IMMUNOHISTOCHEMICAL STUDIES ON THE INTERACTION BETWEEN EHRLICH ASCITES TUMOR CELLS AND NEWCASTLE DISEASE VIRUS*‡

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Plates 18 and 19

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Evidence has been presented which supports the hypothesis that Newcastle disease virus interacts with Ehrlich ascites tumor cells in a manner similar to the reactions of a virus with its susceptible host cells in a normal infection, except that replication of hemagglutinating, complement fixing, or infectious particles could not be demonstrated (2, 3). That virus penetrated into tumor cells was supported only by evidence of an indirect nature. It was, therefore, important to attempt to study this problem by more direct means. The fluorescent antibody technique of Coons and Kaplan offered such a possibility (4). This technique permits visualization of viral antigen within infected cells by union of antigen and labelled specific antibody. Infections by mumps, canine hepatitis, influenza, herpes zoster, varicella, vaccinia, and Egypt 101 viruses have been studied by this method (5–10).

It is the purpose of this paper to present additional evidence in support of the postulate that Newcastle disease virus (NDV), or a portion of this agent, in order to induce cell damage, penetrates the Ehrlich ascites tumor cell. The data to be described suggest that penetration of cells initially involves the loss of the ability of viral antigen to combine with antibody, and that penetration is followed by the synthesis of a viral antigen which appears to be non-infectious, unable to agglutinate chicken erythrocytes, or to fix complement with homologous antibody.

Materials and Methods

Stock Virus Preparation.—The Hickman strain of Newcastle disease virus, hereafter referred to as NDV, was employed. This agent was propagated in the allantoic sacs of 11 day

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old chick embryos; the infecting inoculum was 0.1 ml. of a 10^{-4} dilution of stock virus. The inoculated embryonated eggs were incubated at 35°C. for 36 hours, and allantoic fluid harvested after the eggs were chilled over night at 4°C.

The majority of the experiments described in this report were carried out with a single pool of virus, aliquots of which were stored in sealed glass ampules at −70°C. The infectivity titer of this pool of virus was determined by intra-allantoic inoculation of 0.1 ml. aliquots of serial two-fold dilutions into a total of thirty chick embryos per dilution. After 60 hours of incubation at 35°C., embryos were chilled, allantoic fluids removed, and the presence of virus in each allantoic fluid determined by hemagglutination reaction using a 0.5 per cent suspension of chicken erythrocytes. The infectivity titer was calculated by the method of Reed and Muench (11). The dilution which would infect 50 per cent of inoculated embryos ($E.I.D._{50}$) was found to be $10^{-9.20}$. This estimate had a standard error of $10^{-0.055}$ as determined by the method of Pizzi (12).

Receptor-Destroying Enzyme (RDE).—The material employed was a filtrate of a *Vibrio cholera* culture which had been incubated with constant shaking for 18 hours at room temperature and extracted at 4°C. for 48 hours (13).

Ehrlich Ascites Tumor.—The Ehrlich ascites tumor was received from Dr. K. Sugiura, of the Sloan-Kettering Institute for Cancer Research, New York City. The tumor was maintained by weekly serial passage in CF-1 male mice, 20 to 22 gm., obtained from Carworth Farms, New City, New York. For each passage approximately 2×10^6 cells were inoculated intraperitoneally into each mouse (14). Prior to use in experiments, freshly harvested tumor cells were washed 3 times in at least 20 volumes of a phosphate buffered Tyrode solution, termed modified glucosol (15), as previously described (14). Only suspensions containing less than 5 to 10 per cent red blood cells were employed. Cells were counted in hemocytometers; all cells greater than 12 μ in diameter were considered tumor cells (14). At least 500 cells were counted for each determination. Repeated determinations on the same sample have revealed the standard error of such counts to be approximately ±10 per cent of the number counted.

Preparation of Fluorescein-Labelled Sera.—Specific immune serum was prepared with NDV-infected allantoic fluid in rabbits (16). Prior to conjugation this serum had an hemagglutination-inhibition titer of 1:1024. A crude globulin fraction of this serum was prepared and conjugated with fluorescein isocyanate by methods which have been described (4).[1] In order to avoid non-specific staining of uninfected ascites tumor cells, the conjugated serum was adsorbed twice with mouse liver powder. The procedures for adsorption of serum were identical with those described by Coons and Kaplan (4).

Preparation of Cells for Staining.—After the various experimental manipulations—which will be described in detail below—ascites tumor cells were washed once with modified glucosol at 4°C. The cells were then sedimented in lusteroid tubes by centrifugation at 2,500 R.P.M. for 5 minutes at 4°C. The supernatant fluid was decanted, and the tubes partially immersed in an alcohol-CO_2 ice mixture at −70°C. The frozen pellet of cells could be readily removed for sectioning by gentle distortion of the flexible lusteroid tube. Four μ sections were made in a cryostat (4). In some experiments air-dried smears were used instead of sections. The staining procedure for sections and smears was identical with that described by Coons and Kaplan (4).

Fluorescence Microscope.—The majority of the experiments to be reported were carried out in the laboratory of Dr. Albert H. Coons, with the equipment described by Coons and Kaplan (4). Experiments with unsectioned cells, however, were carried out with an AH-6 (General Electric Company) water-cooled mercury vapor arc as light source. The filtration used was 3.2 cm of $CuSo_4 \cdot 5H_2O$ (25 gm. per 100 ml.) followed by a Corning No. 5840 half thickness, and a Corning No. 5850, 4.1 mm. filter. A standard dark field microscope with non-fluorescing

[1] We are indebted to Dr. A. H. Coons for the preparation of fluorescein labelled antibody.

lenses was employed. This equipment showed little or no blue autofluorescence of unstained cells, but otherwise gave results comparable to those obtained in the other experiments in which a carbon arc light source was employed (4).

Tests for Specificity of Staining.—The immunological specificity of the staining observed in these experiments was indicated by: (*a*) lack of staining of untreated cells and cells treated with normal allantoic fluid; (*b*) disappearance of stainable antigen from the cell membrane when virus was eluted from cells at room temperature with the aid of RDE; and (*c*) inhibition of staining by prior exposure of sections to unconjugated homologous immune serum.

Determination of Quantity of Virus Adsorbed Per Cell (Virus:Cell Ratio).—The average number of infectious viral "particles" adsorbed per cell was determined as follows: after the time allowed for adsorption of virus to cells, the mixture was centrifuged for 5 minutes at 2,500 R.P.M. Hemagglutination titrations were carried out on both the original virus suspension and the supernatant fluid after adsorption. From these titrations the per cent virus adsorbed was calculated. As described above, the virus stock used contained $10^{9.2} \pm 10^{0.05}$ E.I.D.$_{50}$ per 0.1 ml. It was assumed that one "infectious unit" equaled 0.693 "infectious particles." This would be the case if infection of the chick embryo were to result from the presence of a single infectious particle, and if these particles were distributed according to the Poisson distribution.[2]

RESULTS

Fate of Viral Antigen after Combination of NDV with Ehrlich Ascites Tumor Cells in Vitro.—It has been previously reported (17) that when NDV coated Ehrlich ascites tumor cells are brought to 37°, there follow several changes in the nature of the interaction: (*a*) elution of virus from cells cannot occur, and consequently tumor cells lose the apparent capacity to "recover" viability on standing *in vitro*; and (*b*) combination cannot take place between specific NDV antibody and virus previously adsorbed to the tumor cells. In contrast, with virus-cell mixtures held at 0 to 23°C., NDV did elute from tumor cells and apparent "recovery" of the cells' ability to multiply did occur (17). As interference experiments had suggested that NDV actually penetrated these cells, the above finding suggested that virus, or a portion thereof, penetrated cells at 37°C. To obtain evidence relative to this concept the fluorescent-antibody technique was used to observe the fate of viral antigen after virus coated cells had remained at 0 or 37°C.

An average of 160 "infectious viral particles" were adsorbed per tumor cell ($10^{10.5}$ E.I.D.$_{50}$: 1.4×10^8 cells in 2 ml.; per cent adsorption = 99.2). One aliquot of virus coated cells was held for 60 minutes at 0°C. and another for 60 minutes at 37°C. Control cells were mixed with normal allantoic fluid and modified glucosol, respectively. The cells were then frozen, sectioned, and stained.

Control cells were completely unstained. Cell-virus complexes held at 0°C. showed a bright ring of fluorescence located on the periphery of sectioned

[2] From theoretical considerations these assumptions appeared valid. In addition, the estimates derived from them were compatible with the experimental observations in experiments to be described, in which the quantity of virus was diluted so that less than one infectious viral particle per cell was employed.

cells. Those held at 37°C. showed faint rings of yellow-green fluorescence; however, the staining was greatly diminished in intensity. No intracellular staining was detectable. In an effort to increase the sensitivity of this technique, sections were stained twice: first with anti-NDV conjugated serum, and then with fluorescein-conjugated anti-rabbit gamma globulin.[3] This increased the intensity of the staining previously seen, but did not reveal any intracellular staining. The appearance of cells stained in this manner will be seen in Figs. 1 and 2. As control determinations revealed that no virus had eluted at either temperature, it was evident that a portion of the viral antigen had become unable to combine with antibody.

It seemed possible that these antigens had penetrated into the cytoplasm of the cell, and were not visualized owing to dilution of stainable antigen in the cytoplasm. Experiments were therefore carried out in which the virus: cell ratio was increased by the use of concentrated viral suspensions. Even when 1,550 "infectious particles" had been adsorbed per cell and the cells had been stained by both single and double layer methods, no antigen was detectable in the cytoplasm of the treated cells. Furthermore, when these large amounts of virus were used, it was no longer possible to observe the difference between the intensity of fluorescent rings in cells held at 0° or 37°C. Experiments in which virus-cell complexes were incubated for 5 and 15 minutes at 37°C. also failed to reveal intracellular staining.

Experiments were designed to determine whether the bright ring of fluorescence present on the cells mixed with concentrated viral suspensions at 37°C. was superficially adsorbed virus, or whether it represented virus in some form of irreversible union with the cells.

Five ml. of NDV-infected allantoic fluid, $10^{10.2}$ E.I.D.$_{50}$/ml., was added to 6.9×10^7 packed ascites tumor cells. A control cell suspension was mixed with normal allantoic fluid. One aliquot of the virus-cell mixture was held for 45 minutes at 0°C., another was held for 15 minutes at 0°C., and then incubated for 30 minutes at 37°C. All virus-cell mixtures were centrifuged for 5 minutes at 2,500 R.P.M., the supernatant fluids removed for hemagglutination titrations, and the cells resuspended to original volume in RDE diluted 1:3 with calcium borate buffer (3). After 4 hours at 21°C., the cells were again sedimented by centrifugation, the supernatant fluids were saved for hemagglutination titrations in 2.5 per cent sodium citrate solution, and the cells were prepared for sectioning.

Table I summarizes the results of this experiment. It will be noted that exposure to RDE completely removed the layer of antigen that had been seen above. It may thus be concluded that this antigen represented virus which was reversibly adsorbed to the cell surface. From the results of the hemagglutination titrations, however, it is clear that the major portion of the virus adsorbed to tumor cells was not dissociated by RDE whether cell-virus mixtures were held at 0°C. for 45 minutes or at 0°C. for 15 minutes and

[3] Kindly supplied by Dr. Barbara K. Watson.

37°C. for 30 minutes. A greater proportion of the adsorbed NDV, however, was eluted by RDE from the cell-virus mixture held only at 0°C. In neither instance could the irreversibly adsorbed virus be detected as stainable antigen. Similar results were obtained when even greater numbers of viral particles were adsorbed to, but not dissociated from, the tumor cells.

The above series of experiments suggested that the antigenic components of viral particles which entered into irreversible union with Ehrlich ascites tumor cells were unable to combine with antibody. The results of these experi-

TABLE I

Effect of RDE Treatment of Newcastle Disease Virus (NDV) Coated Ascites Tumor Cells on Appearance of Cells Subsequently Stained with Fluorescein Conjugated Anti-NDV Serum

Adsorption				Hemagglutination titers		NDV adsorbed but not eluted‖	Appearance after exposure of sections to conjugate
Mixture		Temperature		Supernate	After incubation of cells with RDE§		
Allantoic fluid*	Cells‡	0°C.	37°C.				
5 ml.		*min.*	*min.*			*per cent*	
NDV infected	6.9×10^7	15	30	1:64	1:16	92	Unstained
" "	" " "	45	None	1:64	1:64	67	"
Uninfected	" " "	15	30	—	—	—	"

* $10^{10.2}$ E.I.D.$_{50}$/ml. Hemagglutination titer = 1:256.

‡ Washed Ehrlich ascites tumor cells.

§ Cells incubated 4 hrs. at 21°C. with 5 ml. RDE diluted 1:3 in Ca borate buffer. Supernate after sedimentation of cells was diluted in 2½ per cent Na citrate for hemagglutination titrations.

‖ $\frac{\text{Hemagglutination units adsorbed} - \text{Hemagglutination units eluted}}{\text{Hemagglutination units adsorbed}} \times 100.$ *e.g.*, $\frac{(256 - 64) - 16}{192} = 92$ per cent.

ments did not indicate whether viral penetration occurred, nor could one conclude from them whether the non-detectable antigen had become degraded or denatured.

Fate of Viral Antigen after Combination of NDV with Ehrlich Ascites Tumor Cells in Vivo.—Not only can NDV inhibit intraperitoneal multiplication of Ehrlich ascites tumor cells in mice—whether virus is adsorbed to cells *in vitro* (18) or inoculated intraperitoneally after tumor growth has become established—but also NDV actually induces lysis of these cells (3). When a large number of viral particles was used, the shortest latent period before lysis was 4 hours; when smaller quantities of virus were employed the period of time before lysis of tumor cells had an approximate inverse relationship to the quantity of virus employed (3). Lysis of these cells by NDV, however, could

not be accomplished *in vitro* (3). These findings suggested that intracellular NDV might only be detected after mixtures of tumor cells and virus were inoculated into the peritoneal cavity of mice. Furthermore, the minimal latent period of 4 hours which preceded lysis of tumor cells suggested the possibility that some form of viral replication might be a stage in the process under study. It was therefore of interest to examine cells to which virus had been adsorbed at various times after inoculation into the peritoneal cavity of mice.

NDV-infected allantoic fluid, $10^{9.5}$ E.I.D.$_{50}$/ml., was concentrated 5:1 by centrifugation in the high speed head of a refrigerated International centrifuge for 1 hour at 25,000 *g*. Mixtures were prepared containing 6.9×10^7 washed tumor cells per ml. of concentrated virus, or normal allantoic fluid. These mixtures were held for 15 minutes at 2°C., after which the mixtures were centrifuged and the cells resuspended to the original concentration in modified glucosol. Hemagglutination titrations of the original virus, and of the supernatant fluid after adsorption, revealed that 87.3 per cent of the virus had been adsorbed, giving rise to an estimate of virus:cell ratio of 140 "infectious particles" per cell. The cell suspensions were inoculated intraperitoneally into mice, each animal receiving approximately 10^8 cells. At intervals mice were sacrificed, cells were removed by lavage, and prepared for sectioning.

Cells harvested 1 hour after inoculation were completely unstained. Three hours after inoculation, however, an entirely new type of staining was observed (Fig. 3). In contrast to the appearance of cells with superficially adsorbed virus (Fig. 1), the fluorescent stain now definitely appeared in the cytoplasm; the majority of cells had crescent or "signet ring" type areas of fluorescence. Cells harvested 5 hours after inoculation had a somewhat increased intensity of staining.

Although cytoplasmic antigen was never detected after incubation of cell-virus mixtures *in vitro*, it appeared possible that the observed antigen might merely be the original infecting virus which had in some unknown manner become "unmasked." To investigate this possibility, experiments similar to the above were carried out with decreasing virus:cell ratios. It was reasoned that if the appearance of stainable antigen were an unmasking process, low ratios would show no staining, and intermediate ones would show an intensity of staining proportional to the number of particles employed.

Fig. 4 presents cells to which approximately 14 "infectious particles" per cell had been adsorbed. The cells were harvested 3 hours after inoculation into mice. It will be noted that these cells had a markedly reduced amount of staining when compared to cells which had adsorbed 140 "infectious particles" (Fig. 3). Seven hours after inoculation, however, these cells had an intensity of staining which was roughly maximal (Fig. 5). The staining in cells which had adsorbed 14 "infectious particles" per cell, however, was noted to be more granular than that observed at higher virus:cell ratios.

In Table II the results of experiments carried out at several virus:cell ratios

are summarized. It will be noted that: (*a*) antigen appeared after a "dark period" during which cells were completely unstained; (*b*) the length of the "dark period" varied inversely with the number of infectious viral particles adsorbed per cell; (*c*) although the ultimate intensity of staining was not equal with all virus:cell ratios employed, the differences were decidedly less than could be accounted for by the quantity of virus adsorbed to the cell; and (*d*) when less than one "infectious particle" per cell was used, only a portion of the cells developed stainable antigen. The latter finding will be seen in Figs. 6 and 7 which represent stained air-dried smears of cells harvested 12

TABLE II

Effect of Number of Viral "Particles" per Cell on Time of Appearance of Antigen

Time	Infectious "particles"/cells*			
	700	140	14	0.5‡
hrs.				
1	±§	0	0	0
3	+++	+++	+	0
5	++++	++++	+++	0
7	N.D.	++++	+++	0
8	N.D.	N.D.	N.D.	+
9	N.D.	N.D.	N.D.	++
10	N.D.	N.D.	N.D.	+++
12	N.D.	N.D.	N.D.	+++

* Cells mixed with virus *in vitro*, held 15 minutes 0°C. and 15 minutes 37°C., then inoculated into mice intraperitoneally.

‡ At time of maximal intensity of staining approximately 10 to 30 per cent of the cells showed yellow-green fluorescence.

§ Intensity of staining is expressed semiquantitatively on a scale of 0 to ++++. N. D.= not done.

hours after inoculation. When 5 "infectious particles" per cell were employed, approximately all the cells were stained (Fig. 6); whereas, when 0.5 "infectious particles" per cell was used, only approximately 10 to 30 per cent of the cells had yellow-green fluorescence (Fig. 7). Some of the definitely stained cells are identified by an *A* directly beneath them, while *B* denotes definitely unstained cells. In this photograph some cells appear equivocal. The distinction was, however, unmistakable when these cells were observed directly with the fluorescence microscope.

Multiplication of NDV in Ehrlich ascites tumor cells could not be detected when infectivity, hemagglutination, and complement-fixation titrations were carried out (3). In fact, these viral properties could not be measured shortly after adsorption of virus to cells. The demonstration of an apparent increase of intracellular viral antigen suggested that at the time of maximal staining

intensity, tests for hemagglutinins and complement-fixing antigens of NDV be repeated with extracts of cells prepared by grinding or sonic disintegration. In no case, however, could hemagglutinins or a rise in complement-fixing antigens be demonstrated even after treatment of extracts with RDE. These data suggest that the viral antigen described lacks the properties of a hemagglutinin or specific complement-fixing antigen and is probably a less complex material.

Types of Specific Intracellular Staining.—The forms of NDV antigen staining observed intracellularly are shown at higher magnifications in Figs. 8 to 10. The cells portrayed were infected with an average of 14 "infectious particles" per cell and were harvested from the peritoneal cavity of mice 7 hours after inoculation. Fig. 8 shows relatively diffuse cytoplasmic staining. Fig. 9 depicts a granular figure that appears to have stalk-like interconnections. Fig. 10 shows various types of granular inclusions. The granular forms of staining of NDV antigen were observed rarely with larger quantities of virus. As these figures represent sectioned cells, they clearly indicate the cytoplasmic location of the viral antigen described in these experiments.

It would be hazardous to attempt further interpretation relative to the significance of these distributions of antibody-binding material as the techniques utilized have been shown to be susceptible to distributional artefact (19). This is especially unfortunate since it will be shown, in a paper to be published shortly, that NDV-infected cells develop cytoplasmic inclusions within which particles one-tenth the mean virus diameter may be seen with the electron microscope (20).

DISCUSSION

It was the purpose of this investigation to answer by more direct means the question: Does Newcastle disease virus penetrate the cells of the Ehrlich ascites tumor in the course of the reaction which leads to hyperplasia and subsequent lysis of these cells? The finding that specific viral antigen appeared in the cytoplasm of cells some time after virus-cell complexes were inoculated into the peritoneal cavities of mice provides an unequivocal answer to this question. Plainly a portion of the original virus must have penetrated to give rise to this antigen.

The data described in this report, however, raise two questions which cannot be finally answered by the methods employed.

The first of these questions pertains to the fate of the original infecting virus. It was shown in these experiments that after viral coated cells were incubated at 37°C., or inoculated into mice, the majority, or all, of the detectable viral antigen disappeared from the cell surface. It had been previously shown that neither hemagglutinating particles, nor particles which can block hemagglutination-inhibiting antibody, elute from the cells under these con-

ditions (17). It is thus likely that whatever happened to the viral antigen occurred at the cell surface or intracellularly. Two possibilities must be considered: (*a*) the infecting viral antigen may have become denatured or disingrated, either before or after penetration; or (*b*) the infecting viral antigen may have become "masked" after penetration by combination with some inhibitor. Although the concepts which have been developed with bacterial viruses may make the first alternative the more attractive one, it is felt that further investigation will be required to distinguish between these hypotheses in the present case.

The second question raised by this investigation pertains to the nature of the cytoplasmic viral antigen which appeared after the "eclipse period" during which no viral antigen could be detected. Again two major alternative explanations must be considered: (*a*) the cytoplasmic antigen may represent the reappearance of initial infecting antigen after "unmasking"; or (*b*) the cytoplasmic antigen may represent newly synthesized viral antigen. The finding that when less than one "infectious particle" per cell was employed only a portion of cells was stained, and these developed an intensity of staining which was not proportionately less than that observed in cells infected with large numbers of viral particles, would support the latter hypothesis. It is difficult to conceive that a single viral particle might give rise to sufficient antigen to account for the intensity of staining observed unless some form of replication were involved. The "unmasking" hypothesis can nevertheless not be entirely ruled out.

If the cytoplasmic antigen is indeed the product of a partial cycle of replication, as seems most likely, this antigen may represent an incomplete viral particle of a previously unrecognized degree of simplicity. Further investigation will be required to determine whether the inability of the antigen to infect, hemagglutinate, or fix complement with homologous antibody, depends on an extreme degree of "incompleteness," or on union of viral particles with an intracellular inhibitor.

It was the original object of these investigations to determine the mechanism whereby NDV killed Ehrlich ascites tumor cells in the absence of viral multiplication. The findings summarized in this report suggest that cell death follows a series of reactions whose initial step is penetration of at least a portion of the virus into the tumor cell. The data suggest that penetration is then followed by an abortive, and incomplete, form of viral replication. This may be the cause of cell death and lysis, or the synthesis of viral antigen may be a concomitant but unnecessary phenomenon.

SUMMARY

Newcastle disease virus infection of Ehrlich ascites tumor cells resulted, after a period of time, in the appearance of intracellular viral antigen which

could be demonstrated by the fluorescent antibody technique. This antigen appeared in the cytoplasm of infected cells only after inoculation of cell-virus mixtures into the peritoneal cavities of mice. The latent period prior to the appearance of antigen depended inversely on the number of viral particles adsorbed onto the cells prior to inoculation. The final intensity of staining appeared not to be proportionate to the number of viral particles adsorbed to each cell. The appearance of this antigen was not correlated with a rise of titer of infectious, hemagglutinating, or complement-fixing virus. Viral antigen was demonstrated on the surface of tumor cells after adsorption of NDV onto these cells at 0°C. At appropriate virus:cell ratios, antigen was noted to disappear from the surface at 37°C. *in vitro*, and *in vivo*, in the absence of demonstrable elution of virus. The appearance of intracellular viral antigen could not be detected *in vitro* when tumor cell-NDV mixtures were incubated at 37°C., even when an average of 1550 "infectious particles" had adsorbed to each cell.

Addendum.—Since this paper was completed a report has been published in which the multiplication of an adapted variant of NDV on Ehrlich ascites cells is described (21). It should be emphasized that these findings in no way conflict with those described for the present system, as this multiplication could occur only with strains of virus which had undergone many serial passages through ascites tumor cells *in vitro*.

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EXPLANATION OF PLATES

Figs. 1 to 5, and Figs. 8 to 10, are photomicrographs of 4 μ sections of Ehrlich ascites tumor cells, treated as described below, and stained with fluorescein conjugated anti-NDV immune serum. The light areas represent the yellow-green fluorescence of the bound conjugate. Figs. 6 and 7 represent similarly treated air-dried whole cell smears.

PLATE 18

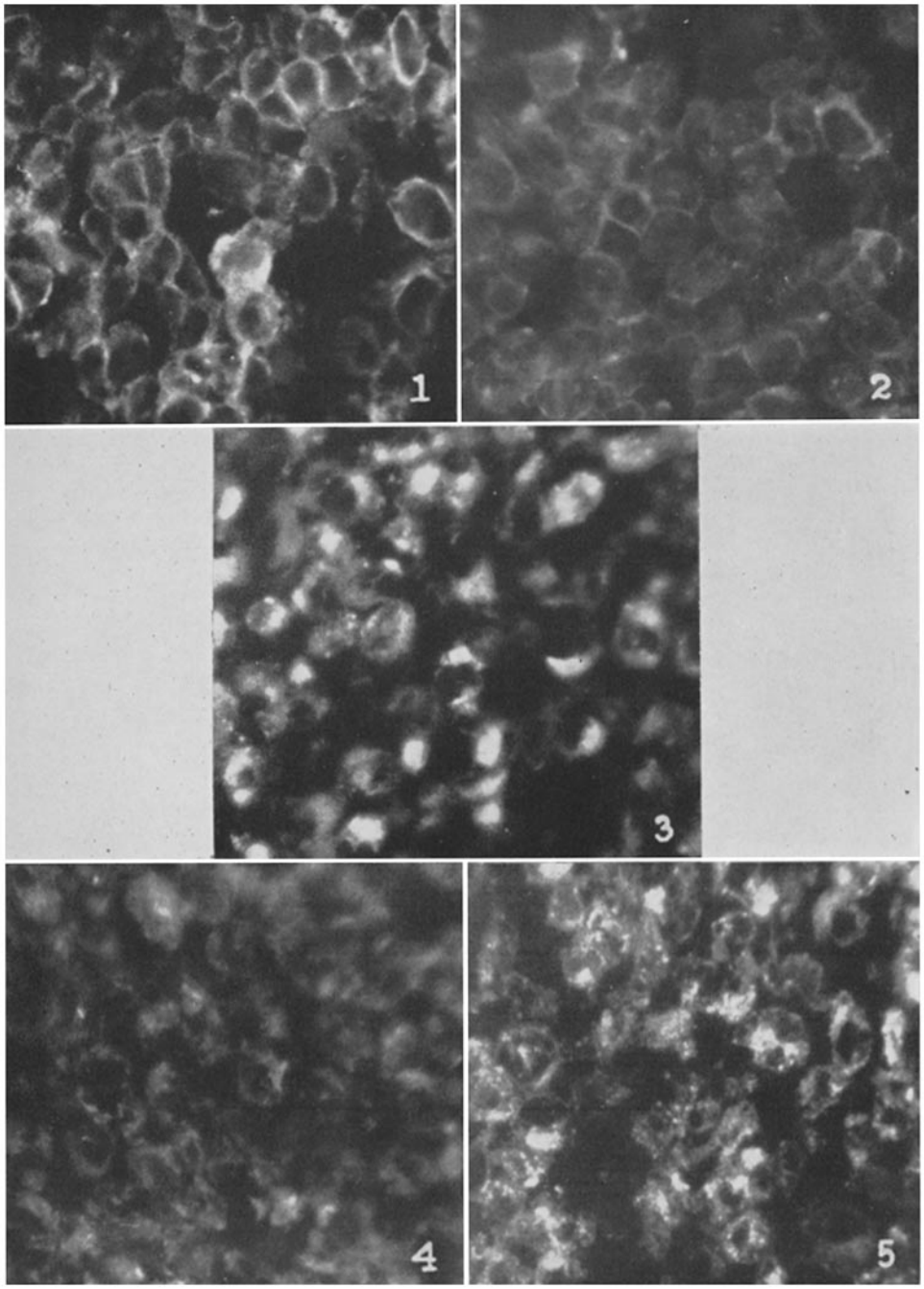
FIG. 1. Cells to which 160 "infectious particles" were adsorbed per cell, and which were then held for 60 minutes at 0°C. × 900.

FIG. 2. Cells to which 160 "infectious particles" were adsorbed per cell as above, but which were then placed at 37°C. for 60 minutes. The fluorescent ring seen in Fig. 1 is present but greatly diminished. No intracellular staining is seen. × 900.

FIG. 3. Cells to which 140 "infectious particles" per cell were adsorbed, and which were then inoculated intraperitoneally into mice and harvested after 3 hours *in vivo*. Cytoplasmic staining is seen. × 900.

FIG. 4. Cells to which 14 "infectious particles" per cell were adsorbed and which were then harvested at the same time as those in Fig. 3. These cells show markedly less intensity of staining. × 900.

FIG. 5. Cells to which 14 "infectious particles" were adsorbed and which were then harvested (as above) 7 hours after inoculation. The intensity of staining is approximately maximal. × 900.



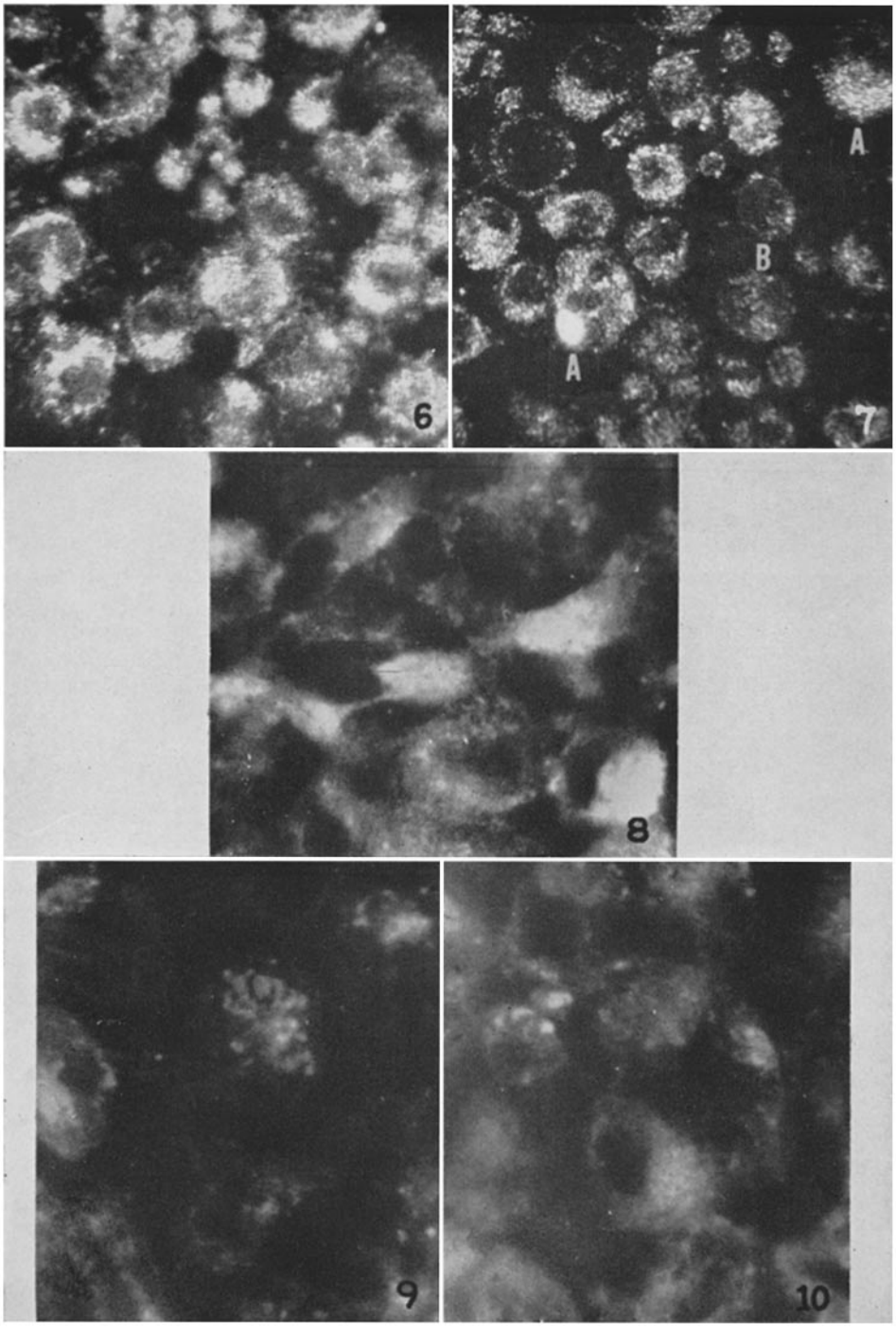
(Prince and Ginsberg: Erhlich ascites tumor cells and Newcastle virus)

PLATE 19

FIG. 6. Air-dried smear of cells which had adsorbed an average of 5 "infectious particles" per cell, and which were harvested 12 hours after intraperitoneal inoculation. Almost all cells show marked fluorescence. × 900.

FIG. 7. As above, except that approximately 0.5 "infectious particles" per cell were adsorbed. Ten to 30 per cent of these cells show marked fluorescence (*A*), whereas the remainder are only slightly stained (*B*). Some cells appear equivocal in this picture, but were distinctly different from the brightly stained cells on direct observation. × 900.

FIGS. 8 to 10. Cells to which 14 "infectious particles" were adsorbed, harvested 7 hours after intraperitoneal inoculation. × 900. Note different types of cytoplasmic localization: diffuse (Fig. 8); granular figures with stalk-like interconnections (Fig. 9); granular inclusions (Fig. 10).



(Prince and Ginsberg: Erhlich ascites tumor cells and Newcastle virus)