SEQUENTIAL CELLULAR CHANGES PRODUCED BY TYPES 5 AND 7 ADENOVIRUSES IN HeLa CELLS AND IN HUMAN AMNIOTIC CELLS

Cytological Studies Aided by Fluorescein-Labelled Antibody*

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Investigations of the biologic characteristics of adenoviruses types 1 to 4 have suggested that natural subdivisions exist within the adenovirus group (1–4). Types 1 and 2 have been found to comprise one subgroup, in that they have similar growth cycles, produce almost identical cytological changes in HeLa cells, and react quantitatively in the same manner with their type-specific neutralizing antibodies. Types 3 and 4, which constitute a second subgroup, resemble each other in the biologic aspects mentioned, yet differ from types 1 and 2. In order to determine whether types 5 and 7 adenoviruses fall into the same subgroups, the cytologic changes they evoked in HeLa cells were studied by light and phase-contrast microscopy. The role of the observed changes in viral development was then investigated by means of the fluorescein-labelled antibody technique for the localization of intracellular antigen (5). Experiments were also undertaken to explore: (*a*) the possibility that the alterations produced by the prototype adenovirus strains might not be representative and that other strains of the same virus types might induce different cellular changes, and (*b*) the possibility that the alterations observed were peculiar to cells of the HeLa strain, which are cells in continuous culture derived from malignant tissue. Additional strains of adenoviruses types 5 and 7 were therefore tested, and the sequence of cytologic changes were followed in human amniotic cells in primary culture.

The results of phase and light microscopic investigations which are here described indicate that type 7 resembles types 3 and 4, and that type 5 con-

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forms in large part to the pattern of infection induced by types 1 and 2. Furthermore, the nuclear alterations produced are fundamentally the same in both cell types studied, and vary little from one strain to another of a given virus type.

The fluorescein-labelled antibody studies confirm the relationship of the nuclear alterations to adenovirus synthesis by demonstrating the presence of specific adenovirus antigen in certain characteristic virus-induced intranuclear structures.

Materials and Methods

Tissue Culture: HeLa Cells.—Stock cells of the HeLa strain (6) were cultivated in Eagle's basal medium (7) supplemented with 10 per cent human serum, as previously described (8). For cytological and fluorescein-labelled antibody studies the cells were grown on coverslip pieces in 16 × 150 mm. screw cap culture tubes. Approximately 50,000 to 75,000 cells, suspended in 0.5 ml. of medium consisting of 40 per cent human serum and 60 per cent Hanks' balanced salt solution (BSS), were used to initiate each culture. Prior to viral infection the culture fluid containing human serum was removed and the cells were washed twice with 2 ml. of Hanks' BSS; all cultures were subsequently maintained in a mixture of 67.5 per cent Scherer's amino acid-vitamin mixture (6), 25 per cent tryptose phosphate broth and 7.5 per cent chicken serum (9), hereafter called maintenance medium.

Amniotic Cells.—The amniotic cell cultures used in approximately one-half the experiments were furnished by Drs. Juan Rivadeneira and Eli Gold, Metropolitan General Hospital, Cleveland. The cells used to make these cultures were obtained by repeated cycles of trypsinization (0.25 per cent trypsin 1:300 in Hanks' balanced salt solution, pH 8.0) of minced amniotic membrane, agitated by means of a magnetic stirrer. The isolated cells were centrifuged at low speeds and resuspended in mixture 199 with 10 per cent added horse serum. One ml. of suspension containing 3 to 4 × 10^5 cells was dispensed to each culture tube, which was then shaken and incubated in a stationary rack at 37°C. The supernatant medium was renewed as necessary, and when the cells had spread to form a uniform sheet on the coverslip, they were infected. Amniotic cell cultures used in the remaining experiments differed only in that the cells were digested from intact amniotic membranes, which were incubated in trypsin at 37°C., and shaken manually to free the cells (10). Cells so prepared grew well in a variety of media, including Eagle's basal medium with 15 per cent horse serum added, and mixture 199 containing additional 20 per cent human or 15 per cent horse serum. All cells grown in fluids containing human serum were washed with Hanks' BSS prior to the addition of maintenance solutions. Both HeLa maintenance medium and mixture 199 containing 5 per cent horse serum afforded good cell maintenance.

Viruses.—Three strains of type 5 adenovirus were employed. The first was the prototype strain, originally obtained from Dr. R. J. Huebner, National Institutes of Health, and subsequently passed in HeLa cultures in this laboratory. Two additional strains of type 5 virus, isolated from members of the Cleveland Family Study group (11) by Dr. W. S. Jordan, Jr., were tested; both had been passed twice in HeLa cells.

Eight strains of adenovirus type 7 were studied, including the prototype "Gomen" strain originally isolated and furnished by Dr. T. O. Berge, Fort Ord, California, 5 strains supplied by Nathalie J. Schmidt, Division of Laboratories, California State Department of Public Health, and 2 provided by Lieutenant Commander B. L. Gundelfinger, NAMRU No. 4, Great Lakes Naval Training Station.

Viral inocula used in the majority of experiments were 0.1 ml. of $10^{-1.5}$ to $10^{-2.0}$ dilutions of stock viruses in Hanks' BSS containing $10^{5.0}$ to $10^{5.25}$ tissue culture infectious doses of type 5 virus and $10^{3.25}$ to $10^{3.50}$ infectious doses of type 7.

Method for Observation of Cellular Changes.—At numerous intervals after infection, varying from 6 hours to 9 days, the coverslip cultures were withdrawn from the culture tubes and prepared for study by the following methods:

Phase-contrast microscopy: For these observations the coverslip with its adherent living cells was inverted in a few drops of medium on a clean glass slide, and the edges were sealed with a mixture of Permount® and paraffin. Cultures were examined immediately under a Zeiss microscope equipped with condenser and objectives providing positive phase-contrast. The preparations were sufficiently thin to allow good visualization with the oil immersion objective, a requirement for observation of many of the details of cytologic change.

Light microscopy: Cultures were fixed for 3 to 5 minutes in 95 per cent ethyl alcohol or in Bouin's fixative, and stained with hematoxylin and eosin or by means of the Feulgen reaction for deoxyribonucleic acid (2).

Fluorescein-labelled antibody: The indirect fluorescent antibody technic was employed (12). Details of the fixation and staining procedure have been described previously (13). Uninfected and infected coverslip cultures were washed in phosphate buffered saline (pH 7.2), dried, fixed in acetone, and again dried for 30 minutes. Cultures were next overlaid with one of the following sera diluted 1:10 in buffered saline: (*a*) rabbit antiserum specific for the virus type under study (3), (*b*) normal rabbit serum, or (*c*) heterologous rabbit antisera to other adenovirus types. The latter two served as controls as did uninfected cultures exposed to type-specific antiserum. Cultures were then washed, and each culture covered with fluorescein-labelled goat globulin containing antibodies directed against rabbit gamma globulin.[1] The washed cultures were mounted in buffered glycerine (approximately pH 7.0) and examined immediately with the fluorescence microscope (13).

All HeLa cell cultures observed showed some non-specific fluorescent staining. Unlike specific fluorescent staining, however, the non-specific reaction was principally confined to cytoplasm and what appeared to be nucleoli, and was of low intensity. Although it was rarely difficult to distinguish the bright, intense specific staining from that which was non-specific, control cultures were prepared with each set of specifically stained, infected cultures to allow direct comparison.

Details of the photomicrography and the fluorescence microscope used, its light source and filters, have been described in an earlier communication (13).

EXPERIMENTAL

Microscopic examination of tissue culture cells infected with adenoviruses types 5 and 7 revealed a variety of characteristic cytologic alterations. In order to ascertain whether the different types of alteration seen represented steps in a sequence of cellular change, or merely varying individual cell responses to infection, it was necessary to employ a viral inoculum which would infect the majority of cells present within a short period of time. Undiluted stock suspensions of adenovirus could not be used because of their marked "toxic" effects on host cells (2, 14, 15). Pilot experiments were therefore necessary to determine for each virus type the optimal infectious dose which approached synchrony of infection without producing such "toxic" effects as rounding and clumping. Cultures infected with an appropriate amount of virus were then examined at many different intervals after infection, and the sequential nature of the cellular

[1] The fluorescein-labelled goat antiserum was generously provided by Drs. Barbara Watson and A. H. Coons, Harvard Medical School, Boston.

changes was established. For convenience of description, the changes noted are divided into stages. The alterations in fixed and stained cells visible by light microscopy are compared with those found in unfixed cells examined by phase-contrast microscopy and also with the appearance and distribution of viral antigen demonstrated by fluorescence microscopy.

Adenovirus Type 5

Light Microscopic Studies of Fixed and Stained Cultures:

HeLa Cells.—The cytologic effects of 3 different strains of type 5 adenovirus were studied in HeLa cell cultures. One strain evoked a sequence of nuclear alterations almost identical with that resulting from infection with adenovirus types 1 and 2 (2). The prototype 5 strain differed in that it produced an additional feature in the majority of infected nuclei: prominent bar-shaped eosinophilic and Feulgen-negative crystals, which have been observed also in fixed and in fresh HeLa cells by interference contrast microscopy (16) and in thin sectioned HeLa cells by electronmicroscopy (17). The third strain occupied an intermediate position in that it caused the formation of bar-shaped crystals in some, but not in the majority, of cells.

Since sequential alterations in HeLa cells following infection with adenovirus types 1 and 2 have already been reported in detail (2), the description below will be limited to the effects of the prototype 5 strain. A viral inoculum of the prototype strain containing $10^{5.25}$ TCD_{50} was sufficient to infect a very high percentage of cells within 5 hours; 24 hours after infection with this quantity of virus, 95 per cent or more of the cells showed characteristic stigmata of infection. As with the other adenovirus types studied to date, the principal characteristic changes apparent by light microscopy occurred in the nuclei of infected cells, and the descriptions will be largely confined to the nuclear alterations.

During the first 8 to 10 hours after viral inoculation, the infected cells revealed no consistent morphologic differences from the uninoculated controls shown in Fig. 1. The nuclei varied considerably in size and usually contained several nucleoli, dispersed granular chromatin, and scattered aggregates of heterochromatin. Mitotic forms were frequent.

Stage 1.—The first changes were evident in a small percentage of cells 8 to 10 hours after infection, when multiple, well defined, eosinophilic inclusions appeared within the nuclei (Fig. 2). The majority of these inclusions were Feulgen-negative; in a few cells the outer rims of the inclusions showed pale positive Feulgen reactions, indicating the presence of deoxyribonucleic acid (DNA). Cells containing these stage 1 intranuclear inclusions were most abundant between 10 and 18 hours after infection.

Stage 2.—The changes typical of this stage were apparent in a small number of nuclei 14 hours after infection and predominated between 18 and 21 hours. The progression into stage 2 was marked by the development of basophilic, faintly Feulgen-positive cores within the formerly eosinophilic inclusions and the appearance of a narrow clear zone beneath the in-

clusion rim. The "chromatin"[2] became rearranged around the inclusions and the nuclei became enlarged (Fig. 2).

Stage 3.—By 17 hours after infection a few nuclei had progressed to stage 3; the percentage of nuclei in this stage reached a maximum at 22 to 24 hours. This stage was characterized by the appearance of small, angular, eosinophilic, Feulgen-negative, crystal-like masses in the spaces among the rounded inclusions and rearranged "chromatin" (Figs. 3 and 4). The nuclei continued to enlarge.

Stage 4.—This stage, which began in a small proportion of nuclei 19 hours after infection, was most frequently encountered at 30 hours. The rounded inclusions were now shrunken and intensely basophilic and Feulgen-positive. The differentiation between the inclusion core and its rim and the surrounding "chromatin" was no longer apparent (see Figs. 4 and 5). The most striking structures present in nuclei in stage 4 were sharply demarcated, crystal-like structures, which had become long and bar-shaped and more brightly stained with eosin than when they first appeared during stage 3. These structures remained Feulgen-negative. In some cells the nuclear background surrounding the "crystals" seemed empty; in others it was composed of glassy, homogeneous, and Feulgen-positive material similar to that found in late stage nuclei infected with adenovirus types 1 and 2 (Fig. 6).

Stage 5.—48 hours after infection the cell sheets were breaking up and detaching from the glass. Rounding and clumping obscured much cytologic detail, but in cells that could be visualized the eosinophilic crystals appeared far less prominent or were absent altogether. Much of the intranuclear material had merged into a dense, darkly stained mass, surrounded by an irregular, palely stained zone. The nuclear membranes were often beaded with dark granules, and the cytoplasm showed severe degenerative changes. Because of the degeneration of the cultures and the cell clumping, the photographs of this stage did not illustrate the cytologic features clearly.

Amniotic Cells.—Uninfected amniotic cell cultures, prepared for study with each set of infected cultures, were of rather uniform appearance. In contrast to HeLa cells, the amniotic cells had large amounts of cytoplasm. The nuclei contained evenly dispersed chromatin and one or more basophilic nucleoli (Fig. 11).

The effects of the prototype strain of adenovirus type 5 were studied in cells from 5 different amnions.[3] In all amniotic cultures studied well defined eosinophilic intranuclear inclusions were visible within 24 hours after infection with $10^{5.25}$ TCD_{50}. As in HeLa cells the eosinophilic inclusions subsequently developed granular basophilic cores (Fig. 12). In cells examined at longer intervals after infection (48 to 96 hours) bar-shaped eosinophilic crystal-like structures were prominent, but were thinner and longer than those seen in HeLa cells (Fig. 13). As the nuclei of infected amniotic cells enlarged, the nuclear membranes frequently disintegrated and the "crystals" extended into the cytoplasm. One cytologic feature was found in many amniotic cells, but not in HeLa cells, in the late stages of infection: basophilic, Feulgen-positive, crystal-

[2] The material resembled normal host chromatin in the hematoxylin- and eosin-stained cells, but was found to contain viral antigen in subsequent fluorescent antibody studies.

[3] There were minor variations from one amnion to another in the qualitative changes produced by the virus, in the rapidity with which changes appeared, and in the proportion of cells affected. The alterations produced in the infected amniotic cells generally resembled those observed in HeLa cells, and are therefore described in less detail.

like masses which resembled those observed in HeLa cells infected with adenoviruses types 3 and 4 (2), and also 7 (see below). These basophilic masses often coexisted in the same cells with bar-shaped eosinophilic crystals and occurred in the cytoplasm as well as in the nucleus.

Phase-Contrast Microscopic Studies of Unfixed HeLa and Amniotic Cells:

Living, unfixed cultures of both HeLa and amniotic cells infected with type 5 virus were examined by phase contrast microscopy under high magnifications. The early, rounded intranuclear inclusions could be seen in infected cultures of both cell types, but only in thinly spread cells under optimal conditions. Sharply outlined bar-shaped "crystals," which were characteristic of stained cells in stages 3 and 4, were also present in unfixed cells.[4] They usually appeared in pale relief, outlined by denser intranuclear matter. In the nuclei and cytoplasm of late stage amniotic cells polygonal crystals were often seen which resembled those characteristic of infection with adenovirus types 3, 4, and 7.

Fluorescent Antibody Investigations:

Experiments were performed to correlate the development of intracellular viral antigen as revealed by the fluorescent antibody technic with the sequential cytologic changes visible by light and phase microscopy.[5] These investigations were undertaken to determine (*a*) whether type-specific viral antigen was produced in detectable amounts before, during, or after stage 1 nuclear changes, (*b*) which of the characteristic inclusions contained viral antigen, and (*c*) whether virus occurred or developed in sites where no morphologic evidence (on the light microscopic level) suggested its presence. In the correlative experiments large numbers of cultures were infected with a constant dilution of virus, and at each time interval to be investigated, one group of cultures was prepared for study by the fluorescent antibody technic, another was observed immediately with a phase contrast microscope, and a third group was fixed and later stained for light microscopic examination.

The results of the comparative studies revealed that the first specific fluores-

[4] The appearance of such "crystals" under interference contrast microscopy has been described in an earlier report (16).

[5] Two objections may be raised regarding the accuracy with which intracellular antigen can be localized by fluorescent antibody stains. First, it has sometimes been difficult for observers to determine whether fluorescent material lies within the nucleus or in the overlying cytoplasm. In the studies reported here this difficulty was not encountered at all until very late in the course of infection; the intranuclear location of the majority of the specific fluorescence was clear. Second, the possibility of false localization by the fluorescent stain itself, a familiar problem in other histochemical methods, has been raised on theoretical grounds (18). In the present investigations with adenoviruses, specific fluorescence occurred with such consistency in sites which would be expected to contain viral antigen on the basis of other studies that false localization seems unlikely.

cent staining to appear corresponded to the early eosinophilic inclusions of stage 1. Some of the inclusions were homogeneously stained; others showed fluorescence only around the rims. At 16 hours, when stage 1 predominated, the staining in most cells was confined to the inclusions; the remainder of the nucleus, the nuclear membrane, and the cytoplasm were unstained (see Fig. 7).

With a shift toward stage 2 at 18 hours, the intensity of fluorescence in many individual infected cells was greater, and specific staining appeared in the nuclear background as well as in the inclusions' rims and cores and their adjacent "chromatin" (Fig. 8). The nuclear membrane was now outlined with fluorescent stain in some of the cells and in occasional cells it was the only specifically stained structure visible.

In cultures studied 24 hours and longer after infection, later stages in the cytologic sequence were observed. The specific staining remained principally intranuclear. Some late stages revealed bright, granular, fluorescent aggregates dispersed throughout the nuclei (Fig. 9); others showed diffuse, homogeneous, specific staining, often concentrated at the nuclear membrane (Fig. 10). There were frequently seen bar-shaped areas completely devoid of fluorescent stain which corresponded exactly in size and shape to the late stage eosinophilic "crystals" present in hematoxylin and eosin-stained preparations (compare Figs. 6 and 10). Bright, sharp-edged streaks of antigen were present in many nuclei, possibly representing the basophilic matter found alongside the eosinophilic crystals. Diffuse cytoplasmic staining was noted in some late stage cells, particularly in areas adjacent to the nucleus.

In cultures 48 or more hours after infection the disruption of cell sheets and the clumping and rounding of infected cells in the late stages of cytopathic change precluded satisfactory localization of specific fluorescence.

Adenovirus Type 7

In the majority of experiments the prototype "Gomen" strain of type 7 was used. Five additional strains were tested in HeLa cells and found to produce cytopathic effects similar to the prototype strain. In general, the cytologic manifestations produced by type 7 adenovirus closely resembled those produced by types 3 and 4, described in detail in a previous report (2). Minor differences which were apparent on detailed study are described below.

Light Microscopic Studies of Fixed and Stained Cultures:

HeLa Cells.—Infection with $10^{3.25}$ TCD_{50} of the prototype 7 adenovirus induced a series of characteristic changes in HeLa cell cultures. Division of the alterations into stages is arbitrary, and the distinction between them is less clear than between the stages of cytologic change observed in cells infected with type 5.

Stage 1.—First noted 8 hours after infection, and dominating the cytologic picture for the next 12 hours, were 3 types of intranuclear inclusions. The first was dense and homogeneous, usually surrounded by a wide halo; inclusions of this type varied from eosinophilic and Feulgen-negative to basophilic and Feulgen-positive (Fig. 17). The second type consisted of small masses of eosinophilic granules, more densely packed than normal chromatin (Fig. 18). The third and most frequent type resembled the inclusions characteristic of the first stage of infection with adenovirus types 1, 2, and 5, in that they were discrete eosinophilic structures with basophilic rims; they differed in that they were less regular in size and shape (Fig. 19). Nucleoli were still present in all nuclei in stage 1.

The intranuclear inclusions described above as typical of stage 1 of adenovirus type 7 infection were infrequently observed during experiments on HeLa cells infected with adenovirus types 3 and 4.

Stage 2.—At 14 hours after infection a small proportion of nuclei were in stage 2, although the number of cells in this stage did not reach its peak until 24 hours. The normal host nuclear material was now almost completely replaced by coalescing inclusions and evenly spaced Feulgen-positive granules which formed a large central nuclear mass (Fig. 18). A rarefied zone appeared beneath the nuclear membrane (Figs. 18 and 20). Nucleoli were still apparent.

Stage 3.—Nuclei of cells in this stage, prominent in cultures studied 30 hours after infection, were increased in size, had widened peripheral rarefied zones and central masses of varying appearance (Fig. 21). In some stage 3 nuclei, the central mass was composed of closely packed granules; in others the central nuclear material was arranged in meshworks or honeycomb configurations. Small, basophilic, Feulgen-positive, crystal-like inclusions were sometimes visible in the compartments of the honeycomb or at the edges of the central mass. During this third stage the nucleoli of most cells disappeared.

Stage 4.—The fourth and final stage of nuclear change was already present in some cells 30 hours after infection, and became the most frequent form in older cultures. The large, distorted nuclei resembled rosettes, with intensely basophilic and Feulgen-positive centers surrounded by wide, irregular and lightly stained peripheral zones (Fig. 22). Large crystal-like masses, also basophilic and Feulgen-positive, were conspicuous features in many of the late stage nuclear rosettes, occurring in both central and peripheral zones.

Amniotic cells.—Cultures from 5 different amnions were infected with the prototype 7 virus and studied microscopically. The cytologic changes induced were quite similar to those described above in HeLa cells, although slower to develop. Twenty-four hours after infection with $10^{3.25}$ TCD_{50}, one-third or fewer of the amniotic cell nuclei revealed typical early virus-induced alterations, including eosinophilic inclusions similar to those found in HeLa cells in stage 1 (Fig. 14). By 48 hours, later stages in the cytologic sequence were visible, identified by cells with markedly enlarged nuclei containing intensely Feulgen-positive central masses. Honeycomb configurations studded with dozens of small "crystals" were frequent (Fig. 15), and large, intensely Feulgen-positive crystals were present in many nuclei 72 and 96 hours after inoculation (Figs. 15 and 16). The nuclear membranes were occasionally disrupted and degenerating. Minor differences were apparent from one amnion to another in certain cytologic features evoked by type 7 virus. The most prominent of these variations were the size of the crystals and width of the peripheral nuclear zones.

Phase Contrast Microscopic Studies of Unfixed HeLa and Amniotic Cells:

In contrast to type 5 adenovirus-infected cells, in which most of the cytologic alterations were best visualized in stained preparations, many of the cellular phenomena following type 7 infection were best illustrated in unfixed cells using phase contrast microscopy. The crystals characteristic of infection with adenovirus types 3, 4, and 7, which were quite vulnerable to distortion during fixation (2), could be observed most satisfactorily in fresh unfixed preparations (13, 16). Because electronmicroscopic evidence indicates that these crystals are composed of viral particles (14, 19, and 20), their formation is of particular interest and will be discussed in detail. The phase microscopic findings were observed in numerous experiments in which a large number of coverslip cultures were examined at high magnifications at 1 or 2 hour intervals during the period of greatest cellular change.

In coverslip cultures of HeLa cells observed between 12 and 14 hours after infection most of the cells appeared unaffected, with thinly spread cytoplasm, delicately outlined nuclear membranes, and rather homogeneous, pale nuclei. The nuclei of some cells showed shadowy areas of increased density, sometimes containing irregular filaments and granules, the first recognizable virus-induced changes.

At 16 to 18 hours after infection distinct intranuclear granules appeared grouped in discrete clusters in some cells, and in others evenly distributed throughout the nucleus or arranged in a fine net or meshwork (Fig. 23). A rarefied peripheral nuclear zone was present in many affected cells. Small sharp edged, crystal-like structures were first detected in a very few nuclei 18 hours after infection in some experiments and at 19 hours in others. The tiny "crystals," which appeared only in nuclei which also contained small granules, were usually scattered through the nuclear substance. The small crystalline structures could not be well visualized in fixed and stained cells.

Between 18 and 22 hours after infection the cytologic picture changed rapidly. A shift occurred from early stages with ill defined areas of increased density and fine granules to mid-stages containing a wide variety of striking crystal-like structures (Figs. 24 and 25). The entire central zone of some nuclei consisted of densely packed small "crystals." In other nuclei the "crystals" occupied the peripheral zone around a finely granular central mass.

From 24 to 48 hours after infection the mid-stages became fewer and the late stages with their large and prominent crystals became dominant. Most nuclei showed 2 zones, corresponding to the center and the periphery of the late stage rosettes. The center consisted of small regular granules or "crystals." The peripheral zones of many nuclei contained massive crystals (Fig. 26). In some cells the peripheral zone was filled with homogenous matter teeming with

motion, which was separated from the central mass by a clear band of uniform width. Observation of the homogeneous, vibrating material indicated that it quite suddenly aggregated into well formed crystalline masses. This crystallization process was observed on numerous occasions in cells infected with type 7 and also with type 4 adenovirus. Unfixed amniotic cells infected with type 7 adenovirus revealed similar cytologic changes.

Fluorescent Antibody Investigations:

As in the study of type 5 adenovirus described above, the indirect fluorescent antibody technic was used to follow the development of type 7 adenovirus in HeLa cells. The design of experiments was also similar: fluorescein-labelled antibody findings were compared with microscopic observations of fixed and fresh infected cells.

The first specific fluorescent staining detected after exposure to type-specific antibody coincided with the fine, evenly distributed Feulgen-positive granules of the second stage in the cytologic sequence (Fig. 27). Repeated examination of cultures in the early stages of type 7 infection failed to reveal the presence of type 7 adenovirus antigen in sites corresponding to the early eosinophilic and predominantly Feulgen-negative inclusions of stage 1. Clear cut specific staining apparently localized to the early inclusions was however observed in type 7 infected cultures exposed to heterotypic antiserum to adenovirus type 5, one of the unusual instances of antigenic cross-reaction found in the course of fluorescent antibody studies with adenoviruses (21).

In cultures harvested 24 hours or more after infection, fluorescent staining within the nuclei occurred in coarse networks and in sharp edged, homogeneously stained masses representing the "crystals" characteristic of infection with type 7 (see Figs. 28 and 29).

As in hematoxylin and eosin-stained and in unfixed cells, the late stage nuclei were divided into two zones. The central zone showed a variable amount of specific fluorescent staining; in some cells the staining occurred as fine, evenly dispersed granules, and in others as small crystal-like aggregates (see Fig. 29). Comparison of late stage cells in hematoxylin and eosin-stained and in fluorescent antibody–stained preparations revealed that a considerable proportion of the basophilic matter in the central nuclear zone was not antigenic. In the peripheral zones of late stage nuclei in fluorescent antibody preparations the specific staining was sometimes diffuse and homogeneous, but more frequently was localized to sharp edged, intensely fluorescent crystal-like masses (see Fig. 30). The pattern of the fluorescent staining was thus found to correspond to the homogeneous, vibrating substance which subsequently crystallized, as well as to the "crystals" themselves.

Throughout the sequence the amount of antigenic material present in in-

dividual nuclei infected with type 7 adenovirus greatly exceeded that occurring in cells infected with adenovirus type 5.

DISCUSSION

The existance of major subdivisions within the adenovirus group, suggested by earlier cytological investigations with adenovirus types 1, 2, 3, and 4 (1–4),

TABLE I

Summary of Cytologic Alterations Produced in HeLa Cells by Adenovirus Types 1, 2, 3, 4, 5 and 7

Cytologic feature	Adenovirus types 1, 2, and 5	Adenovirus types 3, 4, and 7
Nuclear enlargement	Marked	Marked
Multiple, well defined oval or round eosinophilic intranuclear inclusions	Consistent and striking early feature	Frequent, early in type 7 infection; infrequent with types 3 and 4. Inclusions less regular in size and shape than those characteristic of infection with types 1, 2, and 5.
Nucleus divided into rarefied peripheral and heavily stained central zones	Feature limited to late stages	Feature apparent throught most of cytologic sequence
Regular arrays of intranuclear Feulgen-positive granules, networks and honeycomb configurations	Sometimes formed when inclusions merge	Consistent features
Basophilic Feulgen-positive crystals in central mass or in peripheral nuclear zone	Very infrequently observed in late stages	Striking and consistent feature, best visualized in unfixed cells with phase contrast microscope
Bar-shaped eosinophilic and Feulgen-negative crystals	Observed only in cells infected with type 5 (not with 1 or 2)	Not found

has been corroborated by studies of adenoviruses types 5 and 7. On the basis of cytologic effects, adenovirus type 5 may be classified in one subgroup with types 1 and 2, and type 7 in a second with 3 and 4, as illustrated in Table I. Characteristic of infection with members of the first subgroup are multiple, discrete oval or round intranuclear inclusions which undergo a series of morphologic and tinctorial changes. Prominent features of cells infected with members of the second subgroup are regular arrays of granules and large, well formed, Feulgen-positive crystals within the nuclei. In certain respects the changes produced by members of the two subgroups are similar; it should not be inferred that they are wholly different. It should be stressed that division of

the 6 viruses into the same 2 subgroups has been found in investigations of other of their biologic characteristics, including the quantitative neutralization by specific antisera, the length of the latent period of the initial multiplication cycle, and the amount of virus produced per HeLa cell (3, 4, and 22).

In a recent communication Barski reported cytologic studies of adenovirus types 1, 2, 3, 4, 5, and 7, in which "one common appearance was observed in cells infected with adenovirus types 1, 2, and 5, and another nuclear lesion was observed consistently in cells infected with types 3, 4, and 7." Investigation of 20 different adenovirus strains revealed that the nuclear alterations held true to type. He also noted that similar nuclear changes could be demonstrated in rabbit fibroblast cultures (23). Recently Orfila (24) has also reported, from studies of human kidney cultures infected with type 7 adenovirus, that the cytopathic effects resemble those described as characteristic of types 3 and 4. It was shown in the present study that human amniotic cells infected with adenovirus types 5 and 7 exhibited basic patterns of intranuclear change which were very similar to those which occurred in HeLa cells, and that the nuclear alterations caused by the two viruses were consistent from one strain to another with the same virus type.

The reproducibility and consistency with which the specific cellular alterations occur after infection with a given adenovirus type indicate that the capacity to produce such changes in the host cell is a fundamental property of the virus itself. The significance of individual virus-induced inclusions and stages of nuclear change will be considered in more detail in a subsequent paper in which the production of infectious virus and the cytologic changes will be correlated.

The results of the fluorescein-labelled antibody studies which enabled visualization of intracellular viral antigen accumulation serve to confirm and extend previously reported morphologic and cytochemical evidence linking the adenovirus-induced nuclear changes with actual viral synthesis (1, 2, 14, 15, 19, and 20). Specific viral antigen appeared in infected cells at approximately the same time as the onset and development of the intranuclear changes. Furthermore, the sites of fluorescent staining coincided exactly with those of certain characteristic virus-induced structures. In the case of adenovirus type 5 infection, specific fluorescent staining appeared in the characteristic, round, intranuclear inclusions from the time of their formation in stage 1 throughout the subsequent sequential changes. These observations are in accord with those recently reported by Pereira, Allison, and Balfour (25). These authors also demonstrated specific fluorescent staining in the eosinophilic inclusions characteristic of the early stages of infection with type 5 virus.

Although in late stages of infection with type 5 adenovirus diffuse and particulate specific fluorescence occurred in the nuclear substance surrounding the eosinophilic inclusions, the bar-shaped crystals were conspicuously unstained.

The significance of the bar-shaped crystals remains an enigma. That they play a very essential role in viral synthesis seems doubtful, for they do not appear to be a consistent feature of type 5 infection. As described above, the extent to which such crystals developed in HeLa cells varied widely in the 3 strains of type 5 virus tested. Furthermore, their occurrence was not described by Lagermalm *et al.* (26) in an electronmicroscopic study of HeLa cells infected with type 5 adenovirus, although they were quite prominent in HeLa cell cultures studied by Morgan *et al.* (17). According to the latter investigators, the bar-shaped crystals were not composed of viral particles but of very fine particulate matter, possibly representing protein molecules. Cytochemical evidence has indicated that the bar-shaped crystals do not contain detectable amounts of deoxyribonucleic acid (16, 17).

The immunofluorescent studies of adenovirus type 7–infected cells indicated that the early eosinophilic inclusions did not contain type-specific antigen, but one which reacted with antisera to type 5 adenovirus instead. This cross-reaction is of interest because the early inclusions of type 7 infection are morphologically quite similar to those which are characteristic of infection with type 5. The bright, specific fluorescence present in type 7–infected cells coincided strikingly with the regular arrays of Feulgen-positive granules of the early stages and the well defined crystals of the mid and late stages of the cytologic sequence. It is apparent thus that the crystals characteristic of infection with type 7 (and also 3 and 4) are intimately related to viral development, in contrast to the bar-shaped, eosinophilic, crystalline masses which occur in type 5 infected cells. Crystals of the former type developed with great consistency in cells infected with 6 different strains of type 7 virus. According to electronmicroscopic investigations they are composed of virus-like particles of approximately 60 to 65 mμ in diameter (20). Moreover, cytochemical studies have demonstrated that such crystals show intensely positive Feulgen reactions for DNA (2, 27).

In those cell structures which developed as a result of adenovirus infection, a general correspondence was noted between Feulgen-positive staining, indicating the presence of DNA, and specific fluorescence with labelled antibody, denoting the presence of viral antigen. The staining reactions of the principal structures produced as a result of infection with adenovirus types 5 and 7 are summarized in Table II. Although exceptions exist, there is for the most part a correlation between the presence of DNA as revealed by the Feulgen reaction and the presence of specific viral antigen. The exceptions that have been noted are the eosinophilic and Feulgen-negative, yet antigen-containing, stage 1 inclusions of type 5 adenovirus-infected cells and the basophilic, Feulgen-positive, yet non-antigenic matter in the central mass of late stage type 7 infected cells. As would be expected, the Feulgen-positive structures are predominately basophilic.

Studies of herpes simplex virus-infected cells have revealed a similar correspondence between the presence of DNA and the occurrence of viral antigen; the early basophilic and Feulgen-positive intranuclear inclusion bodies contained herpes virus antigen, whereas the late eosinophilic and Feulgen-negative ones did not (28). Herpes simplex-infected cells differed in that the antigen appeared first in the nucleus and later almost exclusively in the cytoplasm; in adenovirus infection, nuclear staining predominated in all stages, even after the peak of infectious virus production had been reached (13).

TABLE II

Summary of Fluorescein-Labelled Antibody and Cytochemical Staining Reactions of Cell Structures and Inclusions Produced by Adenovirus Types 5 and 7

Structure	Fluorescent staining for specific virus antigen	Feulgen reaction for DNA	Hematoxylin-stained	Eosin-stained
Type 5 infected cells				
Multiple round intranuclear inclusions	+	− to +	+ (Periphery, late)	+ (Early)
Homogeneous, glassy nuclear background	+	+	+*	+*
Long, bar-shaped crystals	−	−	−	+
Type 7 infected cells				
Stage 1 inclusions	−‡	(rarely +)	−	+
Regularly aligned granules of central mass	+	+	+	−
Polygonal crystals—in nuclear periphery and in central mass	+	+	+	−
"Matrix" of late stage central mass enclosing crystals or regular granules	−	+	+	−

* Amphophilic.

‡ Stained after reaction with type 5 antiserum but not type 7 antiserum.

The preponderance of nuclear staining in adenovirus-infected cells is not surprising, in view of the light and electronmicroscopic findings which indicate persistence of the majority of crystals and virus-like particles within the nuclei. In the present studies specific fluorescence was detected in the cytoplasm of only a few cells in the late stages of infection and the degree of cytoplasmic fluorescence was always less than the nuclear fluorescence. In contrast, Pereira, Allison, and Balfour (25) using the fluorescent antibody technic in a study of type 5 adenovirus-infected cells demonstrated that approximately one-half of HeLa cells with specific nuclear fluorescence also demonstrated definite but

much weaker cytoplasmic fluorescence. The mechanism by which the specific viral antigen gets from the nucleus to the cytoplasm is unknown. It has been suggested by some investigators (14, 19, 20, and 27) that the occurrence of smaller numbers of crystals and dispersed virus-like particles in the cytoplasm is the result of disruption of the nuclear membrane.

SUMMARY

The sequential cytological changes which develop in tissue culture cells infected with adenovirus types 5 and 7 are described and compared with those produced by adenovirus types 1, 2, 3, and 4. The evidence that is presented indicates that types 1, 2, and 5 belong to one major subdivision of the adenovirus group and types 3, 4, and 7 to another. That the host cell nucleus is the principal site of adenovirus synthesis has been confirmed by fluorescent antibody studies. They have demonstrated the occurrence of type-specific adenovirus antigen in the characteristic intranuclear inclusions and other virus-induced structures reported to contain virus-like particles or shown by electronmicroscopy.

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EXPLANATION OF PLATES

Photomicrographs of HeLa cells and of human amniotic cells infected with adenovirus types 5 and 7. Cells shown in Figs. 1 to 6, 11 to 16, and 17 to 22 were stained with hematoxylin and eosin after fixation in 95 per cent ethyl alcohol; those in Figs. 7 to 10 and Figs. 27 to 30 were fixed in acetone and stained by the indirect fluorescent antibody technic to demonstrate viral antigen; those in Figs. 23 to 26 are unfixed HeLa cells as they appear by phase contrast microscopy.

PLATE 77

FIG. 1. Uninfected HeLa cells, including mitotic figure (*M*) in early prophase. Note multiple nucleoli (*N*), which vary in size and shape. Hematoxylin and eosin. × 1050.

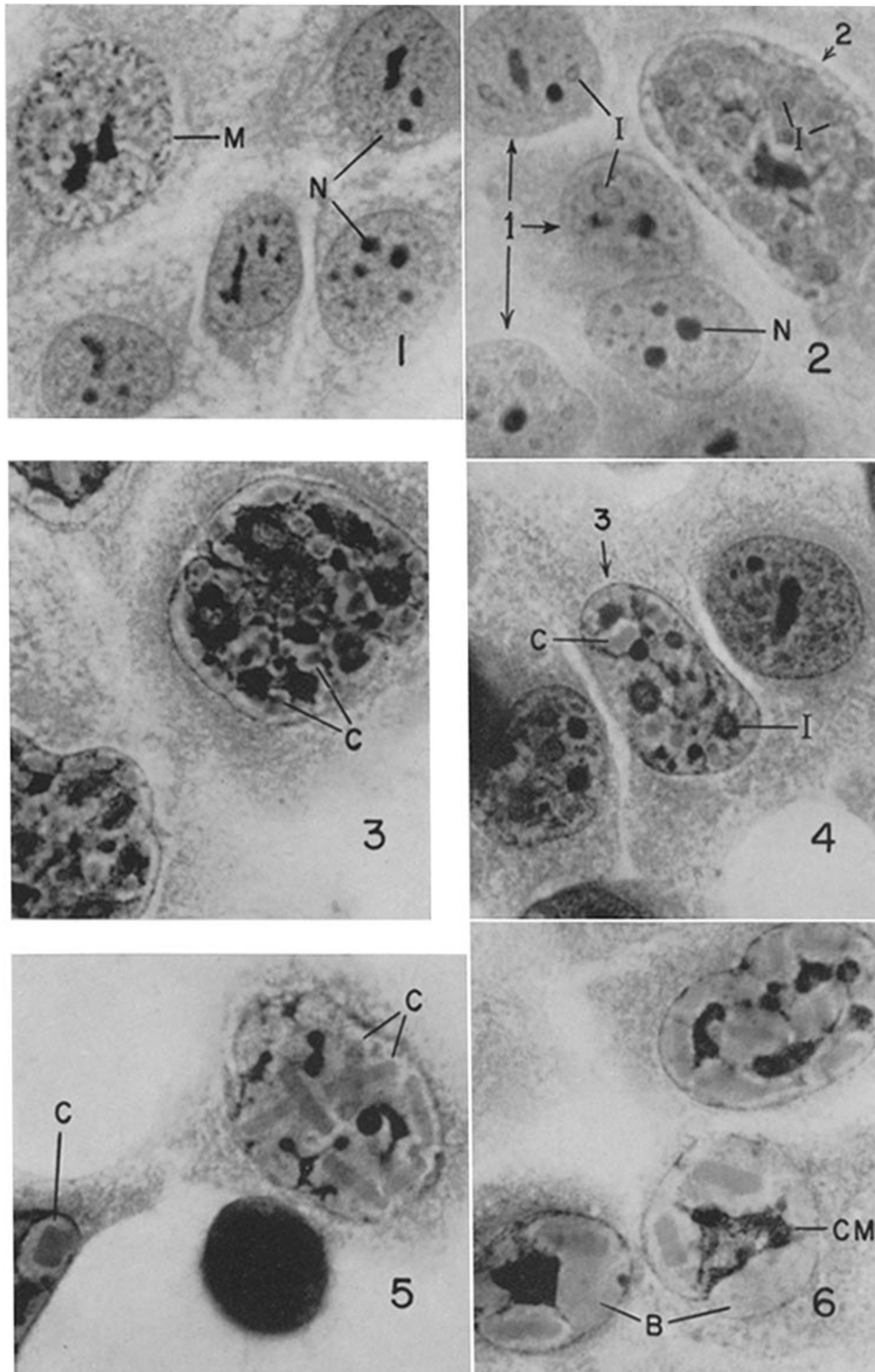
FIG. 2. HeLa cells 16 hours after infection with adenovirus type 5. Nuclei in stage 1 of sequential change (labelled 1) contain numerous round intranuclear inclusions (*I*), which are more lightly stained than the nucleoli (*N*) present in the same cells. Nucleus in stage 2 (labelled 2) shows enlargement and "chromatin" rearrangement around the inclusions (*I*) which now have basophilic granular cores. Hematoxylin and eosin. × 1050.

FIG. 3. HeLa cells 22 hours after infection with type 5, showing stage 3 nuclear changes. The enlarged nucleus contains early, small, eosinophilic, crystal-like structures (*C*). Hematoxylin and eosin. × 1050.

FIG. 4. HeLa cells 20 hours after infection with type 5. Nucleus labelled 3 shows early eosinophilic crystal-like structures (*C*) of stage 3 and darkly stained inclusions (*I*) in which core, rim and surrounding "chromatin" are no longer distinct. Hematoxylin and eosin. × 1050.

FIG. 5. Thirty hours after infection with type 5 virus, showing nucleus in stage 4. Long bar-shaped eosinophilic crystal-like structures (*C*) are prominent features; inclusions are shrunken, basophilic, lacking in detail. Hematoxylin and eosin. × 1050.

FIG. 6. Twenty-four hours after infection. Three nuclei in stage 4 containing elongated crystals. Two nuclei in lower portion of figure contain homogeneously stained background material (*B*) which gives a positive Feulgen reaction. Inclusions are merging into central mass (*CM*). Hematoxylin and eosin. × 1050.



(Boyer *et al.*: Cellular changes in HeLa and amniotic cells)

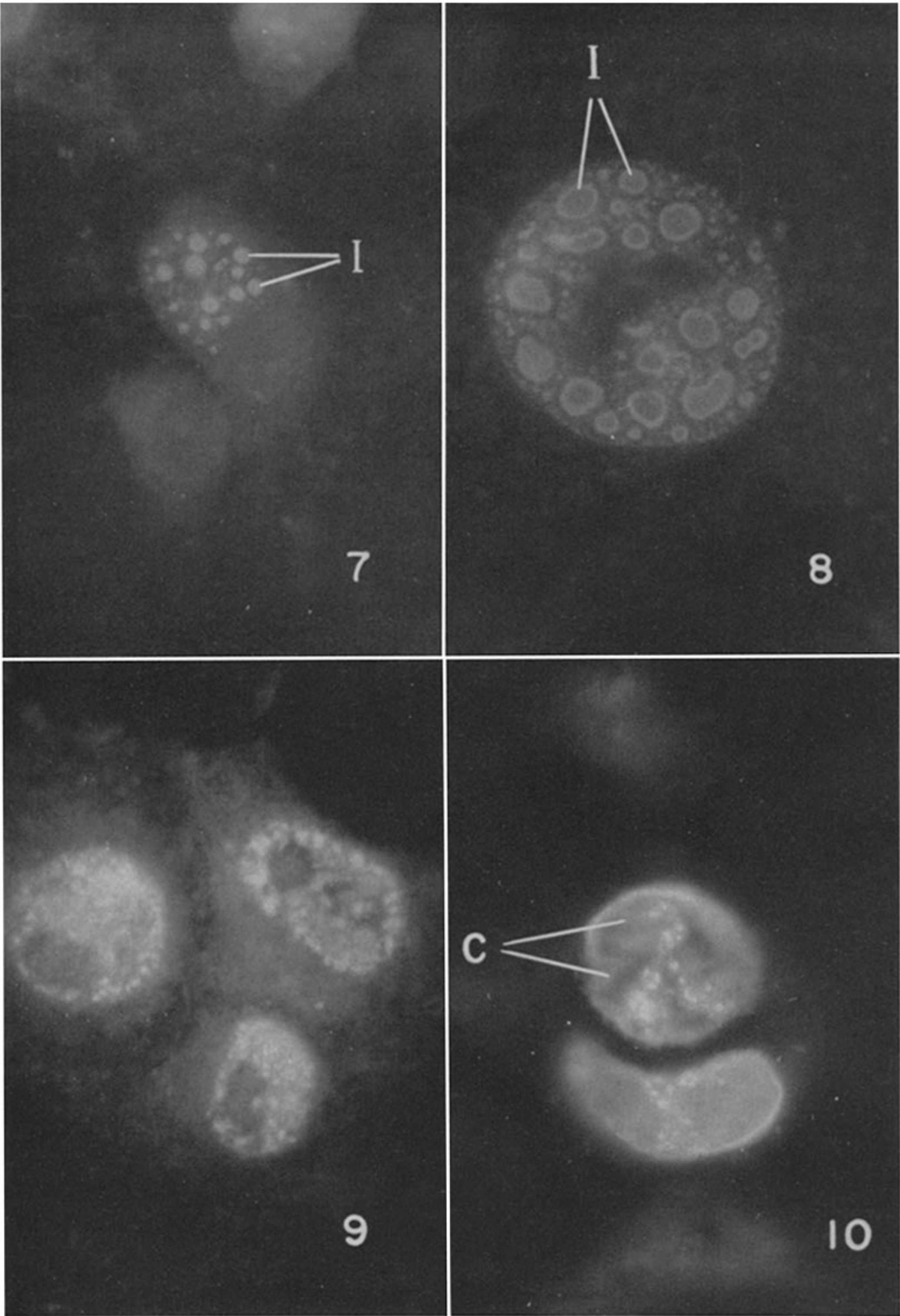
PLATE 78

FIG. 7. HeLa cells 16 hours after infection with type 5 adenovirus, stained by indirect fluorescent antibody technic to demonstrate virus antigen. Note bright staining of multiple rounded intranuclear inclusions (*I*) in center cell, corresponding to stage 1 in sequence of nuclear changes. Outlines of nucleus are not apparent. × 1000.

FIG. 8. HeLa cells from culture 22 hours after infection revealing advancement over stage 1 nucleus shown in Fig. 7. Fluorescent stain appears brightest at inclusions' rims (*I*) and in small aggregates corresponding to rearranged "chromatin" of stage 2. Compare with stage 2 nucleus stained with hematoxylin and eosin in Fig. 2. × 1000.

FIG. 9. HeLa cells from culture 26 hours after infection. Fluorescent staining appears in nuclear background as well as in inclusions. Almost no antigen appears outside nuclear confines. × 1000.

FIG. 10. HeLa cells from culture 30 hours after infection. Note diffuse nature of nuclear staining outlining dark images of bar-shaped crystals (*C*). × 1000.



(Boyer *et al.*: Cellular changes in HeLa and amniotic cells)

PLATE 79

FIG. 11. Uninfected human amniotic cells with abundant amounts of cytoplasm and relatively small nuclei containing evenly dispersed chromatin and several nucleoli (*N*). Hematoxylin and eosin. × 925.

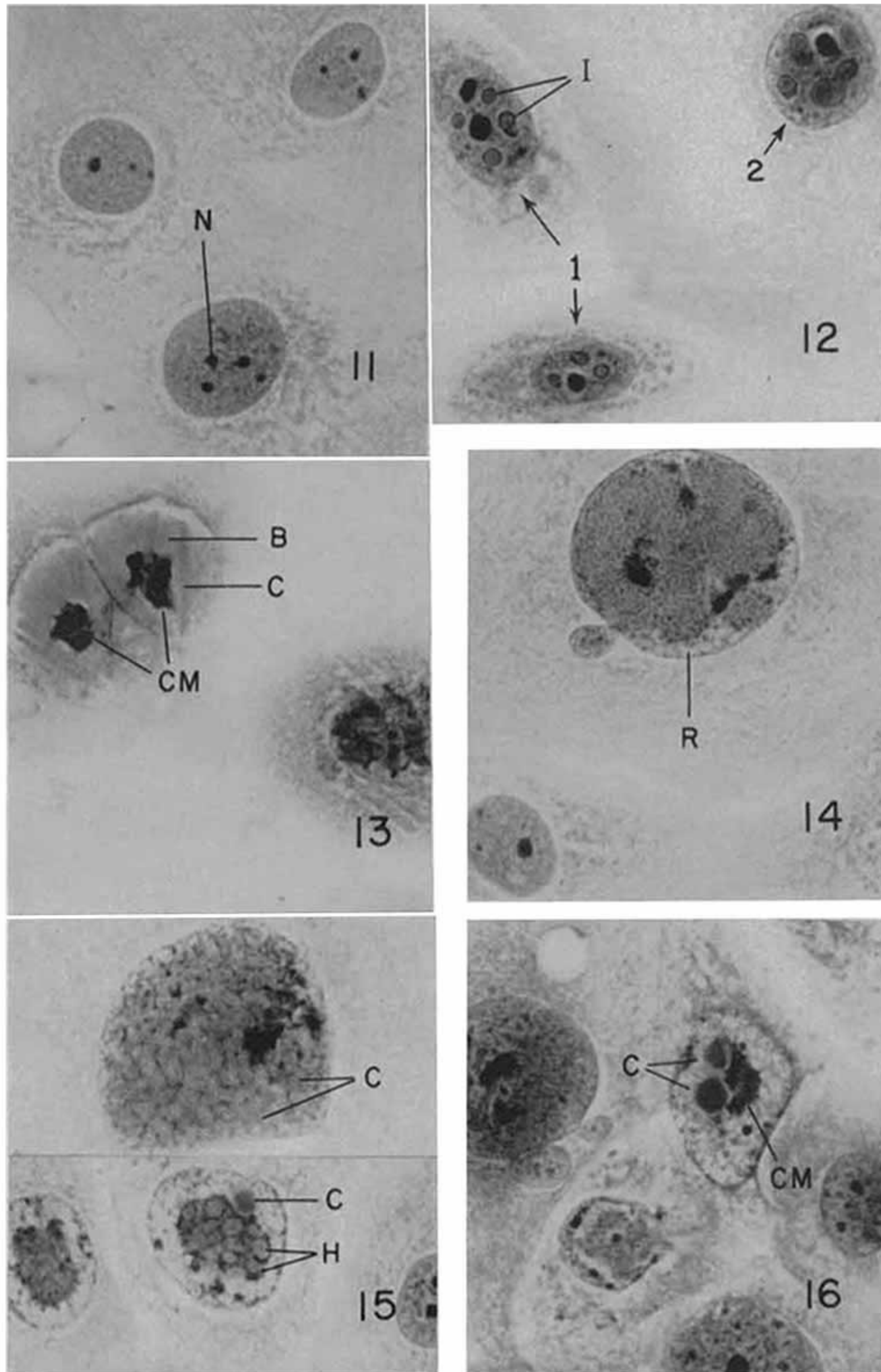
FIG. 12. Amniotic cells 28 hours after infection with type 5 adenovirus. Note well defined eosinophilic inclusions (*I*) in nuclei showing stages 1 and 2 of cytologic sequence. Stage 2 nucleus reveals cores forming within inclusions and "chromatin" rearrangement. Hematoxylin and eosin. × 925.

FIG. 13. Amniotic cells late in the course of infection with type 5 adenovirus (72 hours). Note thin elongated crystals (*C*), homogeneous background material (*B*), and densely stained central mass (*CM*) in two nuclei at left. Nuclear membrane of cell at right has disintegrated and crystals lie in cytoplasm. Hematoxylin and eosin. × 925.

FIG. 14. Cells from amniotic culture early after infection (24 hours) with adenovirus type 7. Nuclear substance has been replaced by coalesced inclusions and granules. A partial zone of rarefaction (*R*) is present beneath the nuclear membrane. Compare with Fig. 18. Hematoxylin and eosin. × 925.

FIG. 15. Cells from amniotic culture showing mid-stages (48 to 72 hours) characteristic of infection with type 7 adenovirus. Nucleus of upper cell exhibits honeycomb configuration; compartments of honeycomb are filled with tiny crystal-like structures (*C*). Two lower cells show more advanced changes. Note coarser honeycomb (*H*) and larger "crystals" (*C*) which are basophilic. Hematoxylin and eosin. × 925.

FIG. 16. Amniotic cells showing late stages (72 to 96 hours) of infection with adenovirus type 7. Note large homogeneous basophilic crystals (*C*) adjacent to central mass (*CM*) of late stage nucleus. Compare with Fig. 22. Hematoxylin and eosin. × 925.



(Boyer *et al.*: Cellular changes in HeLa and amniotic cells)

Plate 80

Fig. 17. HeLa cells 12 hours after infection with adenovirus type 7. Note dense intranuclear inclusions (*I*) surrounded by wide halos. Nucleoli (*N*) are present in same cell. Hematoxylin and eosin. × 1050.

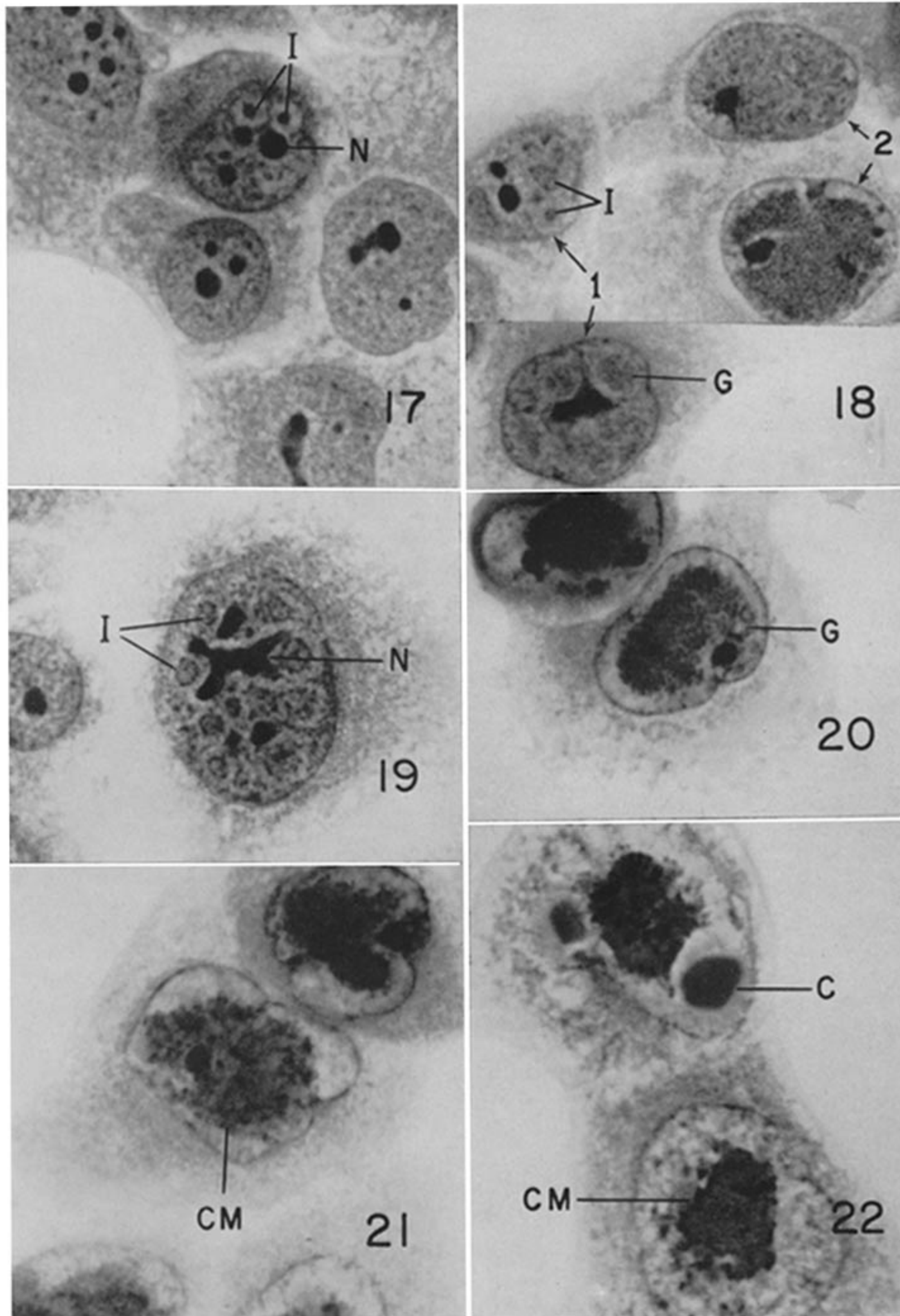
Fig. 18. HeLa cells 18 hours after infection with adenovirus type 7. Two stage 1 and two stage 2 nuclei are apparent. Stage 1 nuclei reveal eosinophilic inclusions (*I*) and clusters of granules (*G*). Stage 2 nuclei are filled with merging inclusions and regular granules forming central zone. Clear zone has begun to appear beneath nuclear membrane of stage 2 nuclei. Hematoxylin and eosin. × 1050.

Fig. 19. Stage 1 cell from culture 14 hours after infection which contains large, irregularly branched nucleolus (*N*) and multiple inclusions (*I*) which resemble those characteristic of stage 1 in infection with adenovirus type 5. Compare with Fig. 2 above. Hematoxylin and eosin. × 1050.

Fig. 20. HeLa cells 30 hours after infection with adenovirus type 7, in transition from stage 2 to stage 3. Peripheral, clear, nuclear zone is wider than in stage 2, and central zone stains more intensely. Note regular array of granules (*G*) in central zone of lower nucleus. Hematoxylin and eosin. × 1050.

Fig. 21. HeLa cells 30 hours after infection with type 7. Material forming central mass (*CM*) of stage 3 nuclei is coarse, often forms honeycomb configuration. Hematoxylin and eosin. × 1050.

Fig. 22. Two stage 4 nuclei in HeLa cell culture 48 hours after infection with adenovirus type 7. Note wide peripheral nuclear zones, containing large, basophilic crystals (*C*). Central mass (*CM*) contains small crystals in upper cell and regularly spaced granules in lower cell. Hematoxylin and eosin. × 1050.



(Boyer *et al.*: Cellular changes in HeLa and amniotic cells)

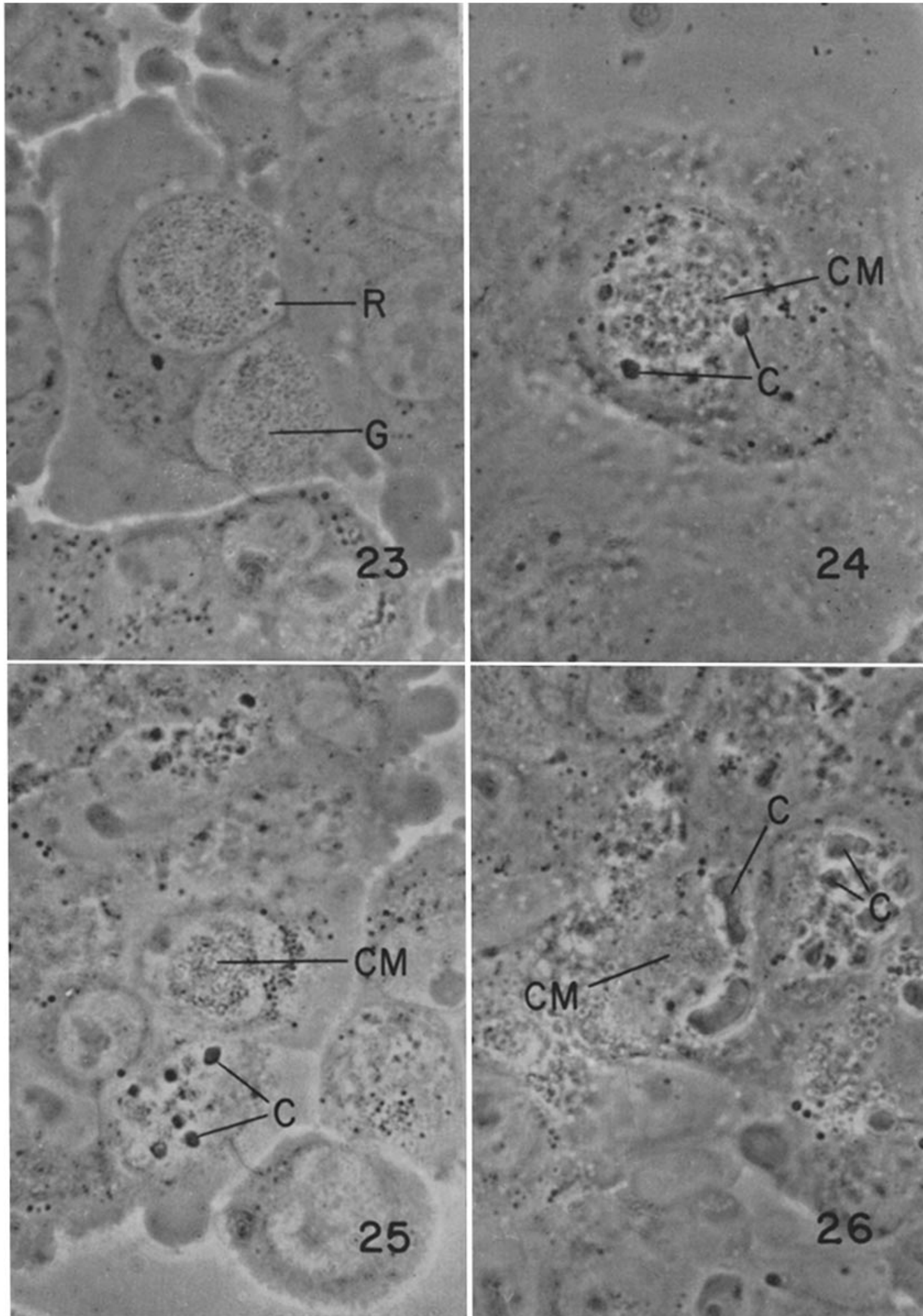
PLATE 81

FIG. 23. Fresh, unfixed HeLa cells photographed under phase contrast microscope 18 hours after infection with type 7 adenovirus. Note zone of rarefaction (*R*) beneath nuclear membrane and central nuclear mass composed of regular granules (*G*). Compare with Figs. 14, 18, and 20. × 1000.

FIG. 24. HeLa cells 22 hours after infection with type 7 virus. Note crystals (*C*) lying at periphery of central mass of nucleus (*CM*). × 1000.

FIG. 25. HeLa cells 24 hours after infection with type 7 adenovirus. One cell contains granular central mass (*CM*) and clear peripheral nuclear zone. Compare with Fig. 21. Lower cell contains numerous large crystals (*C*) dispersed through nucleus. × 1000.

FIG. 26. HeLa cells 48 hours after infection with type 7 virus. Note large, homogeneous crystals (*C*) lying adjacent to round central mass (*CM*) composed of fine granules. Compare with Fig. 22. Nucleus of cell on right contains large number of crystals (*C*). × 1000.



(Boyer *et al.*: Cellular changes in HeLa and amniotic cells)

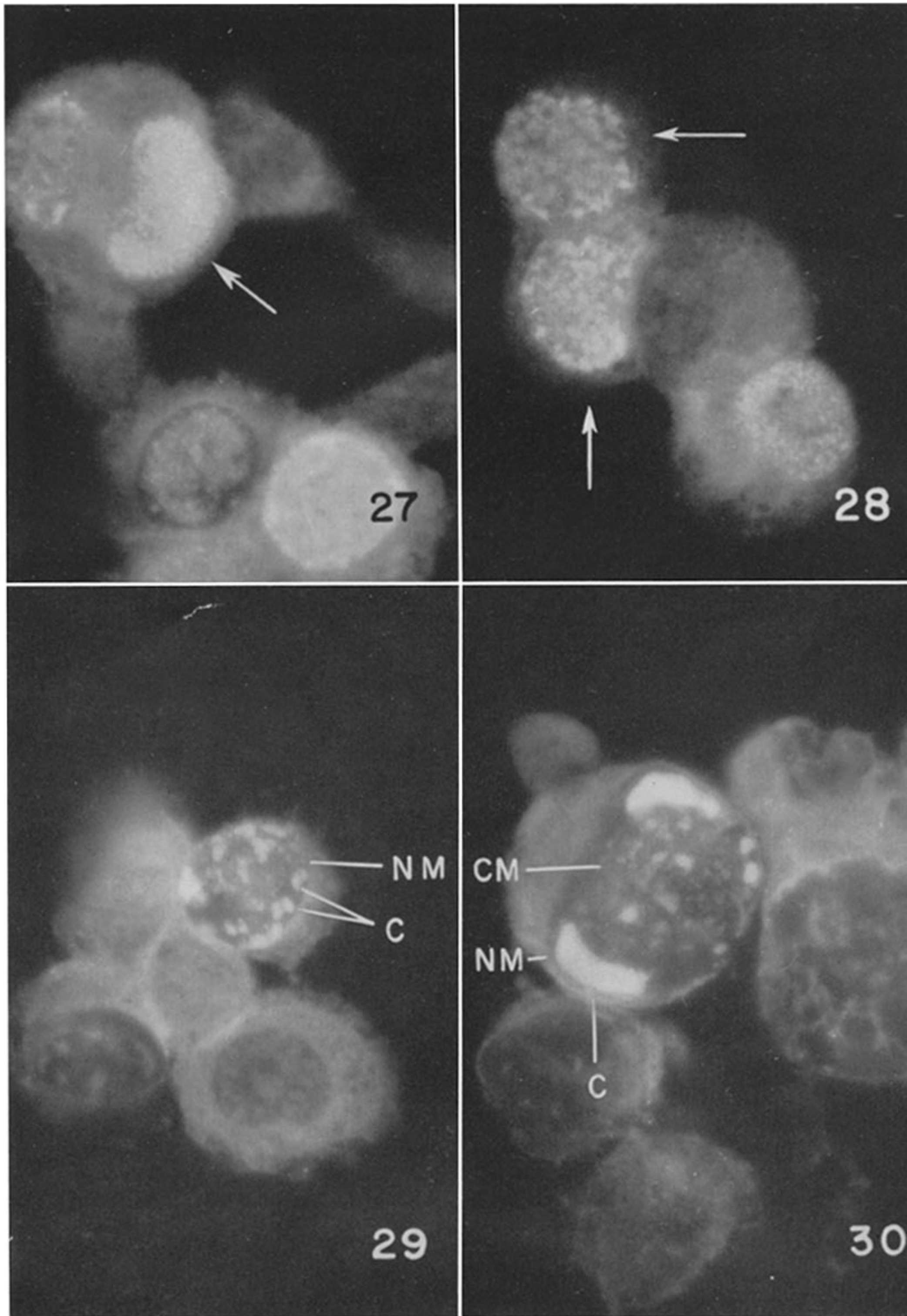
PLATE 82

FIG. 27. HeLa cells 24 hours after infection with type 7 adenovirus, stained by indirect fluorescent antibody technique to demonstrate intracellular virus antigen. Note intense fluorescence in infected nuclei. Cell indicated by arrow reveals diffuse nuclear staining of evenly spaced granules. Compare with stage 2 nuclei in Figs. 18 and 23. × 1000.

FIG. 28. HeLa cells in slightly later stage in sequence of infection. Note bright intranuclear staining (arrows) now appears in coarse aggregates. Compare with central zone of stage 3 nucleus in Fig. 21. × 1000.

FIG. 29. HeLa cell in still later stage, showing sharp edged, brightly stained, crystal-like masses (*C*) in nucleus. Denser central zone of nucleus can barely be distinguished. *NM* indicates site of nuclear membrane. Compare with Figs. 24 and 25. × 1000.

FIG. 30. HeLa cells late in course of type 7 infection (48 hours), including nucleus in stage 4 of sequential alteration. Note large, sharply outlined crystalline masses (*C*) in peripheral nuclear zone and smaller fluorescent aggregates in central mass (*CM*). *NM* indicates site of nuclear membrane. Compare with Figs. 22 and 26. × 1000.



(Boyer *et al.*: Cellular changes in HeLa and amniotic cells)