IMMUNOCYTOLOGICAL EFFECTS OF VARIED INOCULA OF INFLUENZA VIRUS*

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The influenza virus elementary body contains a centrally located, predominantly type-specific "soluble" (S) antigen, and several type and strain-specific surface components (the V antigens), with hemagglutinating (HA) and enzymatic properties (1–5). The S antigen has been identified as a ribonucleoprotein (2, 6–9), and is believed to be the carrier of the genetic information necessary for virus growth. A hemagglutinating, nucleic acid–free protein has also been isolated (2, 6–9). In addition, the elementary body contains several lipoproteins and mucoproteins of host cell origin, some of which appear to be derived from the cell membrane (2, 10–14). Depending upon the method of preparation and storage, the virus particles found in different specimens may differ in structure, physical and chemical properties, and biological activity (5).

The steps in the replication of influenza virus are incompletely understood because of the observed differences in the time and sequence of appearance of the S, V, HA, and infectious properties of the virus in infected cells and in extracellular fluids under various conditions of infection (15–19). The results may vary with (*a*) the kind of host cells in which the virus is propagated, (*b*) the strain and previous history of the seed virus, (*c*) the size and composition of the inoculum, (*d*) the multiplicity of infection, and (*e*) the relative sensitivity of the methods of assay for each of the viral properties studied.

There is evidence that the soluble antigen and the surface components of the virus are synthesized first and that the particles become infectious (ID_{50} measurement) only on passage through the cell membrane (1, 20–23). However, those conditions of infection which lead to an initial increase in the S, V, and HA activities are frequently associated with a decreased yield of infectious virus and with the production of variable amounts of hemagglutinin (1, 20–27). In the extreme, the virus precursors may be formed without the release of any particles from the cells (28, 29). Thus, while the ID_{50}/HA ratio indicates the relative infectivity of the material tested, it provides no

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information concerning the structure or homogeneity of the particles, nor of the events which led to their formation in individual cells.

An earlier report from this laboratory (30) has dealt with the immunofluorescent cytology of influenza virus infection in the amniotic sac of the chick embryo. The results indicated that in most cells the virus antigens appeared first in the nucleus and later in the cytoplasm. Areas were also seen, however, in which the specific reaction occurred only over the exposed edge of the epithelium. It was suggested that the use of appropriately absorbed sera should make it possible to localize the S antigenic component. This has been done by Liu (31), who found that the substance present in the nucleus of some of the fluorescent cells in sections of infected ferret lung was, in fact, soluble antigen. With conjugates specific for hemagglutinin, on the other hand, the predominantly cytoplasmic staining was brightest along the free border of the ciliated cells.

The S and HA analogues of fowl plague virus, a close relative of influenza, have been similarly localized in infected tissue cultures of chick fibroblasts by Breitenfeld and Schäfer (32), chicken macrophages and giant cells by Franklin (33), and Earle's L cells by Franklin and Breitenfeld (34). The antigen corresponding to the S component was found to increase before any other viral activity and was first detected in the nucleus and, with the exception of the L cell cultures, later in the cytoplasm as well. The reaction with the anti-HA serum was first seen in a concentrated area apparently outside the nucleus and subsequently throughout the entire cell, but in some cells it was brightest at the cell margin. In the chick fibroblast cultures, the appearance of hemagglutinin was accompanied by an increase in the infectivity of the extracted cells. No infectious virus was recovered from the L cells, however, and the marginal fluorescence was not seen in these cultures.

While studying dual infection of the chick amnion with several members of the myxovirus group (35), it was observed that variation in the size and relative infectivity of the influenza virus inoculum led to differences in the immunofluorescent cytology as well as in the yield of virus. The results suggested that the larger the quantity of infectious virus produced, the more numerous were the cells with bright staining of the cell margin. In the experiments reported here the inoculum was varied systematically and the intracellular localization of specific fluorescence was followed at intervals in relation to the amount of infectious and hemagglutinating virus recoverable from the amniotic fluid.

Materials and Methods

Seed Virus.—The seed virus was the same egg-adapted PR8 strain of influenza A employed previously (30). It was originally obtained from Dr. John F. Enders, and prior to use was carried through 37 consecutive passages in 0.1 ml amounts of a 10^{-2} dilution in the allantoic sac. The infections were produced with frozen stock stored in a dry ice cabinet.

Inoculation of Eggs and Materials Harvested.—Groups of 10-day-old embryos were inoculated into the amniotic sac with the desired amount of virus, and after 15 minutes of gentle shaking on a shaking machine were placed in the incubator at 35°C. At appropriate time intervals, a representative number of eggs were taken from each group, and the amniotic fluids and membranes harvested. Some of the amniotic fluids from corresponding embryos

were pooled, while others were collected individually. Because of the scope of the experiments, the necessary titrations could not be performed simultaneously. For the sake of uniformity, therefore, the amniotic fluids were quick-frozen in glass-sealed ampules, and stored in dry ice prior to testing. The amniotic membranes were frozen in rubber-stoppered test tubes and maintained at $-10°$ to $-20°C$ until microtomy.

Infectivity Titrations.—After several preliminary titrations, using large numbers of eggs, the amount of virus present in samples of amniotic fluid under various experimental conditions could usually be predicted within 1 $\log_{10}$ dilution. The infectivity tests were performed thereafter in groups of 6 ten-day-old embryos inoculated into the allantoic cavity with 0.1 ml amounts of 4 tenfold dilutions spaced around the expected end-point. The eggs were incubated for 48 hours and, after chilling, tested for the presence of hemagglutinin using 0.5 ml of allantoic fluid from each egg and an equal volume of a 0.25 per cent suspension of chicken red cells. The 50 per cent infectious dose (ID_{50}) was calculated according to Reed and Muench, and the results expressed in $\log_{10}$ units per ml. Since the end-points obtained in comparative titrations of infectivity in the amniotic and allantoic cavities were the same, it was assumed that 1 ID_{50} unit in the amnion corresponded, on the average, to 10 hemagglutinating particles of which one to all may be infectious, as in the allantois (17).

Hemagglutinin Titrations.—The hemagglutinin (HA) titrations, which provide a measure of the total number of particles present, were performed in graded twofold dilutions using the same quantities of test fluid and red cells as in the infectivity determinations. To be comparable with the data reported by others, the results were adjusted so that they expressed the number of hemagglutinating doses of virus for 0.25 ml of a 1 per cent suspension of red cells in $\log_{10}$ units per ml (17). Under these conditions, one hemagglutinating dose corresponds to approximately 10^7 virus particles.

ID_{50}/HA ratios.—The ID_{50}/HA ratios were calculated in order to compare the quality of the virus produced since it has been found that this ratio is 6 when most of the particles present are infectious (17).

Preparation and Absorption of Fluorescein-labeled Antiserum.—An antiserum to the PR8 strain of influenza virus was prepared in rabbits using multiple injections of infected allantoic fluid. The globulin fraction of the serum was extracted and labeled with fluorescein isocyanate according to the procedures previously described (36). To remove the antibodies against the normal cell constituents, the conjugate was absorbed twice with powder prepared from amniotic and allantoic membranes by acetone precipitation (36).

Sectioning and Staining of the Amniotic Membranes.—The frozen amniotic membranes, which consisted of numerous folds of the thin, collapsed sac, were sectioned in the cryostat, stained with fluorescein-labeled immune serum, and observed under the fluorescence microscope (36). The over-all staining and the predominant cytology were recorded and the results compared with the amounts and proportion of infectious and hemagglutinating virus found in the corresponding amniotic fluids.

RESULTS

In comparing the yield of virus under various conditions of infection, it has been customary to express the inoculum in terms of the number of infectious and hemagglutinating particles injected per cell. While no figures are available on the number of cells lining the amniotic cavity, repeated determinations of the allantoic sac have given values of the order of 10^7 to 10^8 (18). Since the volume of amniotic fluid is approximately one-tenth that of the allantoic, a rough estimate of the number of exposed cells in the amniotic cavity would fall between 10^6 and 10^7. In the present studies, the inoculum was varied below and

above 10^6 to 10^7 ID_{50} units so that in one set of experiments the infections were initiated in a varying number of cells while in the other the cells were multiply infected with a varying number of virus particles. The infectious dose used in some instances differed in the proportion of infectious to hemagglutinating particles present.

Infections Initiated in a Varying Number of Cells

In the first set of experiments, the inoculum consisted of pooled allantoic fluid from eggs injected 44 hours previously with 0.1 ml of a 10^{-2} dilution of the stock virus. It titered $10^{7.5}$ ID_{50}'s and $10^{3.1}$ HA units per ml giving an ID_{50}/HA ratio of 4.4. Infection of the amnion was produced using 0.1 ml amounts of undiluted, 10^{-2}, and 10^{-4} dilutions of this preparation. The data shown in Table I are the mean titers of the infectious and hemagglutinating virus recovered from the amniotic fluid and the characteristic cytology observed in the corresponding membranes in at least 6, and sometimes as many as 12, embryos harvested at each of the indicated time intervals.

Events Following Injection of Undiluted Virus

When the largest dose of virus was incubated *in vitro* in 1.0 ml of normal amniotic fluid, its infectivity remained unchanged for 3 hours while the titer of hemagglutinin dropped immediately below the measurable range, returning to its initial level in 1 to 2 hours (presumably after destruction of the hemagglutinin inhibitor present in all tissue fluids). When this amount of virus was injected into the amniotic sac, on the other hand, less than 10 per cent of the infectious and hemagglutinating activities were recoverable from the amniotic fluid in the period from 15 minutes to 3 hours. The missing virus could not be visualized in any of the membranes for at least 2 hours. The first evidence of infection was the appearance of intranuclear viral antigen in a variable number of cells in 4 of the 9 membranes harvested at 3 hours. In some of the cells the localization of the specific fluorescence was distinctly granular (Fig. 1) while in others it filled the entire nucleus (Fig. 2).

After 6 hours, all of the membranes showed varying degrees of involvement which was more extensive than before. The intranuclear fluorescence continued to predominate but it tended to be less discrete because of the appearance of antigenic material in the adjacent cytoplasm, and sometimes throughout the entire cell (Fig. 3). The quantity of infectious virus recovered at this time remained unchanged and was below the level measurable by hemagglutination. The titer of hemagglutinin, on the other hand, increased almost tenfold, indicating that the events observed were associated with the production of "incomplete," or otherwise defective, virus (25, 26).

While the staining of the nuclei, with and without varying degrees of cytoplasmic involvement, was the characteristic feature of the infection, a bright

patch of fluorescence was occasionally seen over the exposed edge of an otherwise unstained cell (Fig. 4 and 5). Later, a more diffuse reaction appeared deeper in the cytoplasm and ultimately, though to a lesser extent, in the nucleus (Fig. 6). In the 12 hour membranes, the number of cells showing this localization tended to vary in proportion to the increase in the residual infectivity of the

TABLE I

Correlation of the Intracellular Localization of Influenza Virus Antigens with the Amount and Kind of Virus Produced in the Amniotic Sac of Chick Embryos Injected with Graded Doses of Allantoic Fluid Seed Virus

Inoculum	Test	Time after inoculation								
		Start	15 min.	1 hr.	2 hrs.	3 hrs.	6 hrs.	12 hrs.	24 hrs.	48 hrs.
Undiluted	ID$_{50}$	6.5	5.5	4.5	4.3	4.8	5.0	5.8	6.8	6.2
	HA	2.1	<1.0–1.3	±1.0	±1.0	±1.0	1.9	2.5	2.8	2.8
	ID$_{50}$/HA	4.4					3.1	3.3	4.0	3.4
	Cytology		C	0	0	n	Nc	Nc	Nc	Nc
						w	cw	cw	cw	cw
10^{-2}	ID$_{50}$	4.5	2.5			2.0	3.5	6.5	8.7	8.4
	HA	0.1	<1.0			<1.0	<1.0	2.2	3.1	3.4
	ID$_{50}$/HA	4.4						4.3	5.6	5.0
	Cytology		0			0	n	CW	CW	CW
							w	NC	NC	NC
10^{-4}	ID$_{50}$	2.5	<1.0				2.7	5.9	8.1	10.0
	HA	−2.1	<1.0				<1.0	<1.0	3.1	4.0
	ID$_{50}$/HA	4.4							5.0	6.0
	Cytology		0				0	w	W	CW
								n	nc	nc

The ID$_{50}$ and HA titers are expressed in log$_{10}$ units/ml. of amniotic fluid.

The concentration of virus present at the start was calculated from the ID$_{50}$ and HA titer of the seed virus taking into account that the volume of amniotic fluid in 10-day-old embryos averaged 1 ml.

Cytology refers to the intracellular localization of the virus antigens in the amniotic membranes, as evidenced by specific staining with fluorescent antibody, and is described as N, n intranuclear, C, c cytoplasmic, W, w cell wall. The capital letters indicate the predominant localization.

ID$_{50}$ unit = 10^1 virus particles.

HA unit = 10^7 virus particles.

All virus infectious when ID$_{50}$/HA = 6.

amniotic fluid from the same egg. This suggested that the order of appearance of the antigenic material in different parts of the cell reflected the kind of virus synthesized.

After the 6th hour of incubation, the staining seen in some portions of the

membranes began to extend into the deeper cell layers. In others, however, it remained confined to the surface epithelium which was frequently punctuated by areas of unreactive cells (Fig. 7 and 8). The increase in new virus, with a higher ID_{50}/HA ratio, reached a peak between the 12th and 24th hour of incubation but the amount of infectious virus recovered failed to exceed the quantity injected and the yield of hemagglutinin was lower than in other experiments. The results were thus consistent with the events which occur under conditions leading to "auto-interference" (15).

Assuming that the number of cells lining the amniotic cavity is closer to 10^6 than 10^7, if one HA unit represents 10^7 virus particles and 1 ID_{50} contains 10 hemagglutinating particles of which one to all may be infectious, the inoculum used in this group of embryos should have been sufficient to expose each cell to at least 1 infectious and to roughly 100 to 1000 non-infectious particles each. The fact that the staining seen throughout the cell population was not uniform, therefore, implied either that the number of exposed cells in the amnion was greater than $10^{6.5}$ or that the infections produced in different cells were governed by factors other than merely the numerical relation between the number of infectious and hemagglutinating particles and the number of cells.

Events Following Injection of the 10^{-2} Dose

When the quantity of virus injected was reduced 100-fold, the titer of hemagglutinins fell below the measurable range, but the proportion of infectious virus remaining was the same as in the group given undiluted virus. The duration of the "constant" period, or interval during which the quantity of unabsorbed virus remained unchanged, was also 3 hours as before. No staining was detectable, however, until the 6th hour of incubation and this coincided with a distinct increase in the infectivity of the amniotic fluid without a measurable change in hemagglutinin. The virus antigens were restricted to a small number of cells in which the specific reaction was seen either over the nucleus and sometimes the adjacent cytoplasm, or as a discrete area of fluorescence at the cell periphery (Fig. 9).

At 12 hours, the amount of infectious virus produced was almost 10 times greater than in the group given the larger inoculum although the titer of hemagglutinin was only slightly lower. The staining seen at this time was less extensive than in the corresponding membranes described previously but the localization was such that all phases of the infection were represented (consistent with more than one cycle of growth). In addition, there was a marked increase in the number of areas with a discrete rimming of the exposed cell border, which again suggested a relation between this cytology and the greater infectivity of the virus produced.

After 24 hours of incubation, the specific fluorescence became detectable throughout most, though not all, of the membrane, and was accompanied by a

proportionate increase in the number of cells with the bright periphery. In some fields, however, the staining of the cell edge appeared "faded" while in others, the cell outlines were poorly defined but the nuclei were paler than the rest of the cell (Fig. 10). Areas were also present in which the cells were undergoing degeneration. The quantity and relative infectivity of the virus produced increased further but the total yield of virus, even after 48 hours of incubation, was not maximal. As before this appeared to be related to the persistence of a certain number of cells which either failed to stain or in which the reaction remained brightest in the nucleus.

While the number of infectious particles injected into this group of embryos was only a fraction of the estimated number of cells, the multiplicity of the non-infectious hemagglutinin was 1 or greater. Under these conditions one might have expected that the uptake of the non-infectious particles would lead to one sequence of events and the uptake of both kinds of particles to another, so that the infection would be restricted to one cycle of growth. This, however, did not appear to be the case, indicating again either that the estimate of the number of cells was wrong or that the quantitative relation between the number of virus particles and the number of cells was not the only factor which governed the course of infection throughout the cell population.

Events Following Injection of the 10^{-4} Dose

In the embryos injected with the smallest inoculum, no virus was detectable either by hemagglutination or infectivity 15 minutes later. The first evidence of infection was the return of infectivity in the amniotic fluid harvested at 6 hours. By the 12th hour, the ID_{50} titer had risen to 5.9, a level just below that measurable by hemagglutination, indicating that the ratio of infectious to hemagglutinating particles was close to 1. At this time a small number of cells was seen with a bright patch of fluorescence over the exposed cell margin, and among them, an occasional cell which either stained throughout or in which the staining was brightest in the nucleus. After 24 hours of incubation, the infection became detectable throughout most of the membrane. The immunocytology at this time was characterized almost exclusively by the presence of a discrete line of brilliant fluorescence which followed the outer edge of the epithelium, occasionally dipping in between individual cells, and in some areas (in which the epithelium was apparently sectioned in a lateral plane), outlining the entire cell margin (Fig. 11). After 48 hours, the staining of the cell periphery was paler and the antigenic material was detectable deeper in the cytoplasm and in some areas, in the underlying cell layers. While a few cells were always seen with a predominantly intranuclear infection, they were smaller in number than in either of the groups of embryos injected with the larger inocula.

From the figures shown, the proportion of infectious to hemagglutinating virus recovered at 24 hours was lower than at 12. After 48 hours, however, the

ID_{50}/HA ratio had returned to 6 indicating that the production of infectious virus was in excess of the increase in the non-infectious hemagglutinin. The total yield of virus was also the largest harvested.

Since the inoculum used in this group of embryos was sufficient to expose a relatively small proportion of cells to either 1 infectious, 1 non-infectious, or both kinds of virus particles, one might have expected that the course of this infection would be the most heterogeneous of all. Whatever the differences at the start, however, they were not apparent during the later stages of virus growth and the results were the reverse of those in the group given undiluted virus. This left little doubt that the course of spread of antigenic material from the outer cell periphery into the interior of the cell was associated with the production of infectious virus.

Infections with Large Multiplicities of Virus

In the second set of experiments, the seed virus was prepared as usual in the allantoic sac using, however, 0.1 ml of a 10^{-4} instead of a 10^{-2} dilution of the stock virus. The new preparation contained $10^{8.6}$ ID_{50}'s, and $10^{3.7}$ HA units per ml, with the resulting ID_{50}/HA ratio of 4.9. To increase the experimental dosage range, a portion of the pooled harvest was concentrated in the centrifuge and resuspended in one-hundredth the original volume. On titration of the concentrate, the ID_{50}, HA and ID_{50}/HA values were $10^{10.7}$, $10^{5.4}$, and 5.3 respectively. Groups of embryos were infected as before using graded inocula adjusted to contain the equivalent of 0.1 ml of 100 times concentrated, 10 times concentrated, reconstituted and uncentrifuged allantoic fluid. The virus recovered from the amniotic fluids and the staining seen in the corresponding membranes in 1 of 3 of the embryos harvested at various time intervals are summarized in Table II.

When compared with the data shown in Table I, the hemagglutinin titer of the reconstituted virus was only twofold greater than that of the undiluted inoculum used in the first set of experiments, but the infectivity was 10 times higher. With the larger proportion of infectious virus, the staining seen 3 hours after inoculation was much more extensive, and by the 6th hour the specific fluorescence was present throughout most of the membrane. The number of cells showing a bright cell border was also larger so that the cytology of this infection was more like that of the group given the 10^{-2} dose and this was reflected in the virus yield. When compared with the other data shown in Table II, the embryos injected with reconstituted virus were the only group in which there was a measurable increase in both infectious and hemagglutinating virus.

While the difference in the hemagglutinin titer of the uncentrifuged and reconstituted virus could not be considered significant, the proportion of cells showing the brighter marginal fluorescence was distinctly smaller in the former. The staining seen after 6 hours of incubation was also not as advanced. The

infectivity of the amniotic fluid did not increase and the yield of hemagglutinin was smaller. These variations implied a hidden difference in the virus measured before and after centrifugation. Similar discrepancies have been encountered in

TABLE II

Correlation of the Intracellular Localization of the Virus Antigens with the Amount and Kind of Virus Produced in the Amniotic Sac of the Chick Embryo with Graded Doses of Centrifuged Virus

Inoculum 0.1 ml.	Test	Time after inoculation					
		Start	15 min.	1 hr.	2 hrs.	3 hrs.	6 hrs.
100 times concentrated	ID_{50}	9.7	8.2	8.6	8.5	7.2	<7.0
	HA	4.4	3.7	4.0	3.7	<3.1	<3.1
	ID_{50}/HA	5.3	4.5	4.6	4.8		
	Cytology		0	N*	N*	N*	
10 times concentrated	ID_{50}	8.7	8.5	7.9	7.9	7.5	7.4
	HA	3.4	3.4	2.8	2.8	2.8	3.1
	ID_{50}/HA	5.3	5.1	5.1	5.1	4.7	4.3
	Cytology		0	C	N	NC cw	NC cw
Reconstituted	ID_{50}	7.7	7.4	6.6	6.8	6.7	7.4
	HA	2.4	1.3	1.3	1.3	1.3	3.1
	ID_{50}/HA	5.3	6.1	5.3	5.5	5.4	4.3
	Cytology		0	0	C	NC CW	NC CW
Original	ID_{50}	7.6	6.8	6.8	7.1	6.9	7.1
	HA	2.7	1.0	1.0	1.6	1.6	2.2
	ID_{50}/HA	4.9	5.8	5.8	5.5	5.3	4.9
	Cytology		0	0	0	Nc cw	NC CW

Legend same as in Table 1.
* Pale nuclear fluorescence in some of the cells.

other experiments in which the use of apparently identical inocula gave rise to distinctly different infections.

With the 10 times-concentrated virus, the localization of the specific fluorescence became detectable at the end of the 2nd hour of incubation at which time it was restricted to the nuclei of a certain proportion of cells. By the 3rd hour, some cells were also seen with a brighter staining of the cell margin but they were much fewer in number than in the group given reconstituted virus. Thus, while the use of a larger proportion of infectious virus led to an increase in the number of cells with peripheral fluorescence, this effect was reversed when the

multiplicity of infection was increased as well. In another experiment in which the amount of infectious virus used was comparable to that of the 10 times concentrated dose but the ID_{50}/HA ratio was 6, the predominant cytology was more like that seen after injection of small inocula. The level of residual virus was lower and, in contrast to the above, there was a measurable increase in both infectious and hemagglutinating virus. The increase in infectious virus, moreover, preceded that of the hemagglutinin and was first detected at 3 hours. This was the only group in which the release of new virus was measurable before the 6th hour of incubation.

The inocula used in this set of experiments caused varying degrees of damage to the embryo which appeared progressively earlier the larger the dose. The amnion became constricted and there was a decrease in the volume and an alteration in the consistence of the amniotic fluid. The staining seen by the 6th hour of incubation usually extended into the deeper cell layers and this was sometimes accompanied by a brighter fluorescence of the exposed edge of the opposite side of the membrane (Fig. 12), as a result of multiplication of the small amount of virus introduced into the allantoic fluid in the course of amniotic inoculation.

After injection of the 100 times–concentrated virus a pale intranuclear reaction was detected in a certain proportion of the cells in some of the membranes harvested at 1, 2, and 3 hours. The embryos of this group, however, survived for only a few hours.

Even if the number of cells lining the amniotic cavity were 10^7, the inocula used in this set of experiments should have led to uniform infections restricted to one cycle of growth throughout the cell population. However, the events visualized intracellularly and, when measurable, the virus produced again varied according to the total number and relative infectivity of the particles injected. In addition, the infections were detectable earlier and progressed more rapidly the larger the size of the dose.

DISCUSSION

It has long been known that variation in the size and composition of the inoculum may have a profound effect on the quantity and quality of the virus yield (20–23, 25, 26). The evidence presented here has indicated that this variation is associated with striking differences (*a*) in the time and order in which the virus antigens appear in different parts of different cells, (*b*) in the relative proportion of cells showing either one or another sequence of events, and (*c*) in the ultimate extent of the involvement throughout the available cell population.

As in the experience of others, the use of small inocula favored the production of infectious virus and this was associated with a predominance of cells in which the virus antigens were first detected in the exposed cell periphery, later deeper in the cytoplasm, and finally, though to a lesser extent, in the nucleus. When the

inoculum was large, the amount of infectious virus produced and the proportion of cells showing this cytology varied according to the total number and relative infectivity of the particles injected.

In general, the higher the multiplicity of infection and the lower the ID_{50}/HA ratio of the virus seed, the greater the quantity of non-infectious virus produced and the more numerous the cells in which the specific fluorescence was first seen in the nucleus, later spreading to the cytoplasm and ultimately throughout the entire cell. This sequence of events has also been observed in studies of fowl plague virus in tissue cultures infected with large inocula (32–34). The conjugates used, however, were specific for either the S or the HA components respectively, and hence did not reveal the entire viral antigen content of the cells. Further, since the cells were not sectioned, initial localization of hemagglutinin in the "endoplasmic reticulum" (32) could not be distinguished from a reaction occurring at the cell surface. The fact that some cells were present with a brighter fluorescence of the cell margin (32) was attributed to the final aggregation of the substance first seen in the nucleus and cytoplasm.

Under conditions which led to "autointerference," some of the cells remained unreactive throughout while the staining of others failed to progress beyond the nuclear or early cytoplasmic stage. Similar results have been reported by Franklin and Breitenfeld (34) in a study of an abortive infection with fowl plague virus in Earle's L cells. In this instance the soluble antigen and hemagglutinin components were formed in approximately the same sequence as in the "normal infection" of chick cells, but the soluble antigen was detectable only in the nucleus and no hemagglutinating particles were released from the cells until they were disrupted. It is also significant that the bright rimming seen in the chick cell cultures did not occur in the L cells.

As the size of the inoculum was increased, the interval between injection and the initial appearance of the specific fluorescence decreased progressively even when the multiplicity of infection was greater than 1. With the largest dose used, a pale intranuclear reaction was visible after 1 hour, but the embryos died shortly thereafter. As evidenced by the staining, the course of infection was also more rapid the larger the dose, indicating that the duration of the "infectious cycle" may be shorter the greater the number of particles infecting an individual cell. This was not apparent from the amniotic fluid titrations, since, with one exception, the level of residual virus stayed "constant" until the 6th hour of incubation. When the amount of virus produced failed to exceed the quantity remaining in the fluid from the inoculum, the intracellular fluorescence provided the only evidence of infection.

The reactions did not appear synchronously or increase uniformly throughout the cell population even after injection of apparently similar inocula, sufficient to expose all of the cells at the start to many infectious and non-infectious particles. This fact implies that the numerical relation between the

virus particles and cells is not the only factor which determines the course of infection. In support are the results of Deibel and Hotchin (37) who compared the proportion of cells containing antigen in tissue cultures of chick embryo, human amnion, and calf kidney cells infected in various ways with two non-cytopathic strains of influenza virus. The number of cells localized was found to vary according to the cell-virus system used, the size of the inoculum, the period of adsorption, and the time of incubation. Differences in the number of stained cells and in the extent of nuclear and cytoplasmic involvement with the W.S. and MEL strains of influenza virus in calf kidney cell cultures have also been observed by Anderson, Armstrong, and Niven (38) using acridine orange. Pereira, Allison, and Balfour (39) have suggested that the asynchrony of infection with adenovirus in HeLa cell cultures may be the result of cell to cell variation in the time required to initiate the infective process. The studies of Franklin (33), and of Wheelock and Tamm (40), on the other hand, indicate that susceptibility to infection may vary with the physiological state of the cell before and after mitosis.

The interpretation of the observations reported here is uncertain. It is possible that the use of a small inoculum leads to the replication of the most infectious particles in a few cells which produce more virus of the same kind. This in turn is taken up by fresh cells as soon as it is released, ultimately resulting in a uniform infection throughout the cell population. The fact that the cytology of this infection is characterized by a predominance of cells in which the virus antigens are first detected at the cell periphery may simply mean that the soluble antigen and hemagglutinin formed in the nucleus and cytoplasm are rapidly converted into infectious virus and do not accumulate in detectable quantity until the cell membrane is damaged sufficiently, thereby creating a "bottleneck in the production line". When the inoculum is large, the events which take place in different cells may be determined by (*a*) the total number and particular set of infectious and non-infectious particles taken up, (*b*) the time and order in which the various particles enter the cell, (*c*) the relative susceptibility of the cell, and (*d*) the opportunity provided for independent replication, recombination, interference, and interferon production (18). It is conceivable that of the particles taken up only one may actually multiply, because of either its earlier entry into the cell or its more rapid rate of growth. The situation would thus be analogous to that of the infection with the small inoculum. The simultaneous replication of several virus particles, on the other hand, may be expected to lead to a more rapid accumulation of the soluble antigen and hemagglutinin components with the initial appearance of the specific fluorescence first in the nucleus and only later in the cytoplasm. This may tax the synthetic capacity of the cell in various ways resulting in the release of defective virus. Finally, if the genetic information necessary for replication is incomplete, or if the cell is overwhelmed by a sufficiently large

number of virus particles, the infection may become arrested at a progressively earlier stage, and in the extreme, lead to a refractory state or to the production of interferon. The events seen under conditions of "autointerference" and after the injection of the largest dose of virus fit these two categories.

SUMMARY

Variation in the amount and quality of influenza virus injected into the amniotic sac of the chick embryo led to differences not only in the yield of virus but also in the immunofluorescent cytology of the infection.

The production of *infectious* virus was associated with a predominance of cells in which the virus antigens were first detected along the cell surface in contact with the amniotic fluid, later deeper in the cytoplasm, and finally, though to a lesser extent, in the nucleus.

When the virus yield was primarily *non-infectious* hemagglutinin the virus antigens appeared in reverse sequence; *i.e.*, nucleus first, then adjacent cytoplasm, and ultimately throughout the entire cell.

Under conditions of "autointerference," the immunofluorescence seen in some of the cells failed to progress beyond the nuclear or early cytoplasmic stage, while many other cells remained unreactive throughout the experimental period.

With the largest dose used a pale intranuclear reaction was localized 1 hour after injection but the embryo died shortly thereafter.

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EXPLANATION OF PLATES

The photomicrographs are of tissue sections of folds of the collapsed amniotic sac in various stages of infection with graded doses of the PR8 strain of influenza virus. They were taken under the fluorescence microscope after the tissues had been treated with fluorescein-labeled immune serum containing antibodies to all the antigenic components of the virus except the one removed by absorption with normal membrane powder.

PLATE 16

FIG. 1. Granular fluorescence of the nuclei 3 hours after infection with the undiluted virus used in the first set of experiments. × 560.

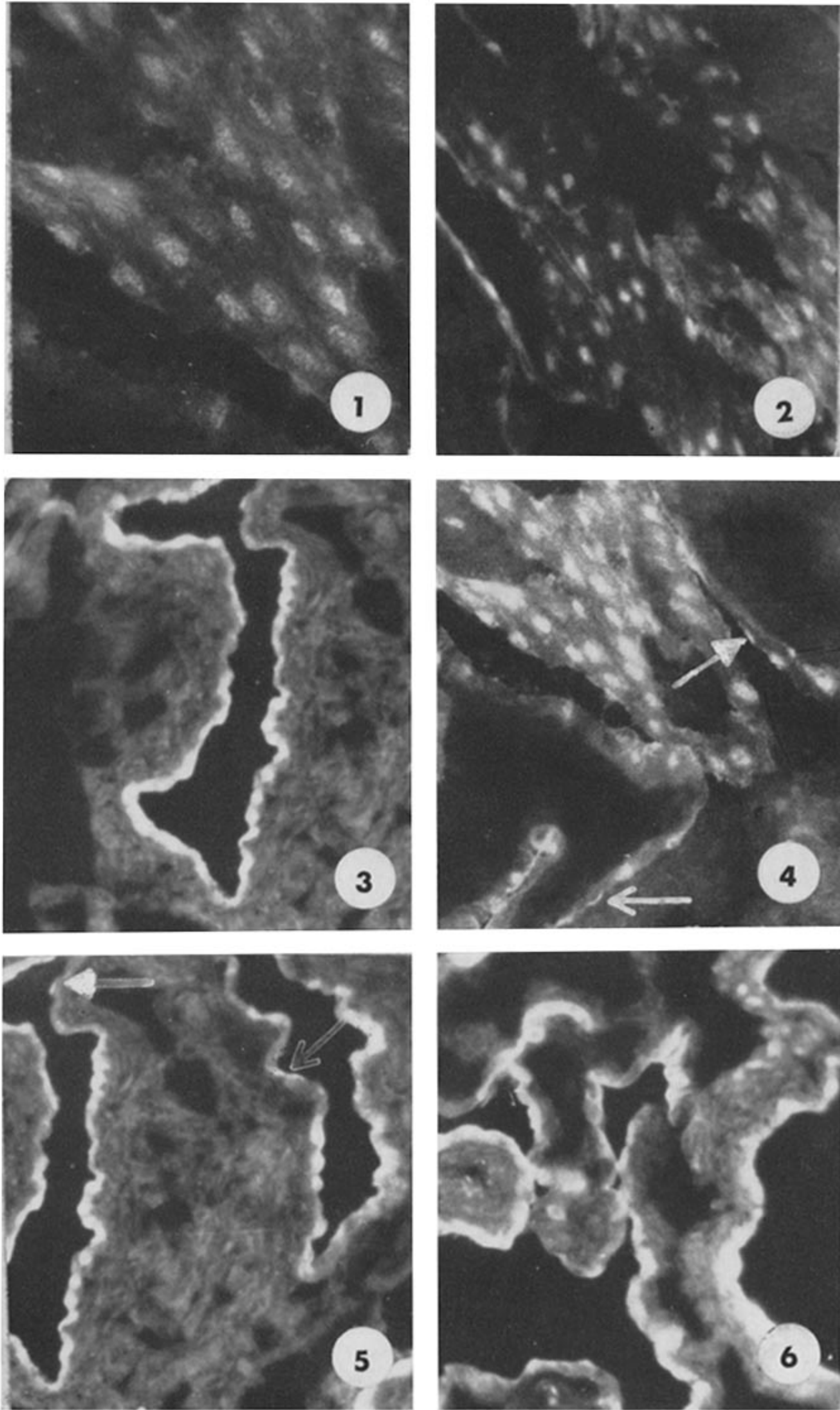
FIG. 2. Diffuse and granular nuclear fluorescence in another area of the same section. × 390.

FIG. 3. Predominantly nuclear fluorescence extending to a varying degree into the area between the nuclei 6 hours after infection with the same inoculum. × 390.

FIG. 4. Another field of the same section as in Figs. 1 and 2 showing 2 areas (arrows) with discrete patches of fluorescence over the outer exposed edge of the epithelium. × 390.

FIG. 5. Another field of the same section as in Fig. 3 showing 2 areas (arrows) with discrete patches of fluorescence over the outer edge of the epithelium. × 390.

FIG. 6. Fluorescence of outer edge of epithelium which appears to be extending inwardly into the cells 12 hours after infection with undiluted virus. × 390.



(Watson: Varied inocula of influenza virus)

PLATE 17

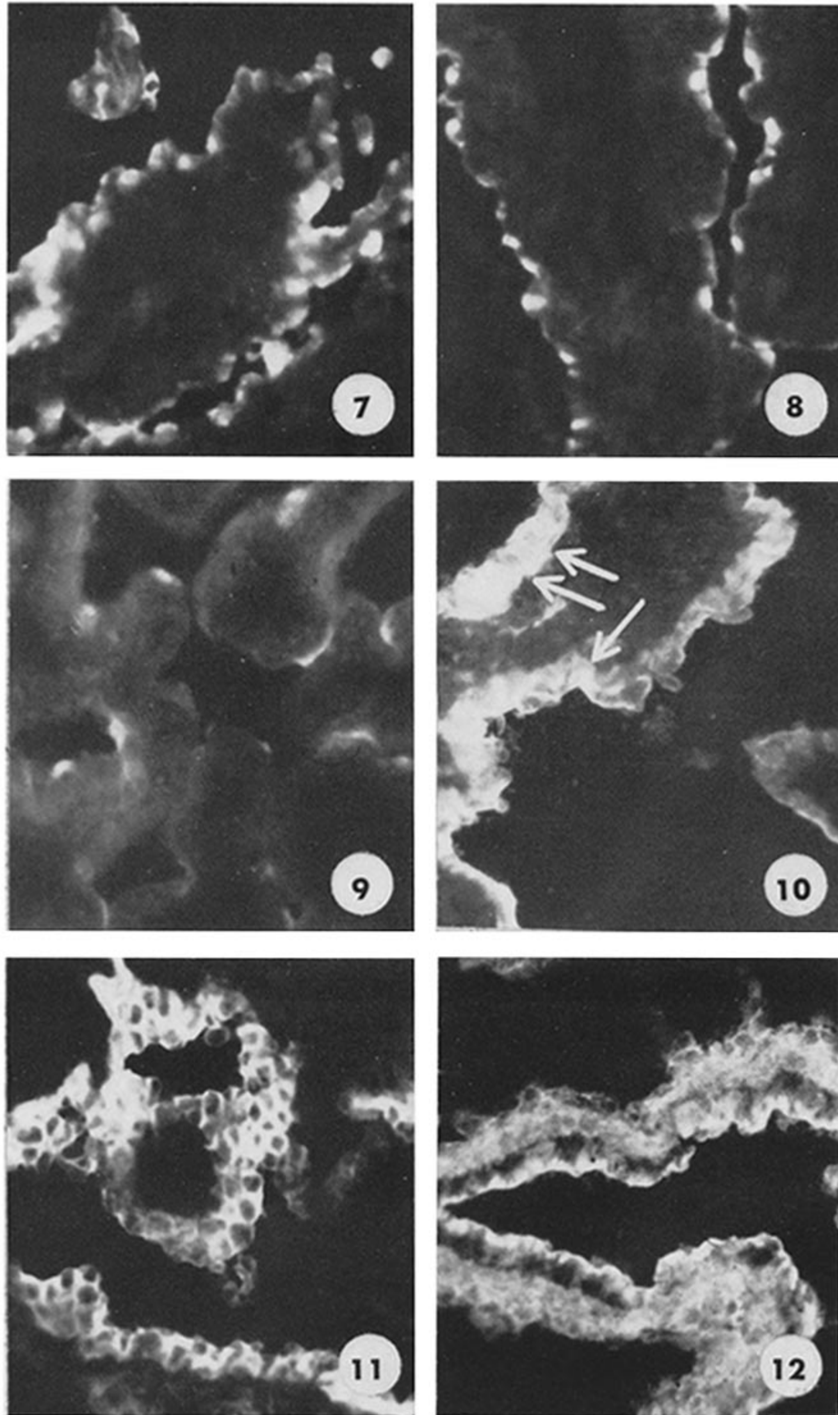
FIGS. 7 and 8. Irregular distribution of the specific fluorescence 24 hours after infection with undiluted virus. $\times$ 390.

FIG. 9. Fluorescence over nucleus in some areas, and outer edge of epithelium in others 6 hours after infection with the 10^{-2} inoculum. $\times$ 560.

FIG. 10. Varying degrees of fluorescence in outer edge of epithelium 24 hours after infection with the 10^{-2} inoculum. Arrows point to cells in which the nuclei appear paler than the cytoplasm. $\times$ 390.

FIG. 11. Individual cells outlined by brilliant fluorescence of cell borders 24 hours after infection with the 10^{-4} inoculum. $\times$ 390.

FIG. 12. Fluorescence of several cell layers on one side of the membrane as well as brighter staining of the opposite surface 12 hours after infection with the centrifuged and reconstituted virus used in the second set of experiments. $\times$ 390.



(Watson: Varied inocula of influenza virus)