Virological, Immunochemical, and Cytochemical Studies of Four HeLa Cell Lines Infected with Two Strains of Influenza Virus

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The production of infectious virus, hemagglutinin, and viral (V) antigens and the changes in ribonucleoprotein (RNP) and lipoprotein metabolism have been studied in four sublines of HeLa cells infected with the PR8 and a PR8 recombinant strain of influenza virus. Much greater amounts of infectious virus and much less hemagglutinin were produced by the PR8 recombinant than by PR8 virus in all four cell lines. Different amounts of infectious virus per infected cell were produced by the recombinant in the four cell lines, whereas very little infectious virus was produced by the PR8 strain in any of the HeLa cells. In all cell lines infected with both strains of virus, "soluble" (S) antigen appeared early in the nucleolus. In cells infected with PR8 recombinant, S antigen subsequently filled the nucleus and later appeared in the cytoplasm. In most cells infected with PR8 virus, nuclear S antigen did not fuse to fill the nucleus, and S antigen was not detected in the cytoplasm. V antigen was observed in the cytoplasm of cells when diffuse nuclear S antigen had formed. The earliest and most frequent change in the RNP of the infected cells was a decrease in stainable RNP spherules (nucleolini) in the nucleolus. This was followed, in a smaller proportion of cells, by the appearance of nuclear and cytoplasmic inclusions containing RNP. There was a characteristic difference in the morphology of the cytoplasmic inclusions produced by the two strains of virus, but the same types of inclusions were observed in all four HeLa lines. A significant increase in lipoprotein was observed only in association with the cytoplasmic inclusions produced by PR8 recombinant virus. There was a striking difference in the proportion of cells with cytochemical changes in RNP in the four cell lines. A significant cytopathic effect (CPE) was observed only in three virus-cell systems in which a high proportion of cells exhibited changes in nucleolar RNP. It is suggested that disappearance of RNP in the nucleolini may be an indication of shutdown of host ribonucleic acid synthesis and that this in turn results in a CPE. Virus infection resulted in a C-mitotic block that was followed by karyorrhexis. Infection of the cell did not always result in the production of infectious virus, in changes in the RNP of the nucleolini, in the development of nuclear or cytoplasmic RNP inclusions, or in CPE. The results suggest that production of infectious virus, shutdown of cellular RNP synthesis with accompanying CPE, and the formation of inclusions appear to be independent events.

Previous studies by Henle et al. (8) have demonstrated that the PR-8 strain of influenza A virus produced a nontransmissible cytopathic effect (CPE) in HeLa cells accompanied by the formation of noninfectious hemagglutinins. This observation has been confirmed with the same or other strains of virus and a number of different cell lines (1, 5, 7, 23). The events taking place have been further studied by application of immunofluorescence techniques, differentiating between the "soluble" (S) and the viral (V) antigens.

Under conditions of infectious virus replication, S antigen was shown to develop in the nucleus (14) and then to penetrate into the cytoplasm (3, 11), whereas V antigen formation was restricted to the cytoplasm. In abortive infections, the S antigen was almost entirely confined to the nucleus throughout the observation period (7, 10), or it faded from the nucleus (15) and did not appear in detectable concentrations in the cytoplasm. The development of V antigen followed a course similar to that described for replication of

infectious viral progeny. The recorded differences in the fate of S antigen and the occasionally observed small yields of infectious viral progeny might be due to the fact that either different strains of virus or different cell lines were employed. That lines of a given cell maintained in different laboratories may no longer be identical was demonstrated by Wong and Kilbourne (38) who showed that a variant of Chang's human conjunctive cells was capable of replicating infectious influenza virus.

In the present investigation, four different sublines of HeLa cells were employed for infection by the standard PR-8 strain leading to an abortive infection or a PR-8 Melbourne (Mpt) recombinant (32) capable of yielding mainly infectious progeny. The relationships among the parameters of viral growth, hemagglutinin production, antigen development, and changes in ribonucleoprotein (RNP) and lipoprotein metabolism were examined.

MATERIALS AND METHODS

Cells. Four lines of HeLa cells were used. One uncloned line from the American Type Culture Collection was designated HeLa W. The second was the S3 clone and was recloned in this laboratory. The third was a clonal line isolated by Finnish workers (HeLa F; 29). The fourth was an uncloned line obtained from J. J. Holland and was designated HeLa JJH. Cells were grown in Eagle's minimal essential medium containing 10% fetal calf serum with added antibiotics (penicillin and streptomycin, 100 units of each per ml, or kanamycin at 25 μ g/ml). After infection, immunochemical studies were made on cells grown in Leighton tubes or, in some experiments, on cells grown in the same manner as for cytochemical studies. Cytochemical studies were performed on cells grown on cover slips in "ring cultures" prepared by sealing small stainless-steel cylinders to the cover slips with a paraffin-wax mixture (35). "Ring cultures" were incubated at 37 C in a humidified atmosphere containing approximately 5% CO₂. Cells were counted by an electronic particle counter (Coulter Electronics Sales Co., Hialeah, Fla.).

Virus. Two strains of influenza viruses, the standard PR8 strain of type A and a PR8 recombinant (PR8-R), were employed. The latter was obtained by recombination of ultraviolet-inactivated Melbourne (Mpt) and an active PR8 Albany strain (32). Virus seeds were prepared by allantoic passages in chick embryos by a routine procedure (9), and infected allantoic fluid served as inocula for the cell cultures. The infectivity titers for both strains were in the order of 10⁹ EID₅₀/ml.

Antiserum. Anti-PR8 serum was obtained from rabbits which were injected five times at weekly intervals with 1 ml of a suspension of PR8-R in Hanks' solution containing 10¹⁰ EID₅₀/ml. One week after the last infection, blood was withdrawn by cardiac puncture and the sera were separated. The antibody titers were determined by neutralization tests in chick embryos, and they ranged from 2 $\times$ 10³ to 4 $\times$ 10³

units/ml against 10⁴ EID₅₀ of PR8-R or PR8 per ml. This serum was used for inactivation and fluorescent-antibody staining of both virus strains.

S antigen. S antigen as well as the corresponding specific guinea pig anti-S serum were prepared according to established techniques (13).

Infection of cells for immunochemical and cytochemical studies. A counted number of HeLa cells in suspension was exposed to PR8 or PR8-R at known input multiplicities and was incubated in a water bath of 37 C for 1 hr. At the end of the incubation period, the preparations were centrifuged at 100 $\times$ g for 10 min. The sedimented cells were washed with medium three times, the second time with the addition of anti-PR8 serum, diluted 1:25 in Hanks' balanced salt solution, in order to remove or neutralize remaining extracellular virus. The cells were then resuspended in medium to 1 $\times$ 10⁵ cells per ml, and 1-ml amounts were seeded into Leighton tubes with or without cover slips.

In the experiments for cytochemical studies, cells were infected as monolayers in "ring cultures" (35) by applying 0.1 ml of virus inoculum for 1 hr at 37 C. Subsequently, the cultures were washed once with 0.5 ml of medium, twice with 0.2 ml of diluted anti-PR8-R serum, and once more with 1 ml of medium. At the time of harvest, media and cells were collected separately and stored at -20 or -70 C for virus assays. The cells from the ring cultures were removed by a solution of ethylenediaminetetraacetic acid (Versene) (24).

Virus assays. The titration of infectivity and hemagglutinin were carried out as previously described (9).

Fluorescent-antibody staining techniques. The γ -globulin fractions of rabbit anti-PR8 serum and guinea pig anti-S serum were coupled to fluorescein isothiocyanate (J. D. Marshall, Jr., et al., *Bacteriol. Proc.*, p. 136, 1958). The labeled γ -globulin was absorbed twice with mouse liver powder before use. Cover slips harvested from Leighton tubes were washed twice in phosphate-buffered saline, dried and fixed in acetone at room temperature, and stored at -20 C. The cover slips were exposed to fluorescein conjugate at 37 C for 1 hr, washed, and mounted on slides in a semipermanent mounting medium (28). V antigen was demonstrated by first treating with unlabeled anti-S serum, followed by staining with labeled anti-PR8 globulin. The percentage of cells showing virus specific immunofluorescence was determined by counting a total of 1,000 cells.

Cytochemical-staining techniques. Studies of nucleic acid metabolism were carried out by use of the toluidine blue-molybdate (TBM) methods (21) with appropriate nuclease digestions to check the specificity of the stain for nucleic acids (18). Preparations were also stained for deoxyribonucleic acid (DNA) by the Feulgen method (26).

Lipids were studied in preparations fixed by Elftman's method (6) for 48 hr and stained by Sudan black. In some instances after fixation, the more soluble neutral lipids were removed by treatment of the fixed cells for 3 min with absolute ethyl alcohol before staining with Sudan black to allow better visualization of the less soluble phospholipid-contain-

ing structures. Lipids were also stained by the Nile blue sulfate method (4) after digestion with ribonuclease to prevent staining of ribonucleic acid (RNA). The specificity of these stains was checked by extraction of cultures with boiling chloroform-methanol for 18 hr. Preparations were also stained after digestion with diastase to remove any glycogen that might be present (20) by the periodic acid Schiff (PAS) method (26) with and without previous extraction of lipids. The amount of reduction in PAS-positive material produced by lipid extraction gave an indication of the amount of PAS-positive lipid (glycolipid or phospholipid) that was present. Residual PAS-positive staining after diastase digestion and after lipid extraction was attributed to glycoprotein or mucoprotein (20).

Preparations were stained for protein-bound groups by the acrolein-Schiff method (34) after fixation for 15 min in formol sublimate (21) at room temperature.

Analysis of cellular changes. Analysis of the incidence of cells containing intranuclear or cytoplasmic inclusions, or both, was made by counting 100 cells in each preparation stained by TBM method C (21) after digestion with deoxyribonuclease (18), except when the incidence was less than 5%, when 1,000 cells were scored. Estimation of the mitotic index was based on analysis of 500 cells in preparations stained by TBM method C.

RESULTS

Production of infectious virus and hemagglutinin.

The amounts of virus and hemagglutinin produced by infection of the four HeLa lines with the two strains of virus are shown in Table 1. Significantly more infectious virus was produced by PR8-R than by PR8 in all four cell lines. Conversely, cells infected with PR8-R produced generally less hemagglutinin than those infected with PR8. Thus, the EID_{50} to hemagglutination (HA) ratios of the PR8 progenies were of a low order, as expected (8), indicating the production of large amounts of noninfectious virus particles. On the other hand, the ratios obtained with PR8-R were close to standard, i.e., more virus particles were infectious.

Analysis of viral replication. Immunochemical and cytochemical studies were performed on cultures 7, 10, and 24 hr after infection with PR8

and PR8-R. The results of virus titrations, the proportion of cells containing V or S antigen, and the total virus yield per culture and per infected cell are presented in Table 2. In the calculations, only cells stained by anti-V and anti-S antibodies were regarded as infected and possibly capable of virus production. The results indicate that much more infectious virus was produced per cell and per culture by PR8-R than by PR8. Furthermore, increasing amounts of infectious virus per cell continued to be produced by PR8-R virus between 10 and 24 hr, whereas the amount of PR8 per cell remained essentially the same during this interval. Different amounts of infectious PR8-R virus were produced per infected cell in the four cell lines, whereas very little infectious PR8 was produced in any of the HeLa cells. The EID_{50} per infected cell was 1- to 30-fold higher in cells infected with PR8-R than in those infected with PR8.

Effect of method of infection. In the experiments reported in Table 1, cells were infected in suspension to achieve the maximal number of infected cells and the highest yield of infectious virus. After infection, when the cells were grown on glass, it was found that some of the HeLa cells did not adhere firmly enough to allow cytochemical studies during the early stages of viral replication. It was necessary, therefore, to infect the cells as established monolayers for the cytochemical investigations. A study of the various parameters of viral infection indicated that the method of infection did not significantly affect the results. The percentage of cells stained by fluorescent antibodies (S and V) and the amount of virus produced per stained cell were of a similar order with each virus in a given cell line, irrespective of whether the cells were infected in suspension or as monolayers.

Evolution of immunochemical and cytochemical changes. The highest percentages of abnormal cells were detected in HeLa F and HeLa JJH lines infected with either strain of virus at an input multiplicity of approximately 100 EID_{50} per cell (Fig. 1 and 2). A similar sequence of development of immunochemical and cytochemical

TABLE 1. *Production of infectious virus and hemagglutinin 24 hr after infection^a*

Determination	HeLa W		HeLa S3		HeLa F		HeLa JJH	
	PR8-R	PR8	PR8-R	PR8	PR8-R	PR8	PR8-R	PR8
EID_{50}/ml (log)	7.1	5.2	6.7	6.0	7.2	4.2	6.0	5.0
HA unit/ml (log) ^b	1.0	1.8	0.7	1.6	1.0	0.7	0.7	1.8
EID_{50}/HA (log)	6.1	3.4	6.0	4.4	6.2	3.5	5.3	3.2

^a Cells were exposed in suspension at an input multiplicity of 10. PR8-R = PR8 recombinant.

^b HA = hemagglutination.

TABLE 2. *Virus yield in four lines of HeLa cells infected with PR8-R and PR8^a*

Cell line ^b	Test	PR8-R				PR8			
		4 hr ^c	7 hr	10 hr	24 hr	4 hr	7 hr	10 hr	24 hr
HeLa S3	EID ₅₀ /ml (log) medium + cells	3.3	4.2	5.5	6.1	3.0	3.0	5.0	5.0
	Percentage of stained cells (S + V)	0	16	25	25	0	2	8	10
	EID ₅₀ per stained cell	—	0.6	7	26	—	—	7	6
HeLa W	EID ₅₀ /ml (log) medium + cells	3.0	4.1	5.7	6.7	3.0	3.0	4.6	4.7
	Percentage of stained cells (S + V)	0	18	41	60	0	10	15	28
	EID ₅₀ per stained cell	—	—	9	64	—	—	2	2
HeLa F	EID ₅₀ /ml (log) medium + cells	3.5	4.2	5.4	6.9	3.0	3.5	4.3	4.7
	Percentage of stained cells (S + V)	0	100	100	100	0	21	22	18
	EID ₅₀ per stained cell	—	0.2	2.6	82	—	0.2	1.1	3.1
HeLa JJH	EID ₅₀ /ml (log) medium + cells	2.4	4.2	4.6	6.6	3.0	4.0	4.4	4.6
	Percentage of stained cells (S + V)	98	100	100	100	0	37	44	25
	EID ₅₀ per stained cell	—	0.1	0.4	32	—	—	1.6	1.4

^a Input multiplicity of infection was approximately 20 EID₅₀ per cell with HeLa S3 and W and 100 EID₅₀ with HeLa F and JJH.

^b Initial concentrations for S3, W, F, and J were 1.8×10^5 , 1.8×10^5 , 8.9×10^4 , and 1.1×10^5 cells per ml, respectively.

^c Refers to time of incubation.

changes was observed in S3 and W cells infected at a multiplicity of 20 EID₅₀ per cell, except that smaller numbers of abnormal cells were observed. The differences between HeLa F and JJH on the one hand and HeLa S3 and W on the other were not due to the different input multiplicities since the proportions of abnormal cells were very different when the four HeLa lines were infected with the same input multiplicity of virus (Fig. 3).

(i) S and V antigens. In all cell lines infected with both strains of virus, the earliest change in a significant number of cells was the appearance of faint but definite staining by S antibody in the nucleolus 4 hr after exposure to virus (SNL; Fig. 1 and 2, Fig. 4 and 5). Subsequently, in cells infected with PR8-R, staining of S-antigen increased in intensity and spread throughout the nucleus until the entire nucleus was filled with bright fluorescent antigen (Fig. 1 and 2, SN), and, in increasing numbers of cells, this was accompanied by S staining in the cytoplasm (SNC; Fig. 1 and 2, Fig. 6). With further incubation, the number of cells with nuclear S staining decreased. At 24 hr after infection, S-staining was only demonstrable in the cytoplasm as a diffuse fluorescence (Fig. 1 and 2, SC).

In many cells infected with PR8 virus, the faint S staining in the nucleolus persisted throughout the

period of observation (Fig. 1 and 2, SNL). In the later stages of incubation, the punctate fluorescence of the nucleolus enlarged in a small proportion of nuclei to form blobs which did not coalesce or increase in staining intensity during the course of time (Fig. 1 and 2, SN; Fig. 7 and 8). The typical brilliant nuclear and cytoplasmic S-staining appeared in a very small percentage of cells infected with PR8 virus (Fig. 1 and 2, SNC).

There was no difference in the localization, formation, or intensity of staining of the V antigen in the cytoplasm in all cell lines infected with both influenza virus strains (Fig. 1 and 2, V; 15). V antigen was almost invariably observed only when S antigen had appeared in the nucleus or cytoplasm, but not when the cells only had faint S staining in the nucleolus (Fig. 1 and 2).

(ii) Changes in the RNP of the nucleoli. At least two different types of RNP can be visualized in the nucleolus of HeLa cells by the TBM method (21). One consists of minute spherules of variable size, or nucleolini, which are stained metachromatically by method B (Fig. 9). The other comprises the remainder of the nucleolus or pars amorphous, in which the nucleolini appear to be embedded; it stains orthochromatically by TBM method B and metachromatically by TBM method C (Fig. 10).

FIG. 1. Immunochemical and cytochemical changes in HeLa F cells infected with PR8 and PR8 recombinant viruses. The following abbreviations indicate the percentages of cells showing a given change: V = V antigen staining; S = brilliant S-antigen staining in nucleus, cytoplasm, or both; SN = brilliant nuclear S-antigen staining; SNC = brilliant nuclear and cytoplasmic S-antigen staining; SC = brilliant cytoplasmic S staining; SNL = faint nucleolar S staining; NL = loss of nucleolar RNA staining; N = intranuclear RNA-containing inclusion; C = cytoplasmic RNA-containing inclusion; and N+C = cells with both nuclear and cytoplasmic inclusions. Input multiplicity in HeLa F and JJH cells was approximately 100.

FIG. 2. Immunochemical and cytochemical changes in HeLa JJH cells infected with PR8 and PR8 recombinant viruses. Abbreviations as in Fig. 1.

In the virus-infected cells, the earliest and most frequent change in the RNP of the nucleoli consisted of a disappearance of stainable RNP in the nucleolini. It is possible that this was preceded by some enlargement of nucleolini (Fig. 11), as has been described in other viral infections (17, 19, 22), but the extreme variability of the size of

the nucleolini in the HeLa cells (16) made this point difficult to assess. The nucleolini became progressively less stained with time, whereas the remainder of the nucleolus (pars amorpha) remained intact (Fig. 12-14), even until final disruption of the cell, when a CPE was evident. The proportion of cells in which staining of RNP in

FIG. 3. Cytological changes in all four HeLa lines infected with PR8-R and PR8 virus 12 hr previously. Abbreviations as in Fig. 1. Input multiplicity was approximately 150.

the nucleolini was deficient or absent in the various cell-virus systems is indicated in Fig. 1-3, NL. The proportion of cells with nucleolar change varied considerably in the four cell lines infected with the same virus and was invariably less with PR8 than with PR8-R in each cell line. With either virus, the proportion of cells in which the nucleolini were affected was in the following descending order of frequency: HeLa JJH, F, S3, and W (Fig. 3).

(iii) Changes in other types of RNP in virus-infected cells. After the decrease in nucleolar RNP, diffusely staining intranuclear RNP appeared in the nuclei of varying numbers of cells as indicated in Fig. 1-3 and Fig. 12. The appearance of this newly formed RNP was followed in preparations treated with deoxyribonuclease and subsequently stained by TBM method C. In such preparations, only nucleolar RNP of the pars amorpha was stained in uninfected control cells so that the newly formed metachromatically stained RNP in the nucleoplasm could be readily identified (Fig. 10 and 13). Since the newly formed RNP was only found in the virus-infected cells, it will be referred to as an intranuclear inclusion. The intranuclear inclusions were more strongly stained in cells infected with recombinant than with PR8 virus. Staining of the inclusions by TBM was entirely due to binding of dye by RNA since digestion with ribonuclease prevented all staining and since hydrolysis by deoxyribonuclease did not reduce the intensity of staining. The inclusions were Feulgen-negative and were stained by the acrolein-Schiff method for protein-bound groups. Intranuclear inclusions appeared later than the

nucleolar changes, and, although the number of cells containing them increased with time, there were always less cells with intranuclear inclusions than with nucleolar abnormalities (Fig. 1-3). Again, intranuclear inclusions were less frequent in cells infected with PR8 than with the recombinant virus, and the same order of frequency in the different lines of HeLa cell was observed as with nucleolar changes (Fig. 1-3).

Cytoplasmic inclusions containing RNP appeared still later and in smaller numbers of cells at first than did the intranuclear inclusions, although 24 hr after infection they were either equally numerous or slightly more numerous than intranuclear inclusions (Fig. 1 and 2). The proportions of cells containing cytoplasmic inclusions varied in the different virus-HeLa cell systems in the same manner as the nucleolar changes and nuclear inclusions, being most numerous with recombinant and much less so with PR8 virus (Fig. 1-3).

There was a striking difference in the morphology of the cytoplasmic inclusions produced by the two viruses, and the same virus produced the same type of inclusion in each of the four HeLa cell lines. In cells infected with PR8-R virus, there were usually one or two inclusions, whereas, with PR8 virus, there were multiple inclusions (Fig. 12-14). Initially, the inclusions produced by recombinant virus were intimately associated with the nuclear membrane, so that it was sometimes difficult to tell whether they were intranuclear or cytoplasmic (Fig. 12). The inclusions in cells infected with PR8 virus were invariably multiple, discrete, and scattered throughout the cytoplasm

FIG. 4. *S* staining of nucleoli of HeLa F cells infected 4 hr previously with PR8 virus. $\times 1,360$.

FIG. 5. *S* staining of nucleoli of HeLa F cells infected 4 hr previously with PR8-R. $\times 1,730$.

FIG. 6. Varying amounts of intranuclear and cytoplasmic *S* staining in HeLa F cells 7 hr after infection with PR8-R. $\times 1,360$.

FIG. 7. Fluorescent *S*-antigen blobs in two nuclei infected 10 hr previously with PR8 virus. The field also includes one of the rare cells in which fusion of the blobs has resulted in diffuse nuclear *S* staining. $\times 1,360$.

FIG. 8. Persistence of nuclear *S* antigen as discrete blobs 24 hr after infection of HeLa F cells with PR8 virus. There is also some *S* staining of the cytoplasm. $\times 1,730$.

(Fig. 13). In preparations stained by TBM method D, both types of inclusion contained dense concentrations of cytoplasmic granular RNP, and the amount of this type of RNP in the rest of the cytoplasm decreased as the inclusions enlarged (Fig. 15-17). Both types of cytoplasmic inclusion were also stained purple by TBM method C (Fig. 12-14). They were strongly stained by the acrolein-Schiff method for protein-bound groups and did not contain any DNA detectable by the Feulgen or TBM methods. In the later stages of development, the inclusions produced by recombinant virus became detached in part from the nucleus, but almost invariably they remained in contact with the nuclear membrane at one or more

points (Fig. 12, 14, and 16). Only one type of inclusion was observed in cells infected with recombinant virus, and the recombinant type of inclusion was rarely observed in cells infected with PR8 virus. Nuclear inclusions and nucleolar changes were always present when cytoplasmic inclusions were observed in cells infected with recombinant virus, but occasionally no nuclear inclusions and normal nucleoli were seen in a cell in which the PR8 type of cytoplasmic inclusion was present.

(iv) *Effects of viral infection on lipoproteins of HeLa cells.* Uninfected HeLa cells contained a variable number of sudanophilic neutral lipid droplets that were stained pink by Nile blue sul-

FIG. 9. Roughly spherical RNP-containing bodies (nucleolini) stained metachromatically (dark in the photograph) in the nucleolus of uninfected HeLa F cells. $\times 1,500$.

FIG. 10. Uninfected HeLa JJH cells treated with deoxyribonuclease, followed by staining by TBM method C. Note the densely staining nucleoli in which there are small unstained vacuoles, corresponding to the nucleolini shown in Fig. 9. There is no staining of chromatin and no diffusely staining RNP in the nucleus. $\times 1,200$.

FIG. 11. Varying degrees of loss of nucleolinar RNP staining in two HeLa W cells infected 23 hr previously with PR8-R. TBM method B. $\times 1,400$.

FIG. 12. Two HeLa JJH cells 24 hr after infection with PR8-R virus and stained by TBM method C. Note the diffusely staining material in the nucleoplasm. The cell on the left also has a typical PR8-R cytoplasmic inclusion in close apposition to the nuclear membrane. $\times 1,400$.

FIG. 13. HeLa JJH cells 21 hr after injection with PR8 virus and stained by TBM method C after digestion with deoxyribonuclease. Note the presence of weakly, but definitely, stained RNP in the nucleoplasm (cf., Fig. 10). The cell on the left contains typical PR8 cytoplasmic inclusions. $\times 1,500$.

FIG. 14. Group of JJH cells infected 24 hr previously with PR8-R virus and stained by TBM method C. All the cells contain intranuclear inclusion material, and cytoplasmic inclusions typical for the recombinant virus are present in three (arrows). Note that in this and other cells in the late stages of infection (Fig. 12 and 13) the pars amorpha of the nucleolus is intact. $\times 1,400$.

FIG. 15. Uninfected HeLa F cells stained by TBM method D. Cytoplasmic granular RNP is diffusely distributed in the cytoplasm except for the nuclear hof (arrows). $\times 1,100$.

FIG. 16. HeLa F cells infected 21 hr previously with PR8-R virus and stained by TBM method D. Note the dense accumulation of cytoplasmic granular RNP in the inclusion and the decrease in this type of RNP in the rest of the cytoplasm. $\times 1,500$.

FIG. 17. HeLa F cell infected 21 hr previously with PR8 virus and stained by TBM method D. There is some reduction in the number of cytoplasmic granules throughout the cytoplasm except in the multiple inclusions (arrows) where they are more densely aggregated. $\times 1,200$.

phate, and were removed after fixation by Elftman's method by brief treatment with ethyl alcohol. In addition, other lipid material, especially in the Golgi zone, was stained blue by Nile blue sulfate and probably represents phospholipid of the Golgi apparatus, lysosomes, and mitochondria. The material in the Golgi zone was also weakly PAS-positive after diastase digestion and still more weakly positive after extraction of lipids. It was also stained by the acrolein-Schiff method for protein-bound groups. As discussed elsewhere (20), this material probably consists of phospholipid and glycoprotein. The morphology of the phospholipid-containing structures in the HeLa cells was best visualized in cells fixed by Elftman's method, treated for 3 min with absolute ethyl alcohol to remove the neutral lipid and stained with Sudan black (Fig. 18). The mitochondria were seen as fine filaments throughout the cytoplasm, and a number of granular lysosomes could be visualized (Fig. 18). The Golgi zone comprised an ill-defined mass of sudanophilic material (Fig. 18). Infection of HeLa cells with PR8-R virus resulted in a variable but definite increase in phospholipid and glycoprotein in the cytoplasm, mainly in the region of the cytoplasmic RNP inclusion (Fig. 19). This lipoprotein body was PAS-positive (Fig. 20), sudanophilic (Fig. 19), and was stained by the acrolein-Schiff method for protein-bound groups and colored blue by Nile blue sulfate. The

mitochondria, lysosomes, and the Golgi apparatus all appeared to merge into a lipoprotein body resembling the RNP inclusion (Fig. 19). Although some increase in PAS-positive lipoprotein occurred in the late stages of cellular degeneration in cells infected with PR8 virus, no localization in the area of the inclusions could be detected (Fig. 21).

Effect of viral infection on mitotic division. Analysis of the mitotic index did not seem practical in those cell-virus systems in which only a small proportion of cells were infected. It was noted, however, that the proportion of C-mitotic abnormalities increased in all the infected cell lines. The commonest abnormality was partial or complete failure of spindle formation with varying degrees of clumping of chromosomes (Fig. 22-24). The effects of viral infection are illustrated in an analysis of mitosis in the experiment depicted in Fig. 2 in which JJH cells were infected with PR8-R (Table 3). Virus infection had no significant effect on mitosis for the first 7 hr. After 10 hr, there was a sharp increase in the number of abnormal metaphases. Viral infection also resulted in a preprophase block after 24 hr (Table 3). There was clumping of the chromosomes in the one cell in prophase in the virus-infected group at 24 hr. Mitotic analysis of F cells infected with recombinant virus 24 hr previously revealed no prophases in 500 cells.

CPE. It was not meaningful to quantitate

FIG. 18. Uninfected JJH cell stained with Sudan black (see text). Long filamentous mitochondria are stained in the periphery of the cytoplasm and there is a more dense accumulation of sudanophilic material around the nucleus and especially in the nuclear hof; this probably consists of lysosomes and Golgi material. $\times 1,100$.

FIG. 19. HeLa JJH cell infected 25 hr previously with PR8-R virus and stained with Sudan black. Note the great increase in sudanophilic material in the juxtannuclear cytoplasm. Compare this with the appearance of the RNP-stained inclusion in Fig. 14. $\times 1,400$.

FIG. 20. HeLa JJH cell infected with PR8-R 24 hr previously and stained by the PAS method. The nucleus (N) is virtually unstained and the PAS-positive inclusion (I) closely resembles that shown in Fig. 12 $\times 1,400$.

FIG. 21. HeLa W cells infected with PR8 virus 44 hr previously and stained by the PAS method. The unstained inclusions are photographed by phase microscopy. $\times 1,100$.

numerically the percentage of degenerating cells in the cultures, because in some instances there were many cells undergoing karyorrhexis, whereas in other instances there were few cells left on the glass and few degenerating cells were seen. In the latter case, presumably, the degenerating cells had come off the glass. The CPE was therefore assessed in part by the number of cells in which the nucleus or cytoplasm or both were fragmented and in part by the reduction in number of cells as compared with the controls. There was no significant CPE except in HeLa JJH infected with

PR8-R and PR8 viruses and in HeLa JJH infected with PR8 virus. In all these cell-virus systems, there was very extensive evidence of cellular destruction, especially in HeLa JJH infected with recombinant virus.

DISCUSSION

The results clearly indicate a striking difference in the cytopathology of the abortive infection of HeLa cells by PR8 and the virus-producing infection by PR8-R strain of influenza virus. Furthermore, the effect of each strain of virus on the

FIG. 22. Partial failure of spindle formation with aggregation of some chromosomes at the metaphase plate and scattering of others towards the centrioles. HeLa JJH infected 10 hr previously with PR8-R TBM method. $\times 1,400$.

FIG. 23. Complete failure of spindle function with irregular scattering and clumping of chromosomes. HeLa JJH infected with PR8-R as in Fig. 22 $\times 1,400$.

FIG. 24. Karyorrhexis of a HeLa JJH cell infected 24 hr previously with PR8-R virus. This probably represents the end result of a C-mitosis like that shown in Fig. 23. $\times 1,400$.

cells was greatly influenced by the line of HeLa cell used. Analysis of the sequence of events as detected by virological, immunochemical, and cytochemical techniques provides some insight into the nature of the cell-virus interactions. The time of appearance and the sites of formation of the S and V antigens did not correspond to those of the abnormal RNP inclusions. The characteristic difference in morphology of the cytoplasmic inclusions produced by the two viruses was not observed with fluorescent-antibody staining. The viral antigens appeared before the RNP inclusions and were present in a larger number of cells. There was no consistent correlation between the percentage of cells developing stainable antigens and the final virus yield per culture or per infected cell. Thus, slightly more recombinant virus was produced per culture by HeLa W than by HeLa JJH, whereas only 60% of HeLa W cells developed stainable antigen as compared with 100% of HeLa JJH. Similarly, the output of recombinant virus at 24 hr was essentially the same per HeLa S3 as per HeLa JJH cell; the former culture contained 25% and the latter, 100% of cells containing stainable antigen (Table 2). It is possible that the higher input multiplicity of infection of HeLa JJH may have caused interference, so that, although all cells were infected, the viral yield was reduced. On the other hand, infection with PR8-R virus produced many more cells containing antigen than did PR8, and the final yield of virus was invariably greater than was that with PR8. The evidence suggests that the development of stainable antigens is an indicator of infection of

the cell but that this infection does not always progress to the stage of production of infectious virus. This poor production of infectious virus by PR8 may be in part due to the smaller number of cells infected and in part to the failure of development of diffuse nuclear antigen and the paucity of cells with complete nuclear and cytoplasmic S antigens. Further evidence to support this concept comes from previous studies of HeLa cells infected with PR8 virus (15) or of L cells infected with fowl plague virus (7) in which no detectable infectious virus was produced and in which S antigen was observed in the nucleus but never in the cytoplasm.

RNP in the nucleolini decreased after the S antigen appeared in the nucleoli but did not decrease in all infected cells, since there were almost invariably more cells with antigen than with decreased nucleolar RNP (Fig. 1 and 2). The one exception was HeLa JJH infected with PR8 virus 24 hr previously (Fig. 2). In this experiment, there was a decrease in the number of antigen-containing cells between 10 and 24 hr after infection, probably due to the CPE. At 10 hr after infection, there were many more cells with antigen than with nucleolar changes. It would appear, therefore, that all infected cells did not develop nucleolar changes. There was no correlation between the percentage of cells in which nucleolar RNP diminished and infectious virus yield per infected cell or per culture. For example, the same amount of infectious PR8-R virus per culture was produced by HeLa F and JJH at

7 hr (Table 2) when nucleolar changes were present in almost twice as many F cells as JJH cells (Fig. 1 and 2). Similarly, the proportion of cells with nucleolar changes was the same in HeLa F and JJH infected with PR8-R virus 24 hr previously (Fig. 1 and 2), whereas almost three times as much infectious virus was produced per infected HeLa-F cell (Table 2). The only significant CPE was observed in the three cell-virus systems in which nucleolar changes were widespread, i.e., HeLa JJH and F infected with PR8-R virus and HeLa JJH infected with PR8 virus (Fig. 1 and 2). The CPE was most extensive in HeLa JJH cells infected with recombinant virus, in which nucleolar changes were most frequent. It is suggested that decrease and disappearance of nucleolar RNP was an indication of shutdown of host cell RNA synthesis and that this in turn contributed to the subsequent degeneration of the cell. In general, the higher the percentage of infected cells, the greater was the number of cells in which nucleolar changes developed. However, infection of the cell did not always lead to nucleolar changes, nor to subsequent destruction of the cell. Of HeLa F cells, 87% was infected by PR8 virus (Fig. 1), but only 12% developed nucleolar changes; there was no significant CPE. Shutdown of host RNA synthesis is known to occur with a number of RNA-containing viruses (27), and there is some evidence that a breakdown or inhibition of host RNA synthesis may occur in chick embryo cells infected with influenza virus (2). Disappearance of nucleolar RNP has been observed in cells infected with herpes, polyoma, and SV40 viruses and in HeLa cells infected with parainfluenza 3 virus (17, 19, 22). In all these infections, a CPE results. Infection of monkey kidney cells with parainfluenza 3 virus produces inclusions, but the cells do not degenerate and the nucleoli are not affected (19). Nucleolar RNP also disappears in HeLa cells in which DNA and subsequently RNA synthesis is inhibited by 5-fluoro-2'-deoxyuridine (19a). Inhibition of synthesis of host cell RNA has been demonstrated in cells infected with herpes virus (30, 33), and inhibition of host cell DNA synthesis occurs in lytic infection with polyoma virus (31). Other studies (19a) of the effects of antimetabolites on the nucleoli suggest that they contain ribosomal or ribosomal precursor RNA and that loss of this type of RNA leads to subsequent disaggregation of polysomes which are stained as the cytoplasmic granular RNP by the TBM method. It is of interest that, in the HeLa cells infected with influenza virus, the cytoplasmic granular RNP decreased and disappeared as the cytoplasmic inclusions developed and that this occurred at a

time when the nucleolar RNP was no longer demonstrable in many of the cells.

There was no correlation between the proportion of cells containing nuclear and cytoplasmic inclusions and the yield of each virus in any of the four cell lines. The variation in yield of infectious virus in the four cell lines cannot be related to the proportion of cells containing nuclear or cytoplasmic RNP inclusions. However, both types of inclusion were much less frequent in all four types of cells infected with PR8 than in the corresponding cells infected with PR8-R. Similarly, PR8 produced very much less infectious virus. It would appear therefore that some of the RNA of the inclusions is probably viral. In view of the very late accumulation of RNP in the inclusions, it seems probable, however, that this material is unused or excess virus-induced RNP. The fact that the inclusions in the cytoplasm consist of dense aggregations of granular cytoplasmic RNA also suggests that they may consist of aggregations of cellular polysomes (19a) and of viral messenger RNA and therefore be sites of synthesis of viral protein. The difference in the type of inclusions produced by the two viruses may also be related to the great difference in virus production. In cells infected with PR8-R virus, the invariable development of inclusion in the nucleus before that in the cytoplasm and the intimate association of the inclusion with the nuclear membrane suggest that at least some of the cytoplasmic RNP may have come from the nucleus. Furthermore, the association of an increased amount of phospholipid and glycoprotein with the RNP inclusion in cells infected with PR8-R virus may be more favorable for the final stage of assembly of the virus, which is known to contain all three components (12). It is of interest that, although the yield of each infectious virus does not differ greatly in four types of HeLa cell, there is considerable variation in the number of infected cells, in the formation of the antigens in the nucleus and cytoplasm, and in the changes in ribonucleoproteins. The results would suggest that the production of infectious virus, shutdown of cellular RNA synthesis and CPE, and the formation of inclusions are independent events.

The development of C-mitotic abnormalities and preprophase block in virus-infected cells has also been observed in HeLa cells infected with herpes virus (22), and chromosome damage has been noted with a number of viruses (25). The only difference between the effect of influenza and herpes virus on mitosis was that the latter permitted the C-mitotic metaphases to progress through a series of abnormal post-C-mitotic anaphase and telophase figures (22). In cells in-

TABLE 3. Analysis of mitosis in HeLa JJH cells infected with PR8-R virus

Time after infection (hr)	Mitosis (%)				Total	Percentage of meta-phases in C-mitosis
	Pro-phase	Meta-phase	Ana-phase	Telo-phase		
4	0.2	1.4	0.8	1.6	4.0	0
7	0.4	1.8	0.2	1.0	3.4	0
10	0.4	2.2	0	0.8	3.4	82
24	0.2	1.0	0	0	1.2	80
4 ^a	1.0	2.0	0.8	1.2	5.0	0
7 ^a	0.2	2.6	0.6	1.0	4.4	0
10 ^a	1.8	1.8	0.4	2.0	6.0	8
24 ^a	0.6	2.6	0.2	0.6	4.0	0

^a Control.

infected with influenza, clumping of chromosomes in C-metaphase progressed to karyorrhexis and disintegration of the cell (Fig. 24), with the result that no abnormal anaphases or telophases were observed after 24 hr (Table 3).

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