

STUDIES ON INFLUENZA INFECTION IN FERRETS BY MEANS
OF FLUORESCHEIN-LABELLED ANTIBODY*, ‡

II. THE ROLE OF "SOLUBLE ANTIGEN" IN NUCLEAR FLUORESCENCE AND CROSS-
REACTIONS

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PLATE 48

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In an accompanying paper (2), it is reported that specific influenza viral antigens can be detected by means of fluorescein-labelled antibody in the ciliated respiratory epithelium of infected ferrets. The nuclei of these infected cells have very bright fluorescence. Further studies by this method showed that there are marked cross-reactions among the three strains of influenza A virus studied. In the present paper, data are presented to show that the "soluble antigen" common to strains of type A influenza virus (3) is responsible for the fluorescence observed in the nuclei of infected cells and also for the heterologous reactions in fluorescent staining.

The presence of a "soluble antigen" in suspensions of mouse lung infected with influenza A virus has already been described (4, 5). By means of ultracentrifugation and complement-fixation tests, Henle and Wiener (6, 7) were able to demonstrate two types of specific particles in allantoic fluid preparations of influenza A and B virus. The larger particle possesses all the attributes of the virus while the smaller particle has no properties of infection or hemagglutination. They designated the larger particle as V antigen and the smaller particle S antigen. The S antigen may be identical with the "soluble antigen" obtained from mouse lungs. Since the V antigen is probably identical with the elementary virus unit, it consists of strain-specific and type-specific elements and is often contaminated with some S antigen. The S antigen, on the other hand, appears to be common to several strains of influenza A virus studied. However, S antigens obtained from influenza A and influenza B viruses are distinct (3).

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‡ A preliminary report was read at the annual meeting of the American Association of Immunologists, April 14, 1954, (1).

Materials and Methods

The virus strains, ferrets, preparation of fluorescein-labelled antibody solutions, methods of collecting specimens, sectioning, and staining were identical with those described in the accompanying paper (2).

EXPERIMENTAL

Antigenic Relations of Three Strains of Influenza A Virus

The antigenic relations of PR8, Fm₁, and Farrington strains of influenza A virus were determined by means of cross-hemagglutination-inhibition tests using monovalent rabbit antisera. All sera were inactivated at 56°C. for 30 minutes. To remove the non-specific inhibitor, each volume of serum was incubated with 4 volumes of undiluted crude cholera filtrate for 18 hours at 37°C. Residual cholera filtrate activity was destroyed by heating the mixture at 56°C. for 50 minutes (8). The sera were then divided into two equal parts. To one

TABLE I
Cross-Hemagglutination Inhibition Tests of Influenza A Viruses

Anti-sera		Tested against antigens of		
		PR8	Fm ₁	Farrington
Before absorption	Anti-PR8	2560*	<20	320
	Anti-Fm ₁	<20	640	<20
	Anti-Far.	160	<20	2560
After absorption	Anti-PR8	1280	<20	160
	Anti-Fm ₁	<20	320	<20
	Anti-Far.	<20	<20	1280

* Reciprocal of serum dilution.

part, each milliliter of serum was absorbed with human erythrocytes coated with influenza virus according to the method of Jensen and Francis (9). Each antiserum was absorbed with the other two heterologous viruses; *i.e.*, the anti-PR8 serum was absorbed with Fm₁ and Farrington viruses. Hemagglutination-inhibition titrations were performed by the technic described as the Standard Reference Test in Influenza Studies (10) using a 0.5 per cent suspension of chicken erythrocytes. The results are summarized in Table I.

Even before absorption of the antisera with virus-coated erythrocytes, the Fm₁ virus showed no cross-reaction by hemagglutination-inhibition with the PR8 and Farrington viruses. The latter two strains were somewhat related. A single absorption of the PR8 antiserum with two units (9) of Farrington virus did not remove the reaction of PR8 serum with Farrington virus, but absorption of Farrington serum with PR8 virus removed the Farrington cross-reaction with PR8 virus.

Effect of Cross-inhibition with Unlabelled Antisera on Fluorescent Antibody Staining.—When infected nasal turbinate sections were stained with fluores-

cein-labelled antibody solutions, it was noted that marked cross-staining occurred among these three strains of influenza A virus. However, no cross-staining between the influenza A and the Lee strain of influenza B viruses was seen. Since pretreatment of sections with homologous unlabelled antiserum usually inhibited the fluorescent staining, it was decided to investigate whether cross-inhibition could be used to determine the strain specificity of fluorescent staining.

Five unlabelled rabbit sera were used for this experiment. The four monovalent antisera against PR8, Fm₁, Farrington, and Lee B viruses were unlabelled portions of the same sera from which fluorescein-labelled antibody solutions had been prepared. The sera were adjusted to equal hemagglutination-inhibition antibody titers. Five slides of frozen sections of nasal turbinates infected with the appropriate influenza virus were fixed in acetone as

TABLE II
Effect of Unlabelled Antisera on Homologous Fluorescent Antibody Staining of Ferret Nasal Turbinates Infected with Influenza Viruses

Sera used for inhibition	Homologous conjugate staining on nasal turbinates infected with			
	Farrington	PR8	Fm ₁	Lee B
Normal	++++*	++++	+++	++++
Anti-PR8	+	+	++	++++
Anti-Far.	++	+++	++	++++
Anti-Fm ₁	++++	++++	++	++++
Anti-Lee B	++++	++++	+++	0

* Degree of fluorescent staining: +++++, maximum staining = no inhibition; +, minimal staining = marked inhibition; 0, no staining = complete inhibition.

described. One slide was exposed to unlabelled normal rabbit serum, and each of the other four was exposed to an unlabelled homologous or heterologous antiserum for 20 to 30 minutes. After this, the unlabelled serum was rinsed off with buffered saline and the slides were then exposed for 10 minutes to a fluorescein-labelled antibody solution homologous for the virus infecting the nasal turbinate. Finally the slides were rinsed in buffered saline and mounted as described. Sections of turbinates infected with each of the four viruses were studied in this way and the results are shown in Table II.

There was no cross-inhibitory effect between influenza A and influenza B viruses and their antisera. Among the three strains of influenza A virus, the PR8 antiserum not only inhibited its own homologous fluorescent staining well, it was also more active than the Farrington antiserum in the inhibition of Farrington-infected tissues. In the Fm₁ series, the inhibitory effect of three influenza A antisera was about equal. Although it proved possible by hemagglutination-inhibition tests to distinguish these three strains of influenza A virus antigenically (Table I), similar inhibitory procedures were not successful in the differentiation of strain specificity in fluorescein-labelled antibody staining.

Difference between Homologous and Heterologous Fluorescent Staining.—When infected tissues were treated with homologous conjugates, both the cytoplasm and the nucleus of the infected cells were stained. The nuclear fluorescence was usually brighter, and sometimes had a network pattern (Figs. 1 and 2). The cytoplasmic fluorescence had a more homogeneous character with its maximum intensity along the ciliated border of the epithelial cells. However, when in-

TABLE III
*Homologous and Heterologous Fluorescent Staining on Influenza A Virus
Infected Nasal Turbinates with Absorbed Conjugates*

Fluorescent anti-sera	Tissues infected		Fluorescent anti-sera absorbed with							
			None	Lee S	PR8 V	PR8 S	Fm ₁ V	Fm ₁ S	Far. V	Far. S
Anti-Fm ₁	PR8	N*	++++‡	++	0	0	0	0	0	0
		C*	+	+	0	0	0	0	0	0
	Fm ₁	N	++++	++++	0	0	0	0	0	0
		C	+++	+++	++	++	0	++	++	++
	Far.	N	+++	++	0	0	0	0	0	0
		C	+	+	0	0	0	0	0	0
Anti-Far.	PR8	N	++++	++++	0	0	0	0	0	0
		C	+	+	0	0	0	0	0	0
	Fm ₁	N	++++	++++	0	0	0	0	0	0
		C	++	++	0	0	0	0	0	0
	Far.	N	++++	+++	+ 0 [§] 0 [§]	0	0 [§]	0	0	0
		C	+++	++	+ + [§] 0 [§]	++	+++	++	0	++
Anti-PR8	PR8	N	++++	++++	0 [§]	0	0	0	0 [§] 0 [§]	0
		C	+++	+++	0 [§]	+++	+++	+++	+++ [§] +++ [§]	+++
	Fm ₁	N	++++	+++	0	0	0	0	0	0
		C	++	++	0	0	0	0	0	0
	Far.	N	+++	+++	0 [§]	0	0	0	0 [§] 0 [§]	0
		C	++++	+++	0 [§]	+++	+++	+++	+++ [§] +++ [§]	+++

* N, nuclear fluorescence; C, cytoplasmic fluorescence.

‡ Degree of fluorescent staining: +++++, maximum; +, minimum; 0, no staining.

§ Numerals indicating number of absorptions.

infected tissues were stained with a heterologous conjugate, mainly the nuclei were fluorescent; the cytoplasm of the infected cells was stained to a lesser degree. This was especially true with the anti-Fm₁ conjugate staining of PR8- and Farrington-infected tissues, where the cytoplasmic fluorescence was minimal (Table III).

Effect of "Soluble Antigen" from Influenza A Virus on the Nuclear Fluorescence and Cross-Fluorescent Reactions.—Since the nuclear fluorescence was seen in infected cells and was also the chief manifestation of cross-fluorescent reac-

tions among the three strains of influenza A virus studied, some antigenic components common to these viruses were evidently responsible for such reactions. A series of reciprocal cross-absorption experiments with the V and S antigens on conjugates and their stainings on homologous and heterologous infected tissues was performed.

In preliminary experiments, the "soluble" S antigen was prepared by centrifugation from chorio-allantoic membranes infected with influenza virus according to the method described by Kirber and Henle (3). The infected chorio-allantoic membranes were ground in a Waring blender to make a 20 per cent saline suspension. 20 ml. of this membrane suspension was first subjected to centrifugation at 20,000 R.P.M. for 20 minutes to remove the virus particles. The supernate was then centrifuged again at 30,000 R.P.M. for 60 minutes. After this, the supernate was discarded and the sediment was resuspended in buffered saline to its original volume and centrifuged at 20,000 R.P.M. for 20 minutes to reduce further any contamination with virus particles. The second supernate was again centrifuged at 30,000 R.P.M. for 60 minutes. At this time, a small pellet was barely visible. The supernate was removed carefully with a capillary pipette to avoid stirring up the sediment until less than 0.1 ml. of fluid was left with the pellet. This wet pellet was mixed with 0.5 ml. of fluorescein-labelled antibody solution for absorption overnight at 4°C.

Subsequently, because a dry preparation of the S antigen was more desirable for absorption to avoid dilution of the fluorescein-labelled antibody, precipitation of the antigenic proteins by ethanol was attempted. 20 ml. of a 20 per cent suspension of infected chorio-allantoic membrane was centrifuged at 20,000 R.P.M. for 45 minutes to remove the influenza virus particles. To the supernate, 95 per cent ethanol was added to a final concentration of 20 per cent (*v/v*) at -3 to -5°C. (11). The precipitate was centrifuged at 6,000 R.P.M. for 5 minutes at 4°C. and the supernatant ethanol was decanted. The precipitate was dried in a desiccator under reduced pressure for about 3 hours at room temperature. For absorption, the precipitate was suspended in 0.5 ml. of fluorescein-labelled antibody solution and left at 4°C. overnight. This precipitate was as effective as the original preparation obtained by centrifugation in removing the cross-fluorescent staining without abolishing homologous fluorescent staining. The results obtained with conjugates absorbed with antigens prepared by either method were indistinguishable. Therefore, both preparations are referred to as the "S antigen." The virus V antigen was prepared by centrifugation of 20 ml. infected undiluted allantoic fluids at 4°C. at 20,000 R.P.M. for 45 minutes. The supernate was discarded and the pellet was used for absorption of 0.5 ml. conjugate as described above.

For each experiment, a series of 24 frozen sections from each turbinate was cut at one time. After the sections were fixed in acetone and dried, they were stained with various absorbed homologous and heterologous conjugates. Sections stained with unabsorbed conjugates were always included as reference for estimating the intensity of fluorescent staining in the nuclei and cytoplasm. The results are summarized in Table III.

It can be seen that absorption with the S antigen from influenza B virus (Lee strain) had no effect on the fluorescent staining either in the nuclei or in the cytoplasm. Absorptions of the conjugates with homologous V antigens completely removed all staining both in the cytoplasm and in the nuclei. Absorptions of the conjugates with heterologous V antigens, or any S antigen, either homologous or heterologous, removed only the nuclear fluorescence and the heterologous reaction (Figs. 3 and 4). The homologous cytoplasmic fluorescence

was sometimes reduced slightly, but the free border of the ciliated cells still showed bright fluorescence.

There is an exception to the above generalization of the results. In the absorption of anti-Farrington conjugate with PR8 V (heterologous), one absorption reduced both the homologous nuclear and cytoplasmic staining significantly and five absorptions abolished the cytoplasmic fluorescence completely. On the other hand, the anti-PR8 conjugate stained both the nuclei and the cytoplasm of Farrington-infected cells very well; the cytoplasmic fluorescence could not be removed by absorption with S or heterologous V antigens. Even 5 successive absorptions with Farrington V antigen did not abolish the capacity in the anti-PR8 conjugate to stain the cytoplasm of Farrington-in-

TABLE IV
Effect of Cross-Absorption with Heterologous V Antigen on Unlabelled Antisera

Anti-sera		Hemagglutination-inhibition tested against antigen of	
		PR8	Farrington
Before absorption	Anti-PR8	2560*	320
	Anti-Far.	80	2560
After absorption	Anti-PR8‡	<40	<40
	Anti-Far.§	<40	<40

* Reciprocal of serum dilution.

‡ Absorbed once with Farrington V Antigen.

§ Absorbed once with PR8 V Antigen.

fect cells. The pattern of fluorescent staining by absorbed anti-PR8 conjugate on PR8 and Farrington infected tissues was identical.

Effect of Cross-Absorptions with V Antigen on Hemagglutination-Inhibition Reactions between PR8 and Farrington Antisera.—Since the absorption of Farrington conjugate with PR8 V antigen abolished its homologous staining and the fact that the PR8 conjugate stained the cytoplasm of Farrington-infected cells very well, the obvious possibility is that there are some common antigenic components possessed by the PR8 and Farrington virus particles. Although cross-absorption with virus-coated human erythrocytes did not show a decrease of homologous hemagglutination-inhibition titer (Table I), this might be due to the fact that the quantity of virus used for absorption was not sufficient. Therefore the effect on the absorption of sera with V antigen was investigated.

0.5 ml. each of the unlabelled PR8 and Farrington sera identical to those used in previous experiments in Table I was absorbed once with heterologous V antigen at 4°C. overnight. The V antigens were from the same preparations as used for absorption of conjugates. By

estimation, about 12 times more virus was used in the absorption here than was used in the virus-coated human erythrocytes.

As shown in Table IV, a single absorption of PR8 and Farrington sera with heterologous V antigens completely removed the homologous as well as the heterologous hemagglutination-inhibition reactions. This suggested that the PR8 and Farrington viruses were very closely related if not identical.

DISCUSSION

Coons, Leduc, and Kaplan (12) have discovered the presence of antigenic material in the nuclei of different cell types in mice receiving intravenous injections of foreign proteins and concluded that it is not an artefact. Crampton and Haurowitz (13) also reported the detection of radioactive proteins in the nuclear fractions of rabbit liver homogenates; and Gitlin *et al.* have demonstrated plasma proteins in the nuclei of different cell types in human tissues (14). The present data furnish another instance of the presence of foreign antigenic material in the nucleus. The nuclei of respiratory epithelial cells contained influenza viral antigens at an apparently higher concentration than that in the cytoplasm, because the nuclear fluorescence was usually brighter than the cytoplasmic fluorescence. In the study of influenza virus infection in chick embryos, Watson and Coons have observed a diffuse type of fluorescent staining which was first detectable in the nuclei and later in the cytoplasm of cells in the chorio-allantoic membrane (15). However, in ferrets, the nuclear fluorescence seemed to be composed of small patches and sometimes had a network pattern. This nuclear fluorescence was also the chief manifestation where heterologous fluorescent antibody was applied to the infected mucosa. Such staining reaction in the nucleus could be due to the actual presence of virus particles in the nucleus of infected cells or to some soluble component of the virus which diffused into the nucleus from the cytoplasm. The fact that absorption of conjugates with "soluble antigen" which was devoid of virus particles, abolished the nuclear staining is consistent with the latter possibility. Since S antigen from any of the three strains of influenza A virus removed from fluorescein-labelled antibody the factor reacting with the material in the nucleus, it is concluded that the presence of "soluble antigen" in the nucleus is responsible for the nuclear fluorescence as well as the cross-fluorescent staining. Many strains of influenza A virus contain a common soluble complement-fixing antigen, according to Kirber and Henle (3).

In electronmicroscopy studies (16, 17), influenza virus particles seem to be formed at the periphery of the cytoplasm in infected cells. This is consistent with the findings here that the cytoplasmic fluorescence had its maximum intensity along the free border of the ciliated cells. When a homologous conjugate was absorbed with "soluble antigen," it no longer stained the nucleus but it still stained the free border of ciliated cells. This seems to offer further

evidence that virus particles are probably localized at the periphery of infected cells.

The cytoplasmic cross-fluorescent reactions by anti-PR8 conjugate on Farrington-infected tissues were probably due to common antigenic components in these two strains of virus, because this cytoplasmic staining remained after the conjugate was absorbed with S antigens or with heterologous V antigens. It is a little difficult to understand why five successive absorptions of the PR8 conjugate with Farrington V antigen did not remove the cross-cytoplasmic staining while only one such absorption of the PR8 antiserum with Farrington V antigen had abolished the heterologous as well as the homologous hemagglutination-inhibition reactions. Possibly a small amount of antibody was sufficient to cause the cytoplasmic staining but a larger or a different type of antibody was responsible for the hemagglutination-inhibition reactions. There is evidence that the hemagglutination-inhibition antibody and the neutralization antibody are not identical in influenza virus (18).

Of the three strains of influenza A virus tested for their cross-reactions, the antigenically dissimilar PR8 and Fm₁ viruses cross-stained markedly. This indicates that it may be possible to use one unabsorbed conjugate for the detection of influenza A epidemics. Subsequent use of conjugates absorbed with S antigens would then perhaps be useful in determining the particular epidemic strain of influenza virus.

SUMMARY

Yellow-green fluorescence representing viral antigens was detected in both the nucleus and cytoplasm of epithelial cells of the respiratory tract in ferrets infected with influenza virus. This nuclear fluorescence was the chief manifestation of cross-fluorescent staining reactions among three strains of influenza A virus studied, PR8, Farrington, and Fm₁. Absorption experiments with influenza viral V and soluble S antigens showed that S antigen was responsible for the presence of fluorescence in the nucleus and for the cross-staining reactions among these strains.

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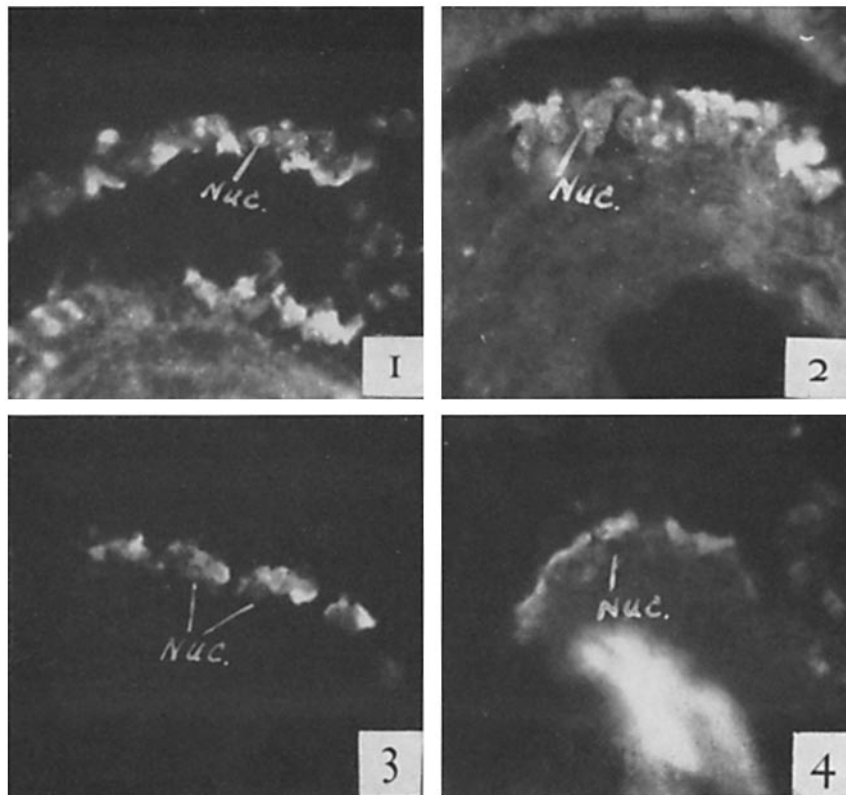
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EXPLANATION OF PLATE 48

FIGS. 1 and 2. Sections from a ferret nasal turbinate infected with PR8 strain of influenza A virus. The sections were stained with unabsorbed homologous fluorescein-labelled antibody solution showing fluorescence in the cytoplasm and nucleus. $\times 280$.

FIGS. 3 and 4. Sections from the same turbinate stained with homologous fluorescein-labelled antibody absorbed with "soluble antigen." The nuclear fluorescence has disappeared but the cytoplasmic fluorescence remains. $\times 280$.



(Liu: Influenza virus infection in ferrets. II)