiment is representative of many similar ones, and while in no case has it been found possible to sediment all the virus at 13,000 r.p.m., even by repeated centrifugation, nevertheless some 90 per cent of the virus can be deposited. It is usually impossible to distinguish between the infectivity of the original suspension and of the resuspended deposit.

The technique described by Eagles & Ledingham (1932) for the purification of vaccinia virus has been followed in the preparation of elementary body suspensions. A 10 per cent suspension of infected mouse lung is prepared by grinding with sand and saline and centrifuging for 30 min. at 4000 r.p.m.; 20 c.c. of this suspension is then centrifuged for 45 min. at 13,000 r.p.m. and the supernatant fluid discarded. The deposit is rubbed up with a glass rod and suspended in 10 c.c. of saline and centrifuged at 4000 r.p.m. for 20 min. to remove flocculated tissue particles. The supernatant fluid is removed and the virus again sedimented by centrifuging at 13,000 r.p.m. for 30 min. The supernatant fluid is discarded and the deposit rubbed up in 2 c.c. of saline and large particles removed by centrifugation at 4000 r.p.m. for 20 min. The resulting suspension has been used for most of the experiments described in this paper.

The suspension is a colourless, slightly opalescent fluid and is free from bacteria. On dark ground examination it shows innumerable minute particles of apparently uniform size. Infectivity tests show that the suspension contains considerable amounts of virus, the infectivity being usually equal to and sometimes greater than that of the original 10 per cent mouse-lung suspension from which it was derived.

The bodies remain suspended for long periods in saline but this stability is somewhat reduced by addition of formaldehyde, while the addition of chloroform results in rapid sedimentation.

Elementary body suspensions are effective immunizing agents and have many advantages for this purpose. They are infective, bacteria-free, and contain little extraneous protein; they can therefore be given in large doses either subcutaneously, intraperitoneally, or intravenously without eliciting undesirable reactions.

Mice were vaccinated by the intraperitoneal inoculation of a standard suspension, 2 doses being given, an initial dose of 0·1 c.c. and a second dose of 0·15 c.c. 4 days later. Eleven days after the second dose the mice were tested for immunity by intranasal inoculation of virus (Table I). It was found

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that the vaccine had conferred complete protection against the homologous virus, and partial protection against the heterologous virus.

Table I. *Protection of mice by intraperitoneal vaccination with living elementary bodies*

	Inoculated with human virus	Inoculated with swine virus
Mice vaccinated with human elementary bodies	0, 0, 0, 0, 0, 0	+/2, +/2, +/2, 0, 0, 0
Mice vaccinated with swine elementary bodies	+, +, +/2, 0, 0, 0	0, 0, 0, 0, 0, 0
Control unvaccinated mice	D_3, D_3, +, +, +, +	D_4, +, +, +, +, +/2

Each figure represents one mouse.
D_3 = mouse dying on third day after inoculation.
D_4 = mouse dying on fourth day after inoculation.
Remaining mice killed on the fourth day:
+ = mouse with severe lesions.
+/2 = mouse with small lesion.
0 = mouse with no lesion.

Immunization of ferrets was also successful, and by the intravenous inoculation of rabbits neutralizing sera of great potency were prepared. After 5 doses of 1 c.c. at intervals of 2–4 days, the resulting serum completely neutralized the virus, as tested by the intranasal mouse technique, in a dilution of 1 : 128, comparing favourably with the immune horse serum (IH_2 of Laidlaw and colleagues, 1935), which neutralized in a dilution of 1 : 64.

These results indicate that the elementary bodies represent either the complete virus or at least the portion which is responsible for infectivity.

The complement-fixing antigen

The antigen used in the complement-fixation reactions which have been described previously by us (F. & H. 1937 and H. & F. 1937), consisted of a saline suspension of desiccated infected mouse lung. An attempt was made to obtain the active fraction by centrifuging the virus suspension at 13,000 r.p.m. for 45 min., a speed which, as is shown above, is sufficient to sediment 90 per cent of the intact virus elementary bodies present. The deposit was resuspended to the original volume, and the complement-fixing powers of the original antigen, supernatant fluid, and resuspended deposit were tested against known positive sera. A representative result is shown in Table II.

Table II. *Separation of complement-fixing antigen by centrifugalization. (Antigens tested against a known positive serum)*

Antigen dilution ...	1 : 1	1 : 2	1 : 4	1 : 8	Antigen control (1 : 1)
Original antigen	0	0	+	+ + + +	+ + + +
Supernatant fluid	0	0	+	+ + + +	+ + + +
Resuspended deposit	+ + +	+ + + +	+ + + +	+ + + +	+ + + +

Serum control + + + +.
+ + + + = complete haemolysis.
+, + +, + + + = intermediate degrees of haemolysis.
0 = no haemolysis.

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The supernatant fluid possessed a complement-fixing power equal to that of the original antigen, while the resuspended deposit had no such activity. It was therefore evident that the effective antigen involved in fixation was not the elementary body itself, as the deposit, which contained some 90 per cent of the virus, had no fixing power. It was also shown that purified elementary body suspensions, while being highly infective, had no complement-fixing power when tested against known positive sera by the ordinary technique.

This indicated that the effective complement-fixing antigen in the mouse-lung extract was some soluble substance present in the supernatant fluid. The filterability of this antigen was tested by filtration of a small quantity of the fluid through a Seitz filter known to retain the virus. In the first experiment it was found that the filtrate was devoid of complement-fixing power. It was, however, thought that this removal of the fixing antigen might be due to adsorption on the filter; the experiment was therefore repeated with a more concentrated mouse-lung extract and successive samples of the filtrate were examined. It was found that only the later samples of the filtrate fixed complement and that their potency was reduced by the process (Table III).

Table III. *Adsorption of complement-fixing antigen on Seitz filter*

Antigen of four times standard strength filtered through Seitz filter, the filtrate being divided into four equal successive fractions, each tested for antigen content by fixation against known positive and negative sera.

Antigen	Serum	Antigen dilutions 1 : 1	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	Antigen titre
Original antigen	Positive	0	0	0	0	+	+ + +	
	Negative	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	1 : 16
Filtrate, 1st fraction	Positive	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	
	Negative	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	Nil
Filtrate, 2nd fraction	Positive	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	
	Negative	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	Nil
Filtrate, 3rd fraction	Positive	0	+ +	+ + + +	+ + + +	+ + + +	+ + + +	
	Negative	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	1 : 1
Filtrate, 4th fraction	Positive	0	0	+	+ + + +	+ + + +	+ + + +	
	Negative	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	1 : 4

Serum controls: positive + + + +, negative + + + +.
+ + + + = complete haemolysis.
+, + +, + + + = intermediate degrees of haemolysis.
0 = no haemolysis.

The resistance to heat of the fixing antigen was next tested. A slight reduction in potency was found as a result of heating for 15 min. at 55° C., but this was probably due to adsorption of the antigen on protein which was precipitated at this temperature. A more marked reduction occurred at 60° C. in 15 min., while the antigen was completely destroyed at 70° C.

The resistance of the antigen to acid and alkali was also studied. The mouse-lung extract had a *p*H of 7·3. Addition of equal volume of *N*/5 NaOH with neutralization after 15 min. resulted in complete destruction of the fixing antigen. The antigen was, however, more resistant to acid, as a similar

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treatment with $N/5$ HCl did not completely inactivate the antigen though its potency was reduced.

If the mouse-lung extract is gradually acidified with 1 per cent acetic acid a precipitate forms which contains practically all the complement-fixing antigen, and this antigen can be recovered from the precipitate by treatment with phosphate buffer of *p*H 7·3. By this means a much purer antigen can be prepared, as the greater part of the material precipitated by acetic acid does not redissolve in the buffer. This residual material is removed by centrifugation, and the supernatant fluid contains most of the antigen originally present in the extract.

The above results indicate that the complement-fixing antigen of the influenza viruses is distinct from the elementary body and is a soluble, filterable, relatively heat-labile substance, which is readily adsorbed on Seitz filters. It is destroyed by alkali but is somewhat resistant to acid, and can be partially purified by precipitation with 1 per cent acetic acid followed by re-solution in buffer at *p*H 7·3.

Since the effective antigen in the desiccated mouse-lung extract was not the elementary body itself but apparently some soluble substance, attempts were made to derive this substance from the elementary body.

It was first shown that although no fixation could be obtained with an elementary body suspension as antigen, if only a short fixation of 1 hour at 37° C. were used, nevertheless weakly positive results could be obtained by more prolonged fixation. The standard suspension was found to be very anticomplementary, and 6 M.H.D. of complement had to be used to overcome this difficulty. Table IV shows a result obtained by fixation for 4 hours at 37° C. followed by 24 hours at 0° C.

Table IV. *Prolonged fixation with elementary body suspension as antigen.* (6 M.H.D. *of complement*)

Serum	Antigen dilution 1 : 1	Antigen dilution 1 : 2	Serum control
Saline	+ + +	+ + + +	—
Human positive	0	0	+ + + +
Human negative	+ +	+ + + +	+ + + +

+ + + = complete haemolysis.
+, + +, + + + = intermediate degrees of haemolysis.
0 = no haemolysis.

The possibility of absorbing the complement-fixing antibody from serum by means of these suspensions was next tested. A human serum, giving complete fixation to a titre of 1 : 4, was absorbed with an equal volume of elementary body suspension, first for 1 hour at 37° C., and secondly for 1 hour at 37° C. followed by 24 hours at 0° C.; after absorption the virus plus adsorbed antibody was removed by Seitz filtration. The original and absorbed sera were then tested for complement fixation against desiccated mouse-lung antigen

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(Table V). The short period of absorption had no effect, but the prolonged absorption resulted in the removal of more than half the antibody present in the serum. In a second experiment with a more concentrated elementary body suspension prolonged absorption resulted in the complete removal of the complement-fixing antibody from the serum.

Table V. *Absorption of complement-fixing antibody by elementary body suspension*

Serum	Serum dilution		
	1:2	1:4	1:8
Original	0	0	+ + +
Absorbed 1 hr. at 37° C.	0	0	+ + + +
Absorbed 1 hr. at 37° C., 24 hr. at 0° C.	+	+ + +	+ + + +

Serum controls + + + +. Antigen control + + + +.
+ + + + = complete haemolysis.
+, + +, + + + = intermediate degrees of haemolysis.
0 = no haemolysis.

These results suggest that elementary body suspensions contained a small amount of the complement-fixing antigen, but whether this was extrinsic in origin, i.e. adsorbed on the surface of the bodies, or intrinsic was not evident.

Attempts were therefore made to disintegrate the bodies in order that, if the complement-fixing antigen were intrinsic in origin, greater quantities would be liberated. The following methods were used:

(1) By allowing the standard suspension to remain at 0° C. for 3 months.

(2) By alternate freezing and thawing the suspension 25 times.

These preparations were tested for complement fixation by the short method with negative results. It must, however, be pointed out that it is very doubtful if these methods of treatment did in actual fact produce any appreciable disintegration of the elementary bodies; on examination by dark-ground illumination no apparent differences were found before and after treatment.

ANTIBODY PRODUCTION

In our previous papers it has been shown that both neutralizing and complement-fixing antibodies appear in human serum in response to an attack of influenza, that antibodies to a relatively low titre are commonly present in normal human sera (presumably the relics of past infection) and that immunity to influenza appears to be related to the presence of these antibodies. There can be no doubt that these antibodies are the specific result of infection by the influenza virus.

Attempts to demonstrate a similar specific response in ferrets had only been possible in the case of the neutralizing antibody as, owing to the presence of a heterophile antibody in ferret serum active against mouse tissue, complement fixation could not be done with an antigen derived from mouse lung.

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It has since been found possible to prepare a satisfactory antigen from infected ferret lung by a method similar to that used for mouse lung, and with this ferret antigen complement fixation with ferret serum has been possible. It has been found that complement-fixing antibodies appear in the serum of ferrets after infection with either the human or swine virus, while the same animals before infection had no such antibody in the serum. The appearance of these antibodies in ferrets is therefore a specific result of infection.

Studies have been made on the appearance of antibodies in artificially immunized animals, as there appeared to be a difference in the response of naturally infected and vaccinated animals. It had been noted that the immune horse sera were almost devoid of complement-fixing antibody although they had a high content of virus-neutralizing antibody.

By subcutaneous and intravenous vaccination with elementary body suspensions powerful neutralizing sera were produced in ferrets and in rabbits. These sera were tested for complement-fixing antibody and no demonstrable antibody was found. Whether this antibody would have appeared on more prolonged immunization was not tested but it was evident that it was not readily elaborated as a result of vaccination with washed elementary body suspensions. It has been demonstrated above that these suspensions contain only minimal amounts of the complement-fixing antigen. Both complement-fixing and neutralizing antibodies were, however, produced when mice were vaccinated with dried mouse-lung suspensions, which contained relatively large amounts of both the elementary bodies and the complement-fixing antigen.

These results suggest that the complement-fixing antibody is most readily produced as a result of infection with virus. It is not stimulated by vaccination unless the vaccine employed contains a large amount of corresponding antigen. The neutralizing antibody is readily produced both by natural infection and artificial immunization.

Discussion

It has been shown previously that while the antigens involved in the neutralization of the human and swine viruses are not identical, each virus possesses a common antigen which is concerned with complement fixation. From experiments reported in this paper it is clear that the actual effective antigen in the desiccated mouse-lung extract used in the complement-fixation test is not the elementary body, but some soluble substance; its origin is not, however, certain.

It may be suggested that this substance is not derived from the virus but is a result of some change in the lung tissue during infection. This must, however, be regarded as most improbable. It is a specific result of the disease process, it produces an antibody response and must therefore be, at least in part, foreign to the infected animal, and further this response occurs not only in mice in which the lung tissue is involved, but also in man and in ferrets where the disease process affects usually only the upper respiratory tract. It

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seems therefore almost certain that the antigen is derived from the virus itself. If this is so, what is the relationship of the antigen to the virus?

The results reported above suggest that the complement-fixing antigen is a soluble substance produced by the virus during multiplication in the tissues in the course of the disease. This would explain the consistent appearance of antibody in infection and the absence of complement-fixing antibody in animals vaccinated with living washed virus; the vaccine contains little or none of the antigen and it is doubtful if multiplication of the virus occurs in the tissues when given by routes other than the intranasal. The negative results obtained when the elementary bodies are used as antigen in the complement-fixation reaction are readily explained; the comparatively feeble fixation which does occur when the reaction is prolonged might be due to the adsorption of a small amount of the complement-fixing antigen on the virus. It also explains the appearance of complement-fixing antigen in tissue cultures, which has been obtained by Smith (1936). The quantity of the substance does not appear to have been very great but this is probably due to the nature of the tissue in which the virus was multiplying and to the degree of multiplication.

The results described in this paper have considerable similarity to those obtained by Craigie & Wishart in their studies on the vaccinia virus. They showed that the effective antigen involved not only in complement fixation but also in precipitation and agglutination was a soluble substance readily separated from the elementary bodies, but its nature and origin were not determined. In our work the soluble antigen has only been demonstrated by complement fixation; we have failed to obtain precipitation, but this may be due to technical difficulties and also to the much lower titres of our immune sera as compared with anti-vaccinial sera.

CONCLUSIONS

1. The influenza virus contains two main antigenic components—the elementary body and a soluble substance.

2. The preparation and properties of elementary body suspensions of the influenza virus are described.

3. The complement-fixing antigen is a soluble substance probably liberated during multiplication of the virus in the tissues.

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