STUDIES ON INFLUENZA VIRUS

THE COMPLEMENT-FIXING ANTIGEN OF INFLUENZA A AND SWINE INFLUENZA VIRUSES

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Complement fixation studies on influenza A virus (1) infections have been conducted chiefly with human sera and antigens derived from influenza mouse lungs. The use of this test to assay ferret immune sera, however, seems to have been hampered by the occurrence of non-specific or heterophile reactions (2) when such sera were tested against mouse lung suspensions. Since complement fixation offered a possible means of studying further the antigenic differences between strains of influenza A virus, a problem of considerable importance in view of the recent evidence that clinical influenza is a disease of diverse etiology (1, 3*a*, *b*), experiments were conducted to evolve a satisfactory test with the ultimate end of applying it to this problem.

Material and Methods

Source of Antigen.—Complement-fixing antigens were prepared from the lungs of mice and ferrets and from the chorioallantoic and amniotic membranes of the developing chick embryo inoculated with influenza virus. One strain, S-1976, of swine influenza virus and the PR8 and W.S. strains of influenza A virus were used.

Albino Swiss mice were inoculated intranasally under ether anesthesia with 0.05 cc. of a 0.1 per cent suspension of infected mouse lung. 3 days after inoculation the animals were killed with chloroform, and the lungs were removed and pooled. Ferrets were inoculated intranasally with 1.0 cc. of a 0.1 per cent suspension of virus-containing mouse or ferret lung, and the lungs were removed at various intervals as subsequently noted.

The procedure described by Nigg, Crowley, and Wilson (4) was used to obtain antigen from the developing chick embryo. 0.1 cc. of a 10 per cent suspension of infected chick tissue was inoculated between the yolk sac and the chorioallantoic membrane of 10 to 12 day old chick embryos. After 2 days' incubation at 37°C. the chorioallantoic and amniotic membranes were removed, washed in saline, and pooled.

Preparation of the Antigen.—Tissues (mouse lung, ferret lung, or chick embryo membranes) containing influenza virus were weighed, ground with alundum, and made into a 10 per cent suspension with saline. The suspension was centrifuged at 2000 R.P.M. for 30 minutes, and the turbid supernate was drawn off, distributed into celluloid tubes,

and centrifuged at 13,000 R.P.M. for 30 minutes in the air-driven angle centrifuge described by Bauer and Pickels (5). The floating fat was removed as completely as possible with a fine pipette, and the relatively clear supernates were pooled and stored at −18°C. until required for use.

Serum.—Immune serum was obtained from ferrets by bleeding from the heart 10 to 12 days after intranasal instillation of virus. In the majority of the complement fixation tests serum obtained prior to infection of the animal was included as a control; when individual preinfection specimens were not included, a pool of normal ferret serum was substituted. The tests were further controlled by the inclusion of pools of human convalescent and normal serum.

Before use all sera were inactivated by heating at 56°C. for 30 minutes.

Complement Fixation Test.—Guinea pig serum for complement was used fresh or stored at −76°C. until required. It was titrated before each test and then diluted so that 0.2 cc. represented the desired number of units. When primary incubation at 37°C. for 1 hour was employed in the test, two *exact* units of complement were used; when this period was to be followed by overnight incubation at 4°C., two and one-half *exact* units were used. Sensitized sheep cells were prepared by mixing equal parts of 5 per cent cells in saline and an amboceptor dilution containing two units of hemolysin per 0.25 cc.

The test was conducted as follows: To 0.2 cc. of antigen dilution was added 0.2 cc. of complement dilution containing the requisite number of units, followed by 0.2 cc. of the serum dilution. After primary incubation 0.5 cc. of sensitized sheep cell suspension was added. The extent of fixation was recorded after secondary incubation for 30 minutes at 37°C. Appropriate controls on the anticomplementary activity of the reagents and on the hemolytic system were included in each test.

EXPERIMENTAL

Presence of Complement-Fixing Antigen in Ferret Lung.—Since the most obvious manner of avoiding non-specific fixation reactions appeared to be the use of antigen and serum from the same animal species, ferret sera in the preliminary experiments were tested with ferret lung influenza antigen.

Several antigens were prepared as described from ferret lungs removed on the 4th, 5th, or 10th day after inoculation of the animals with the PR8 or W.S. strain of virus. Serial twofold dilutions of the antigens were tested against serial twofold or fivefold dilutions of ferret immune and normal serum pools. In some experiments the serum-antigen mixtures were prepared in duplicate, one set for incubation for 1 hour at 37°C., the other for overnight incubation in the refrigerator. Two pools of human serum, normal and convalescent, were included in the test as controls on the antigen.

In none of these preliminary experiments was it possible to demonstrate any unequivocal fixation of complement; in most cases no fixation occurred, but not infrequently non-specific reactions were encountered in which complement was bound equally well in the presence of either normal or immune serum. Since a pool of human convalescent serum of known

complement-fixing titer against influenza mouse lung antigen likewise failed to fix complement in the presence of ferret lung antigen, it appeared that the virus in this host either failed to produce the antigen or did so in amounts not demonstrable by the means used. The latter appeared the more probable situation, since complement-fixing antibodies have been reported to be present in ferret sera (6, 7) and consequently the corresponding antigen should be present in the animal at some time. However, if the virus *per se* possessed the ability to fix complement, ferret lungs removed at a time (*i.e.*, on the 5th day) when their virus content was known to be high should serve as a good source of the antigen. Since they failed to do so and since other work (6, 8) indicates that a soluble antigen is involved in the influenza complement fixation reaction, it seemed reasonable to suppose that in the ferret, unlike the mouse, the virus produced only small amounts of the antigen or that, if the antigen was elaborated in appreciable amounts, concurrent destruction occurred *in situ*, perhaps enzymatically. The latter possibility was investigated first.

A suspension of normal ferret lung and one of normal mouse lung was prepared by grinding the tissues with alundum and sufficient saline to make a final concentration of 20 per cent lung. Both suspensions were centrifuged at 1000 R.P.M. for 5 minutes, and the markedly turbid supernates were drawn off. To 3.5 cc. of each supernate were added 3.5 cc. of PR8 mouse lung antigen, and the mixtures were placed in the refrigerator. After 22 hours in the refrigerator the mixtures were centrifuged at 13,000 R.P.M. for 30 minutes in the air-driven centrifuge, and the clear supernates were taken off and tested immediately against two pools of human convalescent serum. Falling twofold dilutions of both serum and antigen were used with two units of complement and a primary incubation of 1 hour at 37°C. The titer of the mouse lung antigen mixed with normal ferret lung did not differ significantly from that of the aliquot mixed with normal mouse lung.

This experiment afforded no evidence that ferret lung was endowed with any potentialities for destroying the complement-fixing antigen, and it was assumed, on the basis of what has already been stated above, that the ferret responds to infection by producing only small amounts of the antigen.

Four ferrets were inoculated intranasally with approximately 1000 M.I.D. of a ferret passage PR8 strain of virus, and one animal was killed on each of the 4 succeeding days by exsanguination under ether anesthesia. The lungs were made into 10 per cent suspensions in saline immediately after removal of the 4 day specimen, the first three lungs having been stored intact at −76°C. in the interim. The four preparations were serially centrifuged according to the routine described above, and the final supernates were tested at once; serial twofold dilutions of each antigen were used with serial twofold dilutions of pooled human convalescent serum. Overnight incubation in the

refrigerator was employed to facilitate any fixation which might occur; none of the four preparations, however, displayed even the slightest evidence that any complement-deviating antigen was present.

Aside from whether the virus particle itself or some constituent or metabolic product of it is concerned in the complement fixation reaction, the injection into rabbits of tissue suspensions containing the antigen should give rise to specific complement-deviating antibodies.

The remainder of the four ferret lung suspensions was injected into rabbits, two animals for each preparation. Each rabbit received five intraperitoneal injections of 3.0 cc. each, spaced 4 to 5 days apart. Between injections the material was stored at −18°C. and thawed rapidly when required for use; the tubes were refrozen as soon as the requisite amount had been withdrawn. The animals were bled by cardiac puncture 10 days after the last injection. These sera were titered against a PR8 mouse lung antigen (1 hour primary incubation) for their complement-fixing activity, and, to rule out the occurrence of non-specific fixation, also against a control antigen prepared from normal mouse lungs. Subsequently these sera were assayed against 30 fifty per cent mortality doses of PR8 mouse lung virus for their neutralizing capacity (9). Results obtained with four of the sera are presented in Table I.

As shown in Table I, all four ferret lung suspensions appeared to be devoid of complement-fixing antigen when tested directly in the fixation test, yet on injection into rabbits all induced the formation of complement-deviating antibodies; even the lung removed 24 hours after inoculation of virus stimulated a marked response in the rabbit. That these antibodies were specific for the soluble antigen was indicated by the failure of the sera to fix complement in the presence of normal mouse lung specimens. Further, in view of the large amount of material used for immunization, the neutralizing titer of the rabbit sera was surprisingly low as compared with the complement-fixing titer. If it is assumed that the unusually low neutralizing antibody titers were a direct consequence of a relative paucity of virus in the inocula, then it is justifiable also to assume that the relatively high complement-binding titers were due to the stimulus provided by the complement-fixing antigen itself. Since the latter is a soluble antigen, separable and distinct from the virus (6, 8), it appears probable that the disproportionate amounts of the two antibodies produced in the rabbit reflected the concentration of the corresponding two antigens in the inocula; it will be recalled that the inocula were centrifuged at 13,000 R.P.M. for 30 minutes, a procedure which removes approximately 90 per cent of the virus but affects the concentration of the soluble antigen to a lesser extent (8); in addition, storage at −18°C., while useful to preserve the soluble antigen, is not conducive to survival of the virus.

From these data it was concluded that the antigen was actually present in infected ferret lungs, and that with careful and accurate adjustment of the reagents in the test, it might be possible to demonstrate the occurrence of specific fixation, although obviously the system could not be made too sensitive if non-specific reactions were to be avoided.

TABLE I

Results of Complement Fixation Tests with Ferret Lung Antigens and Convalescent Human Serum as Well as with Mouse Lung Antigens and Serum of Rabbits Inoculated with Ferret Lung Antigens

Ferret	Interval between intranasal inoculation* and removal of lung	Complement fixation test: Ferret lung antigen dilution	Complement fixation test: Dilution of serum — Pooled human convalescent 1:2	1:4	1:8	Rabbit immunized with ferret lung	Rabbit immune serum: Neutralizing capacity against PR8 strain	Complement fixation test†: PR8 mouse lung antigen dilution	Dilution of serum 1:4	1:8	1:16	1:32	Normal mouse lung antigen dilution	Dilution of serum 1:4	1:8	1:16
	hrs.						*log*									
1	24	1:2	−	−	−	1	1.50	1:2	++++	++++	++	+	1:2	−	−	−
		1:4	−	−	−			1:4	++++	++++	++	+	1:4	−	−	−
		1:8	−	−	−			1:8	++++	+++	++	+				
								1:16	+++	++	+	−				
2	48	1:2	−	−	−	2	4.80	1:2	++++	++++	+++	++	1:2	++	−	−
		1:4	−	−	−			1:4	++++	+++	+++	+	1:4	−	−	−
		1:8	−	−	−			1:8	++++	++++	++	+				
								1:16	+++	++	++	+				
3	72	1:2	−	−	−	3	4.80	1:2	++++	++++	+++	++	1:2	++	−	−
		1:4	−	−	−			1:4	++++	++++	++	+	1:4	−	−	−
		1:8	−	−	−			1:8	+++	+++	++	+				
								1:16	+++	++	++	−				
4	96	1:2	−	−	−	4	4.10	1:2	++++	++++	++++	+++	1:2	++	−	−
		1:4	−	−	−			1:4	++++	++++	+++	+	1:4	−	−	−
		1:8	−	−	−			1:8	++++	++++	+++	+				
								1:16	++++	+++	++	+				

* Inoculation = 1000 M.I.D. of ferret passage PR8 strain.

† Primary incubation period of 1 hour at 37°C.

Ferret lung antigens were prepared as before and titered in serial twofold dilutions against falling twofold dilutions of ferret sera. It soon became evident that: (*a*) ferret lungs, weakly antigenic at best, varied in antigen content; (*b*) overnight fixation in the cold was necessary; and (*c*) accurate quantitation of reagents was required, especially in the case of complement, small excesses of which were frequently sufficient to obliterate the weak positive reactions obtained. Two and one-half *exact* units were found to give the best results.

Table II presents the results obtained with two ferret sera titrated against

an antigen prepared from a ferret lung removed 4 days after inoculation with the PR8 strain of virus. These results, typical of those obtained with other specimens, show the comparatively slight fixation which occurred with ferret antigens.

Attempts to Detect Complement-Fixing Antigen in Ferret Sera.—Since the complement-fixing antigen was present in the lung, it seemed possible that it might also be found in the blood stream during the early stages of the disease.

It has been reported previously (8) that the complement-fixing antigen in influenza mouse lung suspensions could be salted out with ammonium

TABLE II

Results of Complement Fixation Tests with Influenza A Virus Ferret Lung Antigen and Ferret Sera

Ferret	Serum	Dilution of antigen	Dilution of serum			
			1:4	1:8	1:16	1:32
5	Preinfection	1:2	−	−	−	−
		1:4	−	−	−	−
	Convalescent	1:2	++++	++++	++++	+++
		1:4	+	+	+	+
		1:8	−	−	−	−
6	Convalescent	1:2	++++	++++	++++	++++
		1:4	++	++	++	++
		1:8	+	+	−	−

Sera inactivated ½ hour at 56°C.

Controls on anticomplementary action of serum and antigen negative.

2½ exact units of complement used, with primary incubation for 1 hour at 37°C. and overnight at 4°C.

sulfate and appeared in the "pseudoglobulin" fraction. This method was adopted here as it offered the possibility of concentrating the several fractions.

Four ferrets were inoculated intranasally with approximately 1000 M.I.D. of ferret passage PR8 strain of virus; each day thereafter one animal was exsanguinated. The 1st and 2nd day sera were pooled, and the 3rd and 4th day sera were made into a second pool; equal quantities (6 cc.) of each serum were used in the pools.

To 12 cc. of serum were added 12 cc. of a saturated solution of ammonium sulfate. The mixtures were kept in the cold room overnight and then centrifuged at 13,000 R.P.M. for 15 minutes. The supernates were saved and the precipitates, dissolved in 3 cc. of distilled water, were dialyzed against running water for 48 hours. The "euglobulin" which was precipitated out during this dialysis was removed by centrifugation and dissolved in 5 cc. of saline; the soluble "pseudoglobulin" fractions were brought to 5 cc. and made isotonic with sodium chloride.

The supernate obtained after the initial salting out of the "globulins" was saturated with ammonium sulfate and placed in the cold room for 48 hours. The precipitated "albumin" fraction was collected on filter paper, washed with saturated ammonium sulfate solution, dissolved in 5 cc. of distilled water, and dialyzed for 48 hours against running water; the final albumin fraction (15 cc.) was made isotonic with sodium chloride. A 5 cc. portion of the protein-free filtrate was also dialyzed, and the final volume of approximately 10 cc. was made isotonic.

All fractions were tested in twofold dilutions against a 1:5 dilution of pooled human convalescent serum of known complement-fixing titer; overnight fixation in the cold was employed.

The euglobulin fractions were uniformly strongly anticomplementary. The pseudoglobulin and albumin fractions, and the residual filtrate left after salting out was completed, did not contain any detectable antigen. A similar experiment, using serum obtained on the 3rd day after infection, gave identical results.

The Use of Mouse Lung and Chick Embryo Membranes as a Source of Antigen for Complement Fixation with Ferret Sera.—Mouse lung and chick embryo membranes infected with influenza A virus provide a good source of antigen for use with human sera. Inasmuch as the low antigen titer of ferret lung made it unsuitable for routine use with ferret sera, the use of mouse and chick tissue antigens was investigated.

Antigens were prepared from mouse lungs and from the chorioallantoic and amniotic membranes of developing chick embryos inoculated with the PR8 strain of virus. Twofold dilutions of the antigens were tested against twofold dilutions of ferret serum, the tests being controlled by the inclusion of known positive and negative human sera. Unlike fixation tests employing human sera with these antigens, a primary incubation period of 1 hour was found to be insufficient to produce more than a feeble degree of fixation if any; overnight incubation in the refrigerator was required.

The occasional failure in certain experiments of known immune ferret sera to show any evidence of fixation was traced to the inadvertent use of small excesses of complement. Difficulties from this cause, however, were much less frequent than were those encountered by the occurrence of non-specific fixation, a reaction more apt to occur with mouse lung than with chick embryo tissue. To detect such non-specific reactions, normal ferret sera were included in all tests. It was later found that a majority of these reactions was due to the use of less than optimal doses of complement, and subsequently little difficulty was encountered on this score as experience was gained and exact quantitation of the reagents, and especially complement, was striven for. Occasionally, however, sera were encountered which deviated complement non-specifically, and this tendency could not be overcome in these instances. Ferret immune sera were also tested with antigens prepared from normal mouse and chick embryo tissues; lack of fixation in the presence of these antigens indicated that the reactions occurring with influenza tissues were specific.

In Table III are shown the results of a titration of mouse lung and chick membrane antigens against the sera of three different ferrets. A ferret

TABLE III

Comparison of the Ability of Influe nza Virus Antigens Derived from Three Animal Species to Fix Complement with Ferret Sera

Antigen		Serum																							
		Ferret 7								Ferret 8								Ferret 9							
		Preinfection				Convalescent				Preinfection				Convalescent				Preinfection				Convalescent			
Source	Dilution	1:4	1:8	1:16	1:32	1:4	1:8	1:16	1:32	1:4	1:8	1:16	1:32	1:4	1:8	1:16	1:32	1:4	1:8	1:16	1:32	1:4	1:8	1:16	1:32
Mouse lung	1:2	+	−	−	−	++++	++++	+++	+++	+	−	−	−	++++	++++	++++	++++	+	−	−	−	++++	++++	++++	+++
	1:4	+	−	−	−	++++	++++	++	±	−	−	−	−	++++	++++	++++	++++	±	−	−	−	++++	++++	++++	++
	1:8	−	−	−	−	++++	++++	++++	±	−	−	−	−	++++	++++	++++	++++	−	−	−	−	++++	++++	++++	++++
	1:16	−	−	−	−	++++	++++	++++	+++	−	−	−	−	++++	++++	++++	++++	−	−	−	−	++++	++++	++++	++++
Chick membrane	1:2	++	++	++	+	++++	++++	++++	++++	+	±	−	−	++++	++++	++++	++++	+++	+	+	−	++++	++++	++++	++++
	1:4	+	−	−	−	++++	++++	++++	++++	−	−	−	−	++++	++++	++++	++++	+	±	−	−	++++	++++	++++	++++
	1:8	−	−	−	−	++++	++++	++++	+++	−	−	−	−	++++	++++	++++	++++	±	−	−	−	++++	++++	++++	++++
	1:16	−	−	−	−	++++	++++	++++	++	−	−	−	−	++	++	++	++	−	−	−	−	++++	++++	+++	++
Ferret lung	1:2	±	−	−	−	++++	++++	++++	++++	−	−	−	−	++++	++++	++++	+++	−	−	−	−	++++	++++	++++	++++
	1:4	−	−	−	−	++	++	++	++	−	−	−	−	+	+	+	+	−	−	−	−	+++	+++	+++	+++
	1:8	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+
	1:16	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

antigen is also included for comparison; all three antigens were tested simultaneously with the same reagents so that the experimental conditions were practically identical. It will be noted that the mouse and chick antigens possessed about the same degree of complement-fixing activity and that both were definitely superior to the ferret antigen.

Study of Some of the Factors Possibly Involved in Non-Specific Complement Fixation with Ferret Sera.—Since non-specific fixation was occasionally encountered, it was thought of interest to examine some of the factors which might play a part in such reactions. Since mouse lung was known to contain heterophile antigen, it appeared possible that this might be responsible for non-specific fixation occurring when ferret sera were tested with influenza mouse lung suspensions.

Ferret sera were examined for the presence of heterophile antibodies by the method described by Paul and Bunnell (10). Sera were inactivated at 56°C. for 30 minutes, and serial twofold dilutions in saline from 1:4 to 1:256 were prepared. To 0.5 cc. of the serum dilution was added 0.5 cc. of 1:30 guinea pig complement followed by 0.5 cc. of 2 per cent sheep cells. (The cells were washed three times, packed and stored at 4°C. for from 4 to 7 days before use.) 1 cc. of saline was added to bring the volume to 2.5 cc. The mixtures were well shaken, incubated for 1 hour at 37°C., and the extent of hemolysis was recorded; ++++ was used to designate complete lysis of the cells and − to indicate absence of hemolysis. Correspondingly, intermediate degrees of lysis were recorded by +, ++, and +++.

Table IV shows the concentration of heterophile antibody in five ferret and five human sera chosen at random. The lytic action of the ferret sera was quite marked and, unlike human sera, practically all ferret sera tested exerted a strong lytic action on sheep cells. Ferret sera, therefore, contain large amounts of heterophile antibody which was thought to be responsible, in part at least, for some of the non-specific fixation reactions which had been encountered. Hence, attempts were made to remove the heterophile antibodies from ferret sera.

The technique of Davidsohn (11), with some modifications, was employed. Guinea pig kidneys were rinsed with saline, finely minced with scissors, and washed repeatedly with saline until the washings appeared free of gross blood. The tissue was then ground without abrasive and made into a 20 per cent suspension in saline. Sheep and beef red cells were washed three times in saline, then well packed by centrifugation and taken up in four volumes of saline to make 20 per cent suspensions.

The three suspensions were boiled for 1 hour with frequent stirring; small additions of distilled water were made at intervals to prevent drying of the tissues. When cool, the supernates were poured off and saved while the sediments were triturated in a mortar; these were then taken up in the original supernates, and distilled water was added to replace that lost by evaporation.

Absorption of heterophile antibodies was done as follows: To 0.3 cc. amounts of serum inactivated at 56°C. for 30 minutes were added 0.3 cc. amounts of either boiled sheep or beef cells or guinea pig kidney. The mixtures were incubated at 4°C. for 1 hour with frequent shakings and then centrifuged at 8000 R.P.M. for 10 minutes. The supernates were drawn off and subjected to a second absorption in the same manner. The third absorption was carried out in the cold room overnight, the mixtures being gently agitated in a shaking machine. After centrifugation at 13,000 R.P.M. for 30 minutes, the supernates were removed and tested in parallel with unabsorbed aliquots of serum for heterophile antibody content and for complement-fixing capacity.

The results of the absorption experiments were rather unsatisfactory inasmuch as only in an occasional experiment could an unmistakable reduction in heterophile antibody be demonstrated by the lysis test; in such cases boiled beef or sheep cells, or guinea pig kidney were equally efficient in removing these antibodies. Complement fixation tests were complicated by the fact that even high speed centrifugation of the absorbed sera did not always remove anticomplementary substances within the dilution range in which unabsorbed sera fixed complement. The results of an experiment in which this difficulty was overcome are given in Table V. The unabsorbed, control aliquot did not fix complement when diluted more than 1:32 and failed to do so in lower dilutions when the antigen dilution was greater than 1:8. The absorbed sera differed in that fixation occurred with 75 per cent less antigen (*cf.* sheep and beef cell absorbed serum aliquots) or was stronger at the serum dilution end point of 1:32 (*cf.* guinea pig kidney absorbed aliquot). It appeared, therefore, that the better specific

TABLE IV

Results of Heterophile Antibody Titrations with Ferret Sera and Normal Human Sera

Serum		Dilution of serum*						
Species	Number	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Human	1	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−
	3	++++	++++	++++	++++	+	−	−
	4	−	−	−	−	−	−	−
	5	−	−	−	−	−	−	−
Ferret	10	++++	++++	++++	++++	++++	+	−
	11	++++	++++	++	+	−	−	−
	12	++++	++++	+++	++	−	−	−
	13	++++	++++	++++	++++	++++	+++	+
	14	++++	++++	++++	++	+		

* Complete lysis of sheep cells = ++++; absence of lysis = −.

fixations obtained may have arisen from the removal of the substances responsible for the lytic action of the serum on sheep cells. The aliquots absorbed with all three tissues were found to be devoid of lytic action. Subsequent experiments on the lytic activity of ferret sera on sheep, goat, beef, swine, and monkey erythrocytes indicated that only the cells of the first two species were susceptible to lysis; the others were completely resistant. Accordingly, complement fixation using ferret and human sera against mouse,

TABLE V

Results of Complement Fixation Tests with Ferret Serum Absorbed with Various Heterophile Antigens

Dilution of PR8 mouse lung antigen	Pooled convalescent ferret serum				
	Serum absorbed with	Dilution of serum			
		1:8	1:16	1:32	1:64
1:4	Sheep cells	++++	+	−	−
1:8		++++	++	−	−
1:16		++++	+++	−	−
1:32		+++	++	−	−
1:4	Beef cells	++++	++++	−	−
1:8		++++	++++	−	−
1:16		++++	++++	+	−
1:32		+++	++	−	−
1:4	Guinea pig kidney	++++	++++	++++	−
1:8		++++	++++	++++	−
1:16		+++	++++	++	+
1:32		+	+++	+	−
1:2	Untreated control	++++	+++	−	−
1:4		++++	+++	++	−
1:8		++++	+++	−	−
1:16		−	−	−	−
1:32		−	−	−	−

ferret, and chick influenza antigens and a beef cell hemolytic system was studied. Variations in the amount of complement, primary incubation period, etc., did not demonstrate that the use of beef cells and beef amboceptor possessed any advantages over the usual sheep cell system.

The Complement-Fixing Antigen of Swine Influenza Virus and of Influenza A Virus Strains.—Magill and Francis (12) and Smith and Andrewes (13) have shown by reciprocal cross neutralization tests that strains of influenza A virus, while serologically related, may differ widely in antigenic make-up. On the one hand, many of the strains described in the literature can be placed in one or another of three large groups (12), whereas, on the other

hand, the PR8 and W.S. strains, the so called "intermediate and specific strains," are seemingly unique in that each occupies a niche of its own (12, 13) in the classification scheme. The results of cross immunity tests have tended to confirm these findings (13, 14).

While such tests have demonstrated that strains of the virus may differ markedly one from another, they have yielded no information as to what portion of the virus particle endows it with its strain specific properties. In conducting epidemiologic surveys with the use of the complement fixation test, antigens prepared from widely dissimilar strains have been employed (*e.g.*, W.S. by the English workers, Melbourne by the Australian, PR8 by the American, etc.) with equally good results. As a consequence the complement-fixing antigen has come to be considered generally as "group specific." Such an assumption has not been entirely warranted on the basis of the data available since human sera also possess the capacity to neutralize antigenically diverse strains to about the same extent, and hence would not be expected to reveal serologic differences in the complement-fixing antigens of these strains. Ferrets, on the other hand, can be studied after a single attack of the disease and sera from these animals (13) can be shown by neutralization tests to reflect the differences in the antigenic structures of the infecting strains. The use of ferret serum, therefore, appeared to offer a means of determining whether the strain specific properties of the virus were a function of the infectious moiety or of the soluble (complement-fixing) antigen.

To determine whether serologic differences between strains of influenza A virus were demonstrable by the complement fixation test, five strains of virus isolated from four different epidemics in New York State in 1939 (9) were chosen.

Ferrets were inoculated intranasally with each of these strains and bled 10 to 12 days later. Similar sera from ferrets inoculated with the PR8 or W.S. strains known to be dissimilar antigenically by reciprocal cross neutralization and cross immunity tests as mentioned above were included for comparative purposes. A PR8 mouse lung antigen was used in falling twofold dilutions with similar dilutions of each of these sera. Primary incubation was carried out at 37°C. for 1 hour followed by 4°C. overnight. Neutralization tests, as previously described (9), were done to determine the neutralizing potency of the sera and since, for comparative purposes, the concentration of this antibody is more exactly expressed as neutralizing capacity (15, 16) than as the serum dilution end point, the neutralizing capacities of these sera will be presented here.

The data in Table VI show that no remarkable qualitative differences were demonstrable in the complement-fixing titers of these influenza A virus antisera. In a general way, the apparent quantitative differences

seem to be related to the neutralizing capacities of the sera. It might, however, be possible that the rough correspondence of the complement-

TABLE VI

Results of Cross Complement Fixation Tests with the PR8 Strain and Convalescent Ferret Sera against Various Strains of Influenza A Virus

Ferret number	Recovered from infection with	Neutralizing capacity of serum against PR8	Dilution of PR8 antigen*	Dilution of convalescent ferret serum			
				1:4	1:8	1:16	1:32
	strain	*log*					
15	149	7.27	1:2	++++	++++	++++	+++
			1:4	++++	++++	++	+
			1:8	++++	++++	+	−
16	188	6.62	1:2	++++	++++	++++	++
			1:4	++++	++++	++++	++
			1:8	++++	++++	++++	+
17	236	6.19	1:2	++++	++++	++++	++++
			1:4	++++	++++	++++	+++
			1:8	++++	++++	++	+
18	273	5.73	1:2	++++	++++	+++	++
			1:4	++++	++++	++	−
			1:8	++++	+++	+	−
19	399	4.80	1:2	++++	++++	++	−
			1:4	++++	++	−	−
			1:8	++++	+	−	−
20	W.S.	7.57	1:2	++++	++++	++++	+++
			1:4	++++	++++	++++	++
			1:8	++++	++++	++++	++++
			1:16	++++	++++	++++	++++
21	PR8	6.80	1:2	++++	++++	++++	+++
			1:4	++++	++++	++++	++
			1:8	++++	++++	++++	++++
			1:16	++++	++++	++++	++++

Sera inactivated at 56°C. for 30 minutes.

Primary incubation period was 1 hour at 37°C. and overnight at 4°C.

* Mouse lung antigen.

fixing titers and neutralizing capacities resulted from the fact that all the sera except the PR8 serum were tested against a heterologous and not a homologous strain of virus and soluble antigen.

Ferret immune sera against the PR8 and W.S. strains of influenza A virus and a strain (S-1976) of swine influenza virus were obtained by cardiac puncture 10 to 12 days after

TABLE VII

Results of Cross Complement Fixation Tests with Swine Influenza Virus, the W.S. and PR8 Strains of Influenza A Virus, and Ferret Antisera

Ferret number	Influenza virus inoculated	Serum specimen tested	Neutralizing capacity of serum against*			Dilution of antigen	Dilution of serum tested against													
							Swine antigen					W.S. antigen					PR8 antigen				
			Swine	W.S.	PR8		1:4	1:8	1:16	1:32	1:64	1:4	1:8	1:16	1:32	1:64	1:4	1:8	1:16	1:32	1:64
			log	*log*	*log*																
22	Swine	Prein-fection	0	−	−	1:2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
						1:4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
						1:8	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		Convalescent	4.62	1.93	1.93	1:2	++++	++	+	−	−	++++	++	−	−	−	++++	++	−	−	−
						1:4	++++	+	−	−	−	++++	+++	−	−	−	++++	+	−	−	−
						1:8	++	−	−	−	−	++++	−	−	−	−	++	−	−	−	−
						1:16	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
23	Swine	Prein-fection	0	−	−	1:2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
						1:4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
						1:8	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		Convalescent	5.42	3.02	2.17	1:2	++++	++++	+++	−	−	++++	++++	+	−	−	++++	++++	++	−	−
						1:4	++++	++	+	−	−	++++	+++	+	−	−	++++	++++	−	−	−
						1:8	−	−	−	−	−	+	−	−	−	−	±	−	−	−	−
						1:16	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
24	Swine	Convalescent	5.42	3.22	2.77	1:2	++++	++++	++++	+	−	++++	++++	++	−	−	++++	++++	+++	−	−
						1:4	++++	++++	++++	−	−	++++	++++	++	−	−	++++	++++	+	−	−
						1:8	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−
						1:16	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
25	W.S.	Prein-fection	−	0	0	1:2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
						1:4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
						1:8	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		Convalescent	3.39	6.96	6.57	1:2	++++	++++	++++	++++	+	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
						1:4	++++	++++	+++	+	−	++++	++++	++++	+++	++	++++	++++	++++	+++	+++
						1:8	−	−	−	−	−	+++	+++	++	+	−	+++	++	++	−	−
						1:16	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−

26	W.S.	Convalescent	–	6.55	5.80	1:2	+++	–	–	–	–	++++	+++	+++	–	–	++++	+++	–	–	–
						1:4	++	–	–	–	–	++++	+	–	–	–	++++	++	–	–	–
						1:8	±	–	–	–	–	+	–	–	–	–	+	–	–	–	–
						1:16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
27	PR8	Convalescent	2.90	–	6.55	1:2	++++	++++	++++	–	–	++++	++++	++++	++	–	++++	++++	++++	++	–
						1:4	++++	++	+	–	–	++++	++++	++++	–	–	++++	++++	+++	++	–
						1:8	++	–	–	–	–	++++	++	++	–	–	+++	+	–	–	–
						1:16	–	–	–	–	–	++	–	–	–	–	±	–	–	–	–
28	PR8	Convalescent	–	–	6.55	1:2	++++	++++	++	–	–	++++	++++	++++	++++	–	++++	++++	++++	++++	–
						1:4	++++	++	±	–	–	++++	++++	++++	++	–	++++	++++	++++	+++	–
						1:8	++	–	–	–	–	++++	+++	++	–	–	++++	+++	++	–	–
						1:16	–	–	–	–	–	+	–	–	–	–	+	–	–	–	–

* 0 = no neutralizing antibodies.

– = neutralizing capacity not determined.

inoculation of these strains. The swine virus was included because it is so distantly related antigenically to the PR8 and W.S. strains (12–14).

Mouse lung antigens were prepared from these three strains of virus, and the ferret sera were tested for complement-fixing and neutralizing capacities as described in the section above. The results are summarized in Table VII.

An analysis of Table VII brings out a number of interesting facts. It will be noted that the swine antisera neutralized large quantities of swine virus, much less of the W.S. strain, and still less of the PR8 strain. The serum of ferret 24, for example, was capable of neutralizing 262,000 fifty per cent mortality doses of the homologous (swine) virus, but only 1660 such doses of the W.S. strain and 588 of the PR8 strain. The antigen of the swine virus, however, fixed complement with the heterologous W.S. and PR8 antisera, almost equally as well as with its own homologous antisera. Insufficient quantities of the particular PR8 antisera studied were available to permit complete neutralization experiments, but in view of what is already known (12, 13) concerning these three strains, the results should have been comparable to those obtained with the W.S. strain. In the case of ferret serum 25, as an example, it was found that this serum neutralized 9,120,000 fifty per cent mortality doses of the homologous strain (W.S.), 3,710,000 such doses of the heterologous but related PR8 strain, but only 2180 doses of the distantly related swine virus. By the complement fixation test, on the other hand, no differences in the titers of the PR8 and W.S. antisera were demonstrable whether these were tested against the homologous or heterologous antigen. When tested against the swine antigen, however, these influenza A virus antisera gave distinctly lower levels of fixation.

It is evident that the titers obtained in cross complement fixation tests were unrelated to the capacities of the sera to neutralize markedly heterologous strains. The PR8 and W.S. strains, while related, are known to be different antigenically by cross neutralization tests; by cross complement fixation tests, however, their soluble antigens appeared to be so similar that it proved impossible to distinguish one strain from the other. An antigenic component present in the swine soluble antigen appeared to be present also in the PR8 and W.S. antigens as each of the corresponding antisera fixed complement to a like degree with the swine complement-fixing antigen. This was also borne out by the finding that swine antisera in the presence of swine, PR8, or W.S. antigens fixed complement to the same extent.

DISCUSSION

In studying the complement-fixing antigen of influenza virus, the use of ferrets appeared to possess several advantages. These animals, susceptible to infection with influenza viruses, could be made to pass through an attack of the disease under controlled conditions, which obviously does not hold true for man. Also antibodies arising as a result of the actual disease were thought to approximate more nearly natural conditions than would antibodies induced by artificial immunization.

Because of the difficulties reported (2) to occur with complement fixation tests in which ferret serum was used with influenza antigens obtained from animal species (*e.g.*, the mouse) possessing heterophile antigen, it was essential to investigate this problem first. An attempt was made to employ infected ferret lung as a source of antigen, but the use of this tissue had to be abandoned when it became evident that even under the best conditions its antigen content was very low and not infrequently undemonstrable. A point of interest emerged when infected ferret lungs, containing no demonstrable complement-fixing antigen, were injected in large amounts into rabbits; these animals responded by the production of antisera with a surprisingly low virus-neutralizing capacity but a comparatively good complement-fixing titer; for example, the serum of rabbit 1, which failed in a dilution of 1:2 to neutralize 30 fifty per cent mortality doses of virus, nevertheless gave good fixation of complement. As the lung suspensions had been centrifuged at high speed, which removes considerable virus, and subsequently had been stored at $-18°C.$ and thereafter frequently thawed and refrozen, a procedure favoring inactivation of the virus, the marked differences in the titers of the neutralizing and complement-fixing antibodies suggest that these arose from stimuli afforded by two different and distinct antigens.

The use of infected mouse lungs or developing chick embryo membranes as a source of antigen was attended by a high proportion of non-specific fixations, and some difficulty was encountered by the absence of specific complement fixation in the presence of ferret sera which, *a priori*, would have been expected to react positively. When titration of the complement was made as quantitatively exact as possible, practically all the non-specific reactions disappeared. Full units of complement were not used, as even small excesses of this agent were sufficient to abolish otherwise positive, specific fixation. These points were found to be of the utmost importance if success was to be achieved in fixation systems utilizing ferret sera.

That heterophile antibodies are present in ferret sera was demonstrated

by the ability of the latter to hemolyze sheep erythrocytes. These antibodies, however, did not seem to exercise any major interference with the specificity of the influenza complement-fixing reaction since non-specific fixation could be eliminated with proper quantitative adjustment of the complement.

With a satisfactorily functioning test available, it was possible to investigate the complement-fixing antigens of a strain (S-1976) of swine influenza virus and the PR8 and the W.S. strains of influenza A virus. Antigens derived from mouse lungs infected with these strains and antisera obtained from ferrets recovered from infection with these strains were studied in reciprocal cross complement fixation tests. The ability of the swine antisera to fix complement to approximately the same extent with the heterologous PR8 and W.S. antigens as with the homologous swine antigen suggests the presence of a component common to all three soluble antigens. The reciprocal cross neutralization tests showed how marked were the antigenic differences between the swine virus on the one hand and the PR8 and W.S. strains of influenza A virus on the other.

The PR8 antisera fixed complement equally well in the presence of either the PR8 or W.S. antigens, but to a lesser degree with the swine antigen. The same was true of the W.S. antisera.

These results indicated that the swine virus which, on the basis of cross neutralization and cross immunity tests, is almost distinct from though still somewhat distantly related to the PR8 and W.S. strains of influenza A virus, is quite definitely related to these strains through its soluble, complement-fixing antigen. In fact, the swine antigen does not appear to contain components which are not present in the soluble antigens of either the PR8 or W.S. strains. On the other hand, the antigens of the strains of influenza A virus studied did seem to possess some component or components not present in the swine antigen.

SUMMARY

Influenza complement fixation tests designed for use with ferret serum are described. Complement-fixing antigens derived from influenza ferret lungs were unsatisfactory due to their low content of soluble antigen; those prepared from mouse lungs or developing chick embryo membranes proved to be better antigenically and were reliable when the various reagents in the test were properly adjusted to eliminate non-specific fixation of complement.

The results of cross complement fixation tests indicated that the soluble antigens of the PR8 and W.S. strains of influenza A virus were closely

similar, if not identical. They indicated also that the soluble antigen of swine virus possessed components present in the antigens of the human strains of virus.

BIBLIOGRAPHY

1. Horsfall, F. L., Jr., Lennette, E. H., Rickard, E. R., Andrewes, C. H., Smith, W., and Stuart-Harris, C. H., *Lancet*, 1940, **2**, 413.
2. Fairbrother, R. W., and Hoyle, L., *J. Path. and Bact.*, 1937, **44**, 213.
3*a*. Magill, T. P., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 162.
3*b*. Francis, T., Jr., *Science*, 1940, **92**, 405.
4. Nigg, C., Crowley, J. H., and Wilson, D. E., *Science*, 1940, **91**, 603.
5. Bauer, J. H., and Pickels, E. G., *J. Bact.*, 1936, **31**, 53.
6. Hoyle, L., and Fairbrother, R. W., *J Hyg.*, Cambridge, Eng., 1937, **37**, 512.
7. Lush, D., and Burnet, F. M., *Australian J. Exp. Biol. and Med. Sc.*, 1937, **15**, 375.
8. Lennette, E. H., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1940, **72**, 233.
9. Horsfall, F. L., Jr., Hahn, R. G., and Rickard, E. R., *J. Clin. Inv.*, 1940, **19**, 379.
10. Paul, J. R., and Bunnell, W. W., *Am. J. Med. Sc.*, 1932, **183**, 90.
11. Davidsohn, I., *J. Am. Med. Assn.*, 1937, **108**, 289.
12. Magill, T. P., and Francis, T., Jr., *Brit. J. Exp. Path.*, 1938, **19**, 273.
13. Smith, W., and Andrewes, C. H., *Brit. J. Exp. Path.*, 1938, **19**, 293.
14. Francis, T., Jr., and Magill, T. P., *Brit. J. Exp. Path.*, 1938, **19**, 284.
15. Horsfall, F. L., Jr., *J. Exp. Med.*, 1939, **70**, 209.
16. Horsfall, F. L., Jr., and Lennette, E. H., *J. Exp. Med.*, 1941, **73**, 327.